보건학석사 학위논문

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GH3와 zebrafish (*Danio rerio*) embryo, larvae를 이용한 perfluoroundecanoic acid와 perfluorotridecanoic acid의 갑상선 호르몬 교란 영향 연구

2019년 2월

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이 논문을 석사 학위논문으로 제출함

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Abstract

Thyroid disrupting potency and mechanism of perfluoroundecanoic acid and perfluorotridecanoic acid in GH3 cell line and zebrafish (*Danio rerio*)

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Perfuoroalkyl substances (PFASs) have been used in various household goods such as textile, paper, and food packaging products. The use and environmental detection of long-chain PFASs (C>8) have increased. While several PFASs have been known as thyroid disruptor, knowledge gaps are still present about thyroid disrupting effects of long chain PFASs (C>10). In the present study, rat pituitary cell line (GH3) and zebrafish (*Danio rerio*) embryo/larvae were used to identify the thyroid disrupting effects of two long chain perfluorinated carboxylic acid (PFCAs), i.e., perfluoroundecanoic acid (PFUnDA) and perfluorotridecanoic acid (PFTrDA). For comparison purpose, PFOA was also chosen. In GH3, exposure to PFUnDA and PFTrDA showed upregulation of *Tshβ* gene, suggesting that these PFCAs stimulate thyroid hormone (TH) synthesis. In zebrafish, following 5 days exposure of fertilized embryos to PFTrDA showed upregulation in the genes that related to TH synthesis (*tshβ*, *nkx2.1, nis*, *tpo*, *mct8*) and elimination (*dio1, dio2*). Meanwhile PFOA and PFUnDA exposure upregulated *ugt1ab* gene related to TH elimination. In the fish, morphological changes, such as eyeball size decrease, increased yolk sac size, or swim bladder

deflation did occur for all test chemicals, implying TH reduction in developing zebrafish larvae. According to the results of present study, long chain PFCAs can lead to TH reduction perhaps through different mechanism and their disruption potency may increase with their chain length.

Key words: long chain perfluoroalkyl substaces, PFUnDA, PFTrDA, GH3 cell line, zebrafish, thyroid hormone, morphological change, endocrine disruption

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1. Introduction

Perfluoroalkyl substances (PFASs) have been used in clothing, leather, carpets, frying pan coatings, and food contact papers for their resistance to water and oil (Kannan, 2011; Paul et al., 2008). PFASs have carbon-fluorinated (C-F) bound in their structure which makes those PFASs more persistent and bioaccumulative in environment and organism. Therefore long chain PFASs tend to have more long biological half-life then PFASs with shorter carbon chain (Houde et al., 2006; Kim et al., 2013; Lee et al., 2017). Human serum elimination half-life of PFOS, PFOA and PFHxS were estimated as 5.4, 3.8 and 8.5 years respectively (Olsen et al., 2007). According to previous toxicity studies of PFASs, PFASs can cause adverse effects on organism including liver toxicity, endocrine disruption, immunotoxicity (Guruge et al., 2005; Lau et al., 2004; Lau et al., 2007). Despite their toxicity, a variety of PFASs were detected in environment and human sample because of their diverse use for products. Perfluorooctane sulfonic acid (PFOS) and Perfluorooctanoic acid (PFOA) are most detected PFASs (Loos et al., 2007; Thompson et al., 2011). Because of their detection rates and toxicity, PFOS and PFOA have been regulated for use in many countries. For PFOS, It is listed on Stockholm convention as POPs (persistent organic pollutants). And PFOA were regulated through stewardship program (EPA, 2006; UNEP 2010). Consequently, concentration of regulated PFASs and related PFASs including precursors are generally decreasing in consumer products, environment and human. However the use and environmental levels of other PFASs including long-chain (C>8) PFASs are generally increasing (Bjerregaard-Olesen et al., 2016; Land et al., 2018; Okada et al., 2013; Schröter-Kermani et al., 2013; Toms et al., 2014). Toxicity of the most perfluorinated compounds is closely related to their bioaccumulation and their biological half-life. Several studies reports about the relationships between their toxicity and their chain length, PFASs with longer fluorinated carbon chain tended to be more toxic then shorter chain PFASs (Buck et al., 2011; Goecke-Flora and Reo, 1996; Gomis et al., 2018; Kim et al., 2013). Furthermore, half-life and uptake rates showed the increasing tendency until 13 carbons in their structure (Martin et al., 2003). Unlike other lipophilic persistent organic pollutants tend to be accumulated in lipid, PFASs bind to proteins like albumin in human serum then accumulating in lipid (Jones et al., 2003; Poothong et al.,

2017). This property closely related to maternal-fetal transport through placenta barrier. Previous studies report that transport efficiency of maternal transfer of long chain PFCAs which have more than 10 carbon in their structure is increasing with their chain length (Pan et al., 2016; Zhang et al., 2013a). Not only maternal-fetal transport through placenta barrier property but also long chain PFCAs were detected in human breast milk. Since the maternal exposed PFASs easily transfer to the fetus and infant, early-life stage exposure could be more important in PFSAs exposure. PFASs including PFOS and PFOA are known as thyroid hormone disruptor (Buttenhoff et al., 2002; Butenhoff et al., 2012; Coperchini et al., 2015; Martin et al., 2007; Ren et al., 2015; Weiss et al., 2009). According to the precious reports, PFOS and PFOA reduce the activity of thyroid peroxidase (TPO), essential enzyme for thyroid hormone synthesis (Song et al., 2012). Furthermore, PFOS can reduce the thyroid hormone level through metabolic clearance of liver without any change of thyroid stimulating hormone (TSH) level, thyroid gland histology or TPO activity (Chang et al., 2008; Chang et al., 2009; Coperchini et al., 2017; Yu et al., 2009). Diverse PFASs including long chain PFCAs interrupt the transthyretin (TTR)- thyroxine (T4) binding. Consequently free-T4 will be increased (Weiss et al., 2009). Perfluorodecanoic acid (PFDA) which has 10 carbons exposure also led to thyroid hormone reduction and thyroid gland weights loss in rat (Kelling et al., 1987; Langley et al., 1985). However thyroid hormone disrupting effects have been documented for PFOS and PFOA, significant knowledge gaps are present for thyroid-disrupting effects of other long chain PFCAs.

Perfluoroundecanoic acid(PFUnDA) is C-11 chain PFCAs formed by chemical reaction of fluorotelomer alcohols (FTOH) such as 10:2 FTOH. PFUnDA has been used as alternative chemical for regulated PFASs (Harbison and John, 2015; Young et al., 2007). In the environmental samples, ND-3.52 ng/L of PFUnDA has been detected in water from west coast of Korea (Naile et al., 2010). Also in the sediment samples collected from South Bohai Bostal watersheds, in China, 0.010-0.120 ng/g dw of PFUnDA was detected (Chen et al., 2016). In the human samples, 1.11-4.58 ng/mL of PFUnDA has been detected in Korean pregnant serum and 6.5-32.7 pg/mL in breast milk (Fujii et al., 2012; Ji et al., 2012). Studies have indicated that concentration of PFUnDA in human serum showed correlation with thyroid hormone level and occurrence of thyroid hormone disease (Kim et al., 2016; Tsai et al., 2017; Wang et al., 2014).

Perfluorotridecanoic acid (PFTrDA) is C-13 chain PFCAs. PFTrDA has also been detected in environment and human like PFUnDA. For example, 0.1 ng/L of PFTrDA was detected in parramatta river in Australia (Thampson et al., 2011) and 11.3 pg/L of PFTrDA was detected in seawater of Eastern China (Li et al., 2018). In samples of human, PFTrDA has been detected 0.27-0.57 ng/mL in Korean pregnant serum and 3.3-30.3 pg/mL in breast milk (Fujii et al., 2012; Ji et al., 2012). In general population, serum level of PFTrDA showed negative correlation with total T4 and positive correlation with thyroid stimulating hormone (TSH) (Ji et al., 2012). Also maternal PFTrDA level was correlated with fetal total T4 concentration (Kim et al., 2011). Despite the correlation in previous reports, the effect of these two PFCAs on thyroid function and disruption mechanism has not yet been clearly defined. Pituitary is important organ for synthesis of thyroid hormone through negative feedback system of thyroid hormone regulation in organism. GH3 rat pituitary cell line has been used as tool for screening the thyroid hormone disruption potency of chemical (Kim et al., 2015; Lee et al., 2018). Evaluating the disrupting effects of long chain PFASs related to thyroid hormone regulation is possible by using the GH3 cell line. Zebrafish has been used as suitable model for screening thyroid disrupting chemicals. Especially embryo larvae stage, their size is very small, very rapid development, transparency of developing embryos, easy to maintenance and culture those factors make zebrafish very attractive model to evaluation thyroid hormone disrupting effects in early life stage of organism (Heijlen et al., 2013).

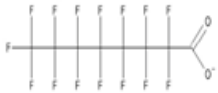
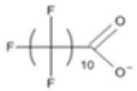
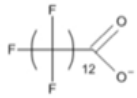
The purpose of the study is to identify the mechanism of thyroid hormone disrupting effects of PFUnDA and PFTrDA and compare disruption potency with regulated PFCAs, PFOA. Using GH3 cell line and zebrafish model. The hypothesis of this study is PFUnDA and PFTrDA may have similar disrupting effects with PFOA which has same carboxylic acid (-COOH) functional group in their structure.

2. Materials and Methods

2.1. Chemicals

PFOA (perfluorooctanoic acid, CAS no. 335-67-1, 96%), PFUnDA (perfluoroundecanoic acid, CAS no. 2058-94-8, 95%), PFTrDA (perfluorotridecanoic acid, CAS no. 72629-94-8, 97%) were purchased from Sigma Aldrich (St. Louis, MO, USA). The physicochemical properties of PFOA, PFUnDA, PFTrDA were discribed in Table 1. Only for in vitro assay, T3 (Triiodothyronine, CAS no. 9002-71-5) was used as positive chemical. T3 was also purchased from Sigma Aldrich. For both fish and cell exposure, ethanol (absolute for analysise EMSURE®, Reag. Ph Eur.) was used as solvent and purchased from EMSURE, Merck (Germany).

Table 1. Physicochemicalproperties of tested PFCAs

Compound	Perfluorooctanoic acid	Perfluoroundecanoic acid	Perfluorotridecanoic acid
Synonyms	PFOA (C=8)	PFUnDA (C=11)	PFTrDA (C=13)
CAS No.	335-67-1	2058-94-8	72629-94-8
Structure			
Molecular formulate	C8HF15O2	C11HF21O2	C13HF25O2
Molecular weight (g/mol)	414.07	564	664.11
LogK_{OW}	10.14	13.37	7.47
Female Half-lives in serum (years)	1.8[a], 2.3[b]	4.5[b]	-
Male Half-lives in serum (years)	1.7[a], 2.8[b], 3.8[c]	12[b]	-

[a] Bartell et al., 2010.

[b] Zhang et al., 2013b.

[c] Olsen et al., 2007.

2.2. GH3 cell culture and exposure

The GH3 Cell line was obtain from Korean cell bank (Seoul, Korea) and maintained at 37℃ with 5% CO2. For culture medium, Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture (Sigma Aldrich) and 10% fetal bovine serum (FBS; Gibco® LifeTechnologies, Carlsbad, CA, USA) were used as described in Kim et al., 2015.

For GH3 exposure, cells were seed 2.5 × 10^5 density in 24-well plates and incubated for 20h using culture medium. In culture medium, it contains FBS which has growth factors and steroid hormones for cell growth. To remove those factors, after 20h from seeding, all culture medium was removed and changed to serum-free medium which contain 1% ITS^+ premix (BD Biosciences, Franklin Lakes, NJ, USA), i.e., insulin (6.25 μg/mL), transferrin (6. 25 μg/mL), selenous acid (6.25 ng/mL), bovine serum albumin (1.25 mg/mL) and linoleic acid (5.35 μg/mL) and then incubated 4h more.

After 4h later, the cells were dose with 3, 10, 30 μM of PFUnDA and 10, 30, 100 μM of PFOA, PFTrDA. Those exposure concentration of PFCAs was determined by previous cytotoxic test. Each exposure medium was made of serum free medium (0.1% v/v ethanol). 1 nM of T3 was used as positive control. Positive control was for quality verification of cell condition. For cell exposure, cells were incubated in triplicate (n=3). After 24h exposure, the cells in each well were collected for RNA extraction.

2.3. Zebrafish embryo larval exposure

Wild type adult zebrafish were obtained from commercial supplier (Greenfish, Seoul, Korea) and maintained in Environmental Toxicology laboratory at Seoul National University in Korea. From sexually matured adult fish were mated for gathering fertilized embryos. 25 embryos were randomly divided into 50 mL beakers with 50 mL exposure media within 4 hours after fertilization.

According to the results of preliminary range finding test, 0, 3, 10, 30 mg/L for PFOA, 0, 0.03, 0.1, 0.3 mg/L for PFUnDA, PFTrDA were determined for the exposure concentration. Each PFCAs was dissolved in ethanol. Exposure media

was made of ethanol stock (0.005% v/v) and dechlorinated water. Each treatment was composed to four replicates. The zebrafish embryos were exposed until 5 days post-fertilization (dpf). During the exposure period, approximately all of the exposure solution was renewed every day. Exposure was conducted in a temperature controlled room (25±1℃) under photoperiod of 14:10 h light:dark. For checking water quality of exposure solution, including pH, conductivity, temperature and dissolved oxygen were measured every day.

The embryo and larval survival (%), hatchability (%), time to hatch (day) were observed every day until 5dpf. At 5 dpf, 5 larvae were randomly collected for morphological change observation and 15 larvae for gene analysis. After collection larvae for gene analysis, larvae were washed with PBS and stored at -80℃.

2.4. RNA Isolation and Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

For RNA extraction in GH3 cells, exposure medium was removed and cells were washed twice with ice-cold PBS (without $Ca^{2+}$and Mg^{2+}) and lysed with lysis buffer (Qiagen, Hilden, Germany). The mRNA was extracted immediately using RNeasy mini kit (Qiagen).

Zebrafish larvae were homogenized using tissue grinder with lysis buffer (Qiagen). After grinding each samples per replicate were centrifuged at 15000 ×g for 3 min. Supernatant was collected in e-tube for RNA extraction. mRNA was immediately extracted using RNeasy minikit (Qiagen). The mRNA concentration and quality (260/280 ratio>1.8) were verified with Nanodrop ND-1000 spectrometer (Nanodrop Technologies, Wilmington, DE, USA). After measurement, all of the mRNA samples were matched to same concentration (diluted to working concentration<1000 ng/μL). And complementary DNAs were synthesized by using cDNA synthesis kit (BioRad, Hercules, CA, USA) For quantitative- real-time PCR (qRT-PCR), 20 μL reaction mix was prepared. The composition of reaction mix, 10 μL of Light cycler® 480 SYBR Green I master mix (Roche Diagnostics ltd., Lewes, UK) 1.8 μL of each PCR primer, 2 mL cDNA were diluted with 4.4 mL of nano-free water.

Cyclopiline for GH3, *18s rna* for zebrafish were used as housekeeping gene. Those gene has been used as housekeeping genes in a number of studies. The primer sequences of GH3 and zebrafish used in this study is shown in Table S1. The thermo cycle profiles was preincubation at 95℃ for 10 min, 40 cycles of amplification at 95℃ for 10 sec, 60℃ for 20 sec and 72℃for 20 sec. For quantification the results of PCR, threshold cycle (Ct) was determined for each reaction. Each Ct values for target gene were normalized to housekeeping genes using $2^{-\Delta\Delta Ct}$ methods (Livak and Schmittigen, 2011).

2.5. Zebrafish larval stage morphological change observation

Table 2. Criteria of determine occurrence of morphological change (malformation) of 5 dpf zebrafish

Malformation	Criteria
Partial hatch	Embryos started to develop but did not hatched completely from chorion
Body size	Relatively small body size compared to normal (at least 10%)
Bent spine	Bend in distal/lateral spine (>10°)
Malformed tail/fin	Irregular development of tail/fin - shape of the tail/ fin - Irregular pigmentation pattern
Eye/head size	Larger/smaller eye (optic vesicle) size than normal (at least 10%)
Jaw development	Reduced development of upper and lower jaw
Heart development	Alteration of size of heart chamber
Yolk sac edema	Accumulation of pellucid fluid in yolk sac, making larger than normal (at least 30%)
Swim bladder inflation	- Posterior swim bladder inflation/deflation - Difference in swim bladder surface area size (at least 30%)

At 5 dpf, randomly collected 5 larvae per replicate were used for morphological change observation. The detailed criteria for determine occurrence of malformation were shown in Table 2. Each criteria was made based on previous studies about zebrafish larvae morphological changes observation (Panzica-Kelly et al., 2010; Brannen et al., 2010; Barrow, 2013; Corrales et al., 2014). Using same larvae, Their body length, eyeball size, swim bladder size, yolk sac size and heart malformation were measured by an image processing program, ImageJ® (Fig. S1). Deflated swim bladders were excluded for their size measurement.

2.6. Statistical analysis

The data normality and homogeneity of variances were analyzed by Shapiro-wilk's test and Levene's test respectively. For comparison among treatment, One-way analysis of variance (ANOVA) with Dunnett's test was used. Spearman's rank correlation was used for trend analysis. In all statistical analysis, p-value <0.05 was considered to be significant. All data are expressed as mean value with standard error of mean (SEM). IBM SPSS 20.0 for Windows (SPSS Inc., Chicago, IL, USA) was used in data analysis.

3. Results

3.1. Alteration of gene expression in GH3

Gene expression results of GH3 cells, exposed to PFOA, PFUnDA, PFTrDA and positive chemical, T3 is shown in Fig. 1.

T3 led to significant down-regulation of *thyroid-stimulating hormone beta* (*Tsh β*). In contrast, Exposure to PFUnDA and PFTrDA showed increasing trends in *Tshβ* gene transcription level (p=0.002 for PFUnDA and p=0.009 for PFTrDA). All of the PFCAs exposure led to down regulation of *deiodinase type*1 (*Dio*1). However there were no statistically significant differences. Exposure to PFOA led to significant down regulation in *deiodinase type*2 (*Dio2*). However PFUnDA and PFTrDA exposure showed little increase in the transcription level of *Dio*2. But only PFUnDA showed statistically significance in gene expression trend.

Exposure to PFUnDA, PFTrDA did not show meaningful change of gene expression in thyroid hormone receptor genes including *thyroid hormone receptor alpha* and *beta* (*Tra* and *Trβ*) this tendency was same as PFOA.

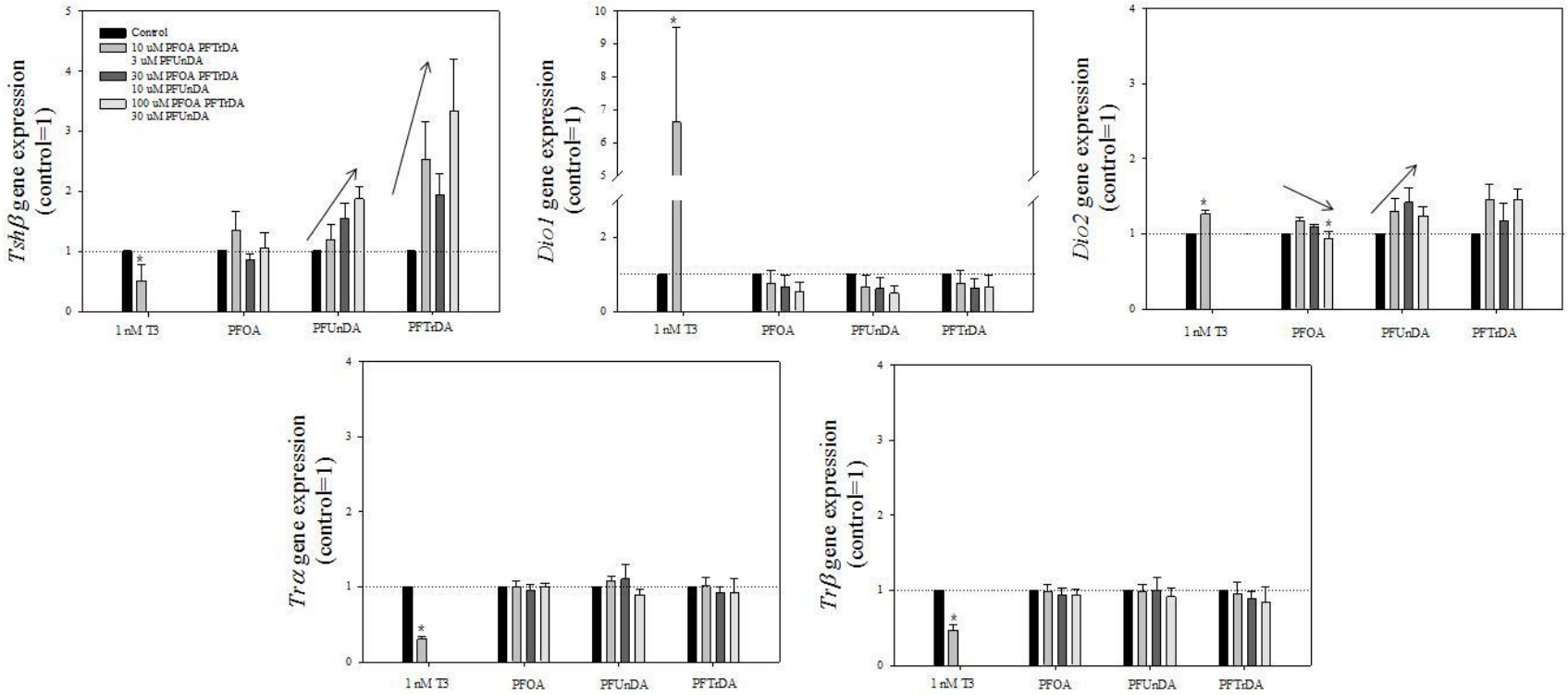


Figure 1. Gene expression in GH3 exposed to PFOA, PFUnDA and PFTrDA. The results are shown as the mean ± SEM of three replicates. Asterisks(∗) indicate significant differences (∗$p < .05$, ∗∗$p < .01$, ∗∗∗$p < .001$) from solvent control (0.1% EtOH). Each arrow indicates significant trend by Spearman's rank correlation analysis.

3.2. Zebrafish embryo larval exposure

3.2.1. Zebrafish embryo larval survival and development effect

The embryo and larval survival (%), hatchability (%), time to hatch (day) were not significantly affected by tested PFCAs in all treatment group (Table 3). Decreasing tendency was observed in embryo and larval survival, hatchability, time to hatch in all of PFTrDA exposed group compare to control group. Exposure to PFOA tends to impede the hatching time of zebrafish. But was no statistical significances.

Table 3. Effect of PFOA, PFUnDA, PFTrDA on embryo and larval survival (%), hatchability (%), time to hatch (day) in zebrafish until 5 dpf

PFCAs concentration	Control	PFOA (mg/L)			PFUnDA (mg/L)			PFTrDA (mg/L)		
	0	3	10	30	0.03	0.1	0.3	0.03	0.1	0.3
Total survival (%)[a]	96.8 ± 1.1	93.3 ± 9.9	94.3 ± 3.5	96.3 ± 4.7	94.3 ± 2.3	97.7 ± 2.5	96.7 ± 0.6	95.3 ± 3.8	94.3 ± 8.1	92.7 ± 5.1
Embryo survival (%)[b]	96.8 ± 3.0	93.3 ± 9.9	94.7 ± 3.5	96.3 ± 4.7	96.0 ± 2.1	98.0 ± 2.6	98.3 ± 0.6	95.3 ± 3.8	94.3 ± 8.1	93.3 ± 5.0
Larvae survival (%)[c]	98.8 ± 1.7	100 ± 0.0	99.7 ± 0.6	100 ± 0.0	99.7 ± 0.6	99.3 ± 0.6	99.3 ± 0.6	100 ± 0.0	100 ± 0.0	99.7 ± 0.6
Hatchability (%)[d]	99.4 ± 0.5	100 ± 0.0	99.7 ± 0.6	100 ± 0.0	100 ± 0.0	98.7 ± 1.5	100 ± 0.0	94.0 ± 3.0	94.7 ± 8.4	95.3 ± 7.2
Time to hatch (day)[e]	3.0 ± 0.2	3.1 ± 0.3	3.2 ± 0.2	3.1 ± 0.2	2.9 ± 0.6	2.8 ± 0.4	2.9 ± 0.4	2.9 ± 0.4	3.0 ± 0.1	2.9 ± 0.2

[a] Total survival (%) is percentage of survived larvae and embryo among the all exposed embryos.

[b] Embryo survival (%) is percentage of survived embryo among all exposed embryo.

[c] Larvae survival (%) is percentage of survived larvae among hatched.

[d] Hatchability (%) is percentage of hatched among all exposed embryos.

[e] Time to hatch (day) is the day of hatching.

3.2.2. Alteration of genes expression related to thyroid hormone regulation in zebrafish larvae

Although PFOA and PFUnDA did not affect *tshβ* and *thyroid stimulating hormone receptor* (*tshr*) gene, PFTrDA upregulated *tshβ* and *tshr* gene transcription level (Fig. 2). Both trends were significant. The gene transcription levels related to thyroid development and thyroid hormone synthesis, PFTrDA increased gene expression of *sodium-iodide symporter* (*nis*) *thyroid peroxidase* (*tpo*) and *monocarboxylate transporter8* (*mct8*). But these genes did not show meaningful statistical significant differences in PFOA, PFUnDA exposed groups. *thyroglobulin* (*tg*) was down regulated by following exposure to PFUnDA. Thyroid hormone receptor gene, *trß* of zebrafish larvae showed decreasing trends in PFOA (p for trend= 0.000) and PFUnDA (p for trend= 0.017) treatment group. PFTrDA exposed group showed increasing tendency in *ttr* gene expression. But there was no statistical significant. Transcription levels of genes related to thyroid hormone metabolism, *dio1* gene was significantly up-regulated by exposure to PFOA and PFTrDA exposed group. PFUnDA showed only increasing trend in their gene transcription level (p for trend= 0.013). Despite the significant up-regulation of *dio2* was observed by exposure to PFTrDA, PFOA and PFUnDA did not show any effect on transcription level of *dio2*. The expression of *uridine diphosphate glucuronosyltransferase* (*ugt1ab*) was significantly up-regulated by exposure to PFOA and PFUnDA. PFOA also significantly up regulated the gene transcription level of *cytosolic sulfotransferase* (*sult1 st5*) gene.

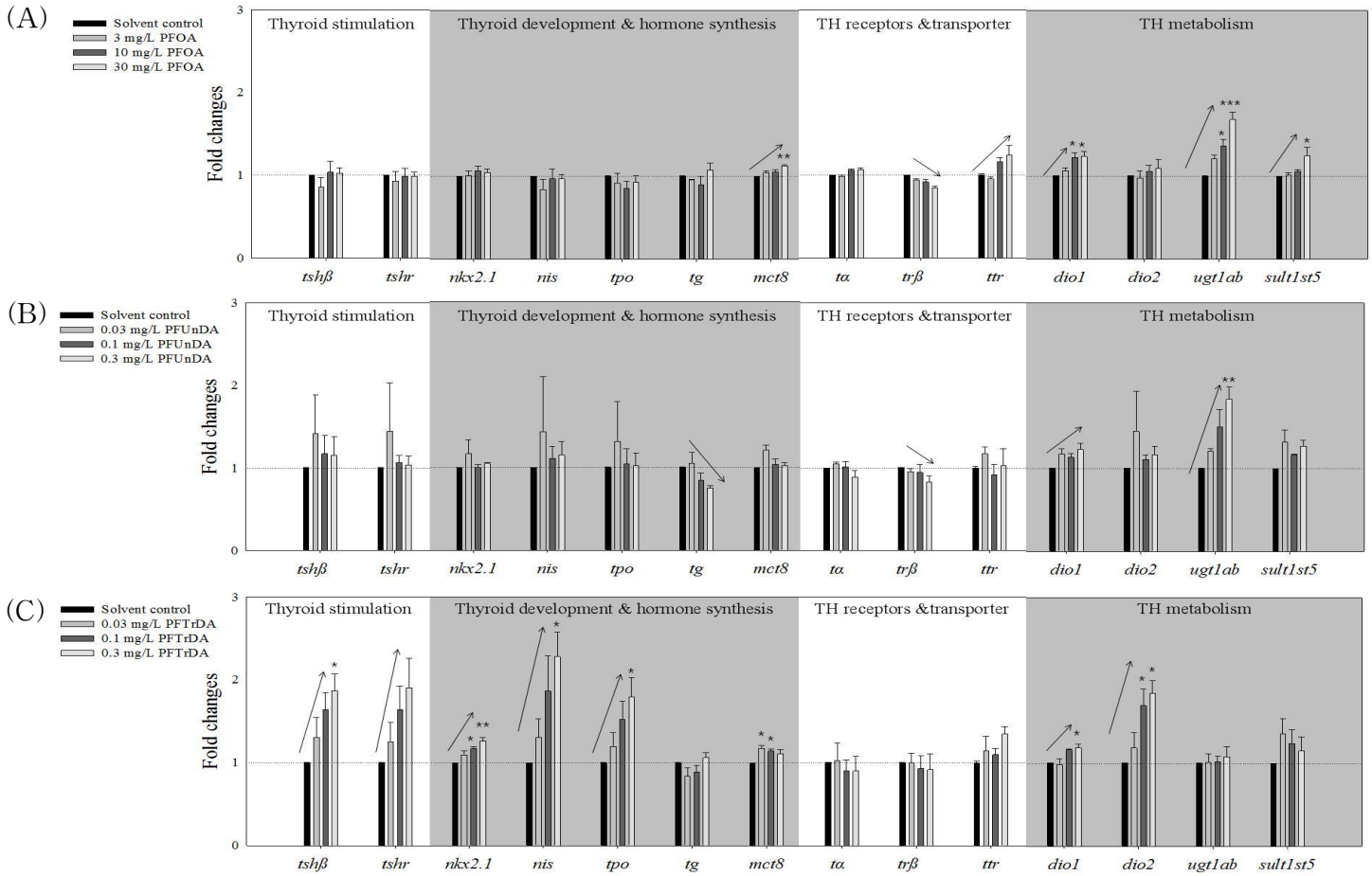


Figure 2. Gene expression in whole body of zebrafish until 5 dpf exposed to (A) PFOA, (B) PFUnDA and (C) PFTrDA. The results are shown as the mean ± SEM of four replicates and each replicates included 15 larvae. Asterisks(∗) indicate significant differences (∗$p < .05$, ∗∗$p < .01$, ∗∗∗$p < .001$) from solvent control (0.005% EtOH) by ANOVA analysis. Each arrow indicates significant trend by Spearman's rank correlation analysis.

3.2.3. Morphological change of zebrafish

Morphological changes occurred only in 20% of normal condition zebrafish larvae, without any exposure to PFCAs zebrafish. However more than 40% of zebrafish larvae exposed to each PFCAs suffered morphological alterations during their development (Fig. 3).

Anterior swim bladder deflation was observed in all of the treatment group including control group. However incidence rate of swim bladder deflation in PFCAs treatment groups was higher than control group except 0.3 mg/L of PFTrDA exposed group (Fig. 4). There was no dose-dependent response in swim bladder inflation status. Although PFOA and PFTrDA did not have any effect on their body length (Fig. 5A), PFUnDA exposed group showed decreasing trend in their relative body length (p for trend=0.05). In relative size of yolk sac (Fig. 5B), all of PFCAs significantly increased yolk sac size and their increasing trend was also significant (p=0.04 for PFOA, p=0.011 for PFUnDA and p=0.001 for PFTrDA). Exposure to PFCAs induced significant decrease trends in their eye size (Fig. 3C). Significant decrease of eye size was observable in 10, 30 mg/L of PFOA and 0.3 mg/L of PFUnDA. All of the PFTrDA exposed groups showed significant decrease in eye size regardless of exposed concentrations. There were no significant changes in swim bladder size of all PFCAs treatment groups.

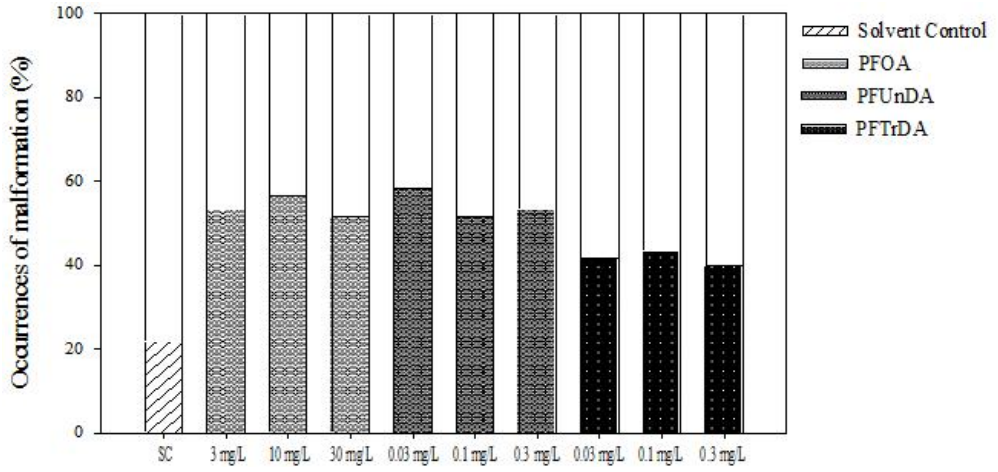


Figure 3. Occurrences of malformation 5 dpf zebrafish exposed to PFOA, PFUnDA and PFTrDA. Each treatment groups consisted of 4 replicates and each replicates included 5 larvae.

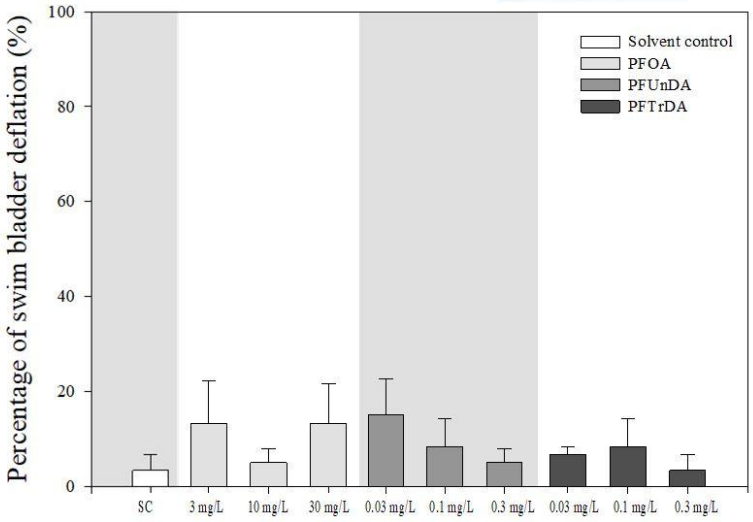


Figure 4. Percentage of swim bladder deflation of 5 dpf zebrafish exposed to PFOA, PFUnDA and PFTrDA. The results are shown as the mean ± SEM of 4 replicates and each replicates included 5 larvae.

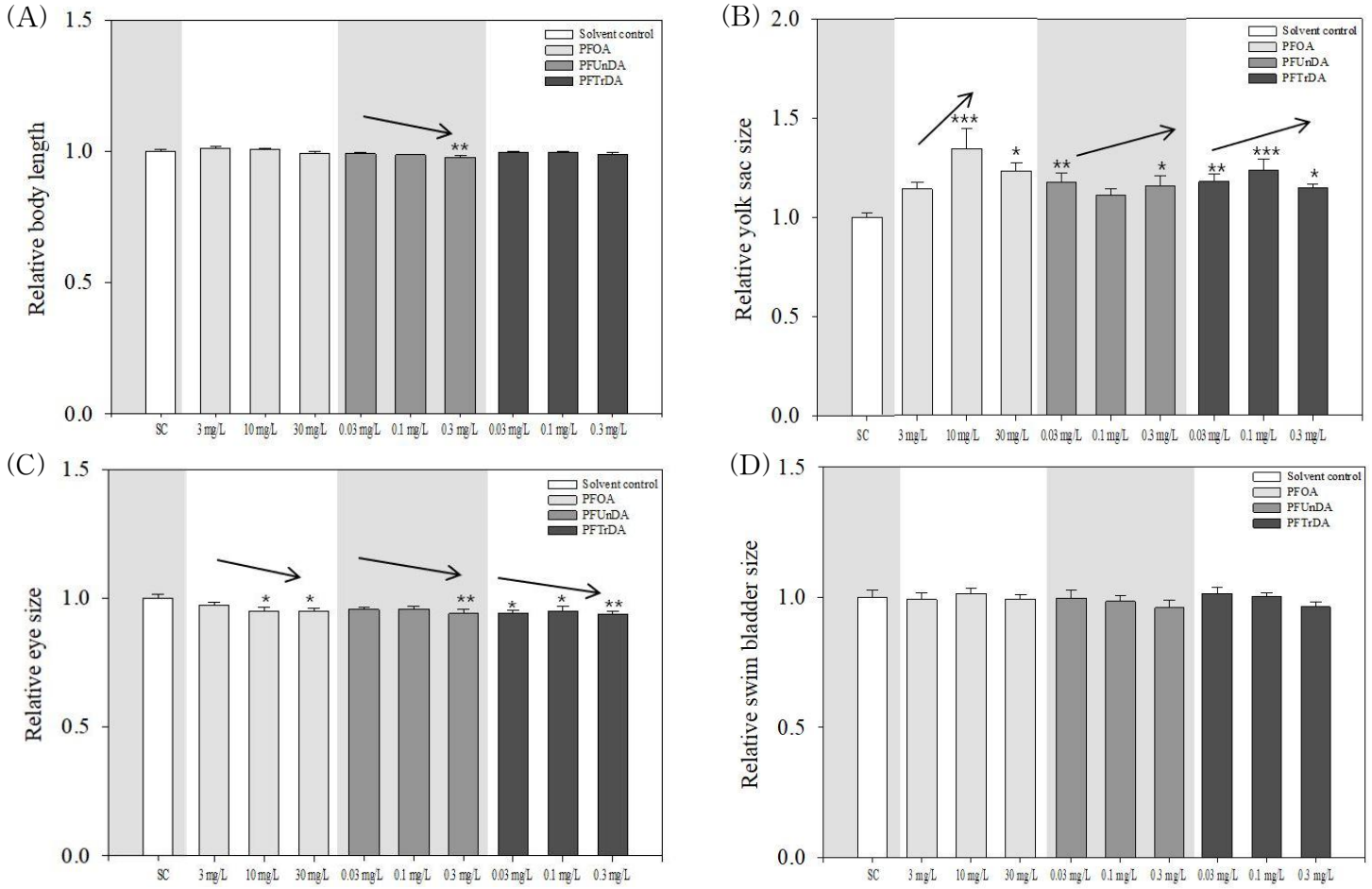


Figure 5. Relative (A) body length, (B) yolk sac area, (C) eye size and (D) swim bladder size of zebrafish until 5 dpf exposure to PFOA, PFUnDA and PFTrDA. The results are shown as the mean ± SEM of 4 replicates and each replicates included 5 larvae. Asterisks(∗) indicate significant differences (∗$p < .05$, ∗∗$p < .01$, ∗∗∗$p < .001$) from solvent control (0.005% EtOH) by ANOVA analysis. Each arrow indicates significant trend by Spearman's rank correlation analysis.

4. Discussion

According to the gene expression changes in GH3 cells and the zebrafish embryo/larvae, long chain PFCAs (C>10) exposure can also disrupt thyroid hormone regulation system, similar to other PFASs (Chang et al., 2009; Martin et al., 2007; Yu et al., 2009; Zhang et al., 2018). In addition, the results of the present study suggest that the disruption potency and mode of action (MoA) of these long chain PFCAs might be different depending on their carbon chain length.

4.1. Thyroid disrupting potentials of long chain PFCAs in GH3 cell

In GH3 assay, longer chain PFCAs showed more fold changes in the transcripts for several key genes. Following exposure to the same dose of 10 uM, PFTrDA showed more severe upregulation of *Tshβ* gene than PFUnDA (Fig. 1), while both compounds showed significant upregulation trends in *Tshβ* gene (p = 0.002 for PFUnDA and p=0.009 for PFTrDA). In contrast, PFOA exposure did not show meaningful changes in transcription level of *Tshβ* gene. As expected, T3 induced significant downregulation in *Tshβ* gene. TSH is secreted to blood vessel by pituitary thyrotrophs (Szkudlinski et al., 2002), and play a role in negative feedback of thyroid hormones (Kim et al., 2015). Therefore, upregulation of *Tshβ* gene implies that exposure to PFUnDA and PFTrDA may lead to stimulation of the thyroid hormone synthesis by the thyroid gland.

PFOA exposure led to a downregulating trend of *Dio2* gene (p for trend = 0.037), but the fold change was small (0.8<folds). On the other hand, exposure to PFUnDA upregulated *Dio2* gene expression (p for trend = 0.037). The *Dio2* gene is expressed predominantly in anterior pituitary (Gereben et al., 2008; Larsen, 2009; Murk et al., 2013) and converts T4 to T3 by outer ring deiodination in the peripheral tissue (Gereben et al., 2008). It is interesting that PFUnDA upregulates both *Dio2* and *Tshβ* genes in the pituitary cell, as T3 in the pituitary gland should downregulate *Tshβ* gene expression.

4.2. Thyroid disrupting potentials of long chain PFCAs in zebrafish embryos and larvae

There is lack of the studies about thyroid disrupting effects of long chain PFCAs using *in vitro* and *in vivo* models (Coperchini et al., 2017). The observation of gene expression changes in zebrafish larvae suggests that longer chain PFCAs may have greater relative disturbance potency, and perhaps different mechanisms of thyroid hormone disruption.

Gene expression in zebrafish following exposure to PFUnDA showed similar disrupting mechanism with PFOA. PFUnDA and PFOA exposure did not show significant changes in *tshβ* and *tshr* gene expression. These genes are related to thyroid stimulation to synthesis thyroid hormone. Also other genes related to thyroid development and thyroid hormone synthesis did not show meaningful changes in their transcription level. However significant upregulation was observed in *ugt1ab* (p for trend = 0.000) and *sult1 st5* (p for trend = 0.003) gene in PFOA exposed group. Like PFOA, upregulation of *ugt1ab* gene was also observed in PFUnDA treatment group. This gene is related to glucuronidation and excretion of T4 from liver. Glucuronidation is the first step of the enterohepatic cycle of metabolism of thyroid hormone (Murk et al., 2013; Wu et al., 2005). These trends in gene expression imply that exposed to PFOA and PFUnDA could accelerate the elimination of thyroid hormone through glucurinudation. According to the previous study, PFOS also promotes the activity of UDP-glucuronosyltransferases (UGT1A1) (Yu et al., 2009). Thus, Zhang et al, found the upregulation of *ugt1ab* gene in zebrafish larvae by following exposure to perfluorododecanoic acid (PFDoDA) untill 4 dpf (Zhang et al., 2018). According to the previous studies and present study, inducing glucuronidation activity could be an important response of PFASs exposure. Thyroid hormone elimination tendency in gene expression of zebrafish larvae also can be connected to morphological response. PFOA and PFUnDA exposure induced significant decrease in eyeball size and increase of yolk size (Fig. 3). Swim bladder deflation (Fig. S1) was also observed. All of these morphological changes indicate thyroid hormone reduction (Bohnsack and Kahana, 2013; Heijlen et al., 2014; Houbrechts et al., 2016; Godfrey et al., 2017; Zheng et al., 2011). Thyroid disrupting potential of PFUnDA have also been suggested in

epidemiological studies. PFUnDA level is negatively related to thyroid hormone level in serum of target population. Wang et al. reported that high level of PFUnDA in serum was associated with lower free T4 and total T4 concentrations among the pregnant women in their third trimester. Also, total T3 and total T3 levels in cord serum were low as well (Wang et al., 2014). Berg et al. showed negative association between PFUnDA and Free T3 (Berg et al., 2015). Other genes that related to thyroid hormone metabolism, all of PFCAs upregulated the expression of *dio1* gene. *dio1* gene expression was not significant in GH3 assay, while in zebrafish, they showed significant upregulation. Liver is the major organ to synthesis DIO1 (Murk et al., 2013). Thus zebrafish, *in vivo* assay result is more persuasive than *in vitro* assay using by pituitary cell line, GH3. For *dio2* gene, PFUnDA and PFOA did not showed meaningful changes compared to PFTrDA expsure. Following exposure to PFOA and PFUnDA, *trβ* showed more severe decreasing trends then *tra*. This tendency also found in previous study using PFDoDA (Zhang et al., 2018). They found that the gene expression of 4 dpf zebrafish exposed to 1.2 and 6.0 mg/L of PFDoDA until 4 dpf led to significant down regulation of *trβ*. But *tra* was significantly down regulated only in 6.0 mg/L of PFDoDA. PFCAs may have more strong effect on *trß* then *tra*. For the transport of thyroid hormone, Only PFOA showed significant up-regulation trend in *ttr*. Other PFCAs also showed increasing trends, but there were no statistical significances. This response seems to increase the transfer efficiency of thyroid hormone to other tissue that thyroid hormone is needed.

PFTrDA showed different pattern in gene expression that can explain thyroid hormone disruption mechanism and potency compared to PFUnDA. Unlike PFOA and PFUnDA, PFTrDA significantly upregulated not only the genes that related to thyroid stimulation (*tshβ, tshr*) but also other genes that related to thyroid development and hormone synthesis (*nkx2.1, nis, mct8*). Especially, steep upregulation was observed in *nis* and *tpo*. NIS is the membrane glycoprotein located in basolateral side of thyroid gland, their function is to concentrate the iodide to uptake and synthesis thyroid hormone (Dohan et al., 2003; Murk et al., 2013; Lee et al., 2017). Through these results, exposure to PFTrDA may promote iodide uptake and coupling of iodide to the thyroglobulin to synthesis thyroid hormone. However, gene that related to thyroid hormone metabolism, *dio2* gene was upregulated especially in PFTrDA treatment group. DIO2 is

related to ORD of T4. So exposure to PFTrDA may promote thyroid hormone to more active form. Being active form of thyroid hormones can be eliminated them more easily (Murk et al., 2013). For this reason, thyroid hormone reduction was expected due to PFTrDA exposure. Also in morphological observation, we found the responses that related to thyroid hormone reduction. Furthermore, the results of gene expression of GH3 which is free from biological regulation of hormone in *in vivo* level indicated the thyroid hormone reduction effect of PFTrDA. Therefore, the upregulation of genes that related to thyroid hormone synthesis may show negative feedback response of thyroid hormone regulation system. Not only the results of present study, previous reports of epidemiology studies also found the negative relationship between PFTrDA and thyroid hormone level like PFUnDA. In normal population in Korea, PFTrDA level was negatively correlated to the total T4 and positively correlated with TSH level (Ji et al., 2012). Maternal PFTrDA level also negatively correlated to fetal total T3 and total T4 (Kim et al., 2011). Exposure to PFUnDA and PFTrDA showed tendency that lowering T4 and T3 level in population. And this corresponds with present study results.

Many studies including the present study report that the toxicity of PFASs increases with their carbon chain length, possibly due to their longer biological half-life. Exposure to PFCAs may led to increasing internal concentration and eventually reach to the saturation thresholds (Buck et al., 2011; Goecke-Flora and Reo, 1996; Gomis et al., 2018; Kim et al., 2013). Our observations of higher disruption potency of PFTrDA than PFUnDA in gene expression in both GH3 cell and zebrafish larva also show the same trend. It is noteworthy that the exposure concentration range of PFOA is two orders or magnitudes higher than those of PFUnDA or PFTrDA in zebrafish embryolarval exposure (Fig. 2).

Even though thyroid hormones were not measured in the present study, several morphological endpoints that are related to certain extent to thyroid hormones also support that these long chain PFCAs may affect thyroid hormone balances. Following the exposure, all PFCAs showed significant decrease in eyeball size, increase in yolk sac size (Fig. 3), and swim bladder deflation (Fig. S1). Eyeball size is related to thyroid hormone disruption. Thyroid hormone deficiency and decrease of eyeball size have been reported in literature (Bohnsack and Kahana, 2013; Heijlen et al., 2014), even though decreasing eyeball size related with

excessive synthesis of thyroid hormone was also reported (Houbrechts et al., 2016). The number of larvae that did not normally formed swim bladder was increased in all of exposed group (Fig S2). Deflation of swim bladder may occur because of decline in thyroid hormones that results in a decline in the production of surfactant proteins. Less surfactant protein leads to lowered surface tension of the swim bladder and hence deflation (Godfrey et al., 2017; Zheng et al., 2011). Therefore, the swim bladder deflation status supports the thyroid hormone reduction potentials of PFOA, PFUnDA and PFTrDA. During early development, thyroid hormone activity is also important in the transition period that yolk sac absorption to exogenous feeding of fish (Vergauwen et al., 2018). Yolk sac size change could suggest that the disruption may be occurred in transporting the essential proteins from yolk sac for growth and also it indicates the development of edema due to fluid accumulation (Jantzen et al., 2016). For these reasons, those morphological changes can explain the reduction of thyroid hormone in developing zebrafish.

5. Conclusion

PFUnDA and PFTrDA showed similar disrupting effects on gene expression of rat pituitary cell. However for the *in vivo* level, using by gene expression assay of developing zebrafish larvae, PFUnDA and PFOA tends to have similar disrupting mechanism. But PFTrDA showed different disrupting effects. Even though thyroid hormones were not measured in the present study, gene expression and morphological changes of zebrafish larvae indicate thyroid hormone reduction potency of PFUnDA and PFTrDA. Also the results suggest the toxicity of PFCAs increased with their chain length. For understanding more about the thyroid related effects, long term study is required to identify the long term effect of PFUnDA and PFTrDA.

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Supplementary Information

Table S1. Primer sequences of GH3, zebrafish used in this study

Gene			Primersequences(5′-3′)		Accession no.
			Forward	Reverse	
GH3	*Cyclophilin*	*housekeeping gene*	tctgagcactggggagaaag	atgccaggacctgtatgctt	M19533.1
	Tshβ	*thyroid stimulating hormone β*	acagaacggtggaaataccg	tctgtggcttggtgcagtag	NM_013116.2
	Tra	*thyroid receptor hormone a*	tatcacttgtgagggctgca	cacagcgatgcacttcttga	NM_031134.2
	Trβ	*thyroid receptor hormoneβ*	atgttttgtgagctgccctg	catgcccaggtcaaagatcg	J03933.1
	Dio1	*deiodinase type 1*	gtggtggtggacacaatgcag	ttgtagttccaagggccaggttta	NM_021653
	Dio2	*deiodinase type 2*	cagctttctcctagacgcct	gcaaagtcaagaaggtggca	NM_031720.3
Zebrafish	*18srrna*	*housekeeping gene*	acgcgagatggagcaataac	cctcgttgatgggaaacagt	FJ915075
	tshr	*thyroid stimulating hormone receptor*	gcgccaaccctttctgtat	ctcgtttgctcctgtttgct	EF487539.1
	tshb	*thyroid stimulating hormone*	gcagatcctcacttcacctacc	gcacaggtttggagcatctca	AY135147
	slc5a5	*sodium-iodide symporter*	ggtggcatgaaggctgtaat	gatacgggatccattgttgg	NM_00108391.1
	tpo	*thyroid peroxidase*	gatcatcacccgtctcctttc	tcctgctcgacttctcctttc	XM_017351696.1
	tg	*thyroglobulin*	ttgctctgtggttcaaagcc	agtcggtgttgctcagaaga	KU662327.1
	nkx2.1	*NK2 homeobox-1*	aggacggtaaaccgtgtcag	caccatgctgctcgtgtact	BC162296.1
	mct8	*monocarboxylate transporter8*	tgatgtccatgatgattcc	ccataatcgtgatgaaacag	NM_001258230
	tra	*thyroid receptor hormonea*	aagtggatataatccgaacataggc	acgccaatgccacttcctt	XM_005163986.4
	trβ	*thyroid receptor hormoneβ*	tgggagatgatacgggttgt	ataggtgccgatccaatgtc	NM_131340

dio1	*deiodinase type 1*	gttcaaacagcttgtcaaggact	agcaagcctctcctccaagtt	BC076008
dio2	*deiodinase type 2*	ttctccttgcctcctcagtg	agccacctccgaacatcttt	NM_212789.3
ugt1ab	*uridine diphosphate glucuronosyltransferase*	ccaccaagtctttccgtgtt	gcagtccttcacaggcttc	NM_213422
sult1 st5	*cytosolic sulfotransferase*	gtgcgcatgccgtttttaga	cgggccacatatataaccttgc	KU662327.1
ttr	*transthyretin*	gcacaacttgatcacggagc	tgtggtgtacgagaaaggc	NM_001005598.2

Fig S1. Example of morphological change measurement using by an image processing program, ImageJ®.

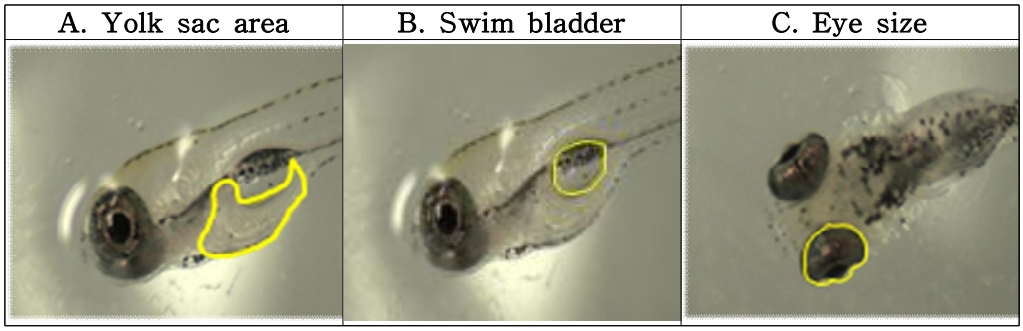
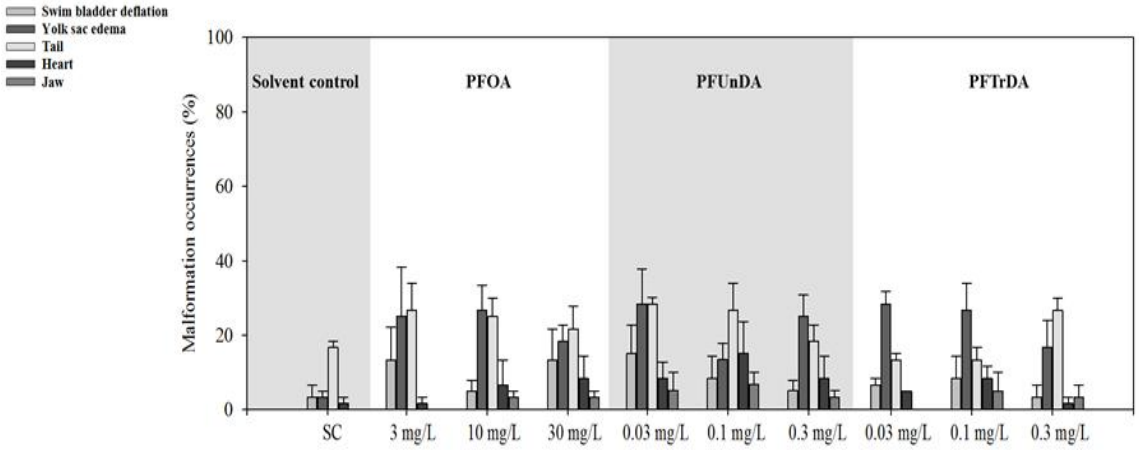


Fig S2. Malformation occurrences of zebrafish exposed to PFOA, PFUnDA and PFTrDA.



국문 초록

GH3 세포주와 zebrafish (*Danio rerio*) embryo, larvae를 이용한 perfluoroundecanoic acid와 perfluorotridecanoic acid의 갑상선 호르몬 교란 영향 연구

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과불화화합물은 열과 화학적 반응에 매우 안정적이어서 섬유산업, 제지산업, 음식포장제 등 다양한 생활용품에 사용되고 있다. 이들은 내분비계 교란을 포함하여 다양한 건강 영향이 보고되고 있어 최근 선진국을 중심으로 규제가 이루어지고 있다. 그 결과, 환경과 인체시료에서 최근 규제대상 과불화화합물의 검출수준은 감소하는 경향을 보이고 있다. 하지만 이들을 제외한 긴 사슬 과불화합물은 긴 잔류성을 지니고 있음에도 불구하고 일부 물질은 대체물질로 사용이 되어 환경과 인체시료에서 꾸준한 검출 수준을 보이고 있으며 심지어는 증가하는 경향을 보인다. 이들은 개체 출생 이전 초기 발달 시기부터 태반을 통해 모체에서 태아로 전달이 되는 특징을 가지며 또한 출생 이후 모유를 통하여 계속적인 노출이 이루어진다. 발달시기의 갑상선호르몬의 적절한 수준 유지는 개체의 정상적인 발달에 필수적인데 규제 대상 물질의 갑상선 교란 영향을 다룬 연구와 비교하여 긴 사슬 과불화화합물의 갑상선 교란성에 대한 연구는 매우 부족한 실정이다.

따라서 본 연구에서는 랫드의 뇌하수체 세포주인 GH3와 제브라피쉬 수정란을 이용하여 기존 규제대상 물질인 PFOA와 일부 긴 사슬 과불화화합물인 PFUnDA와 PFTrDA가 갑상선 조절체계에 미치는 영향과 그 기전을 비교, 파악하고자 하였다. GH3 세포주에서는 갑상선호르몬의 생성 조절에 관여하는 유전자의 발현변화를 관찰 하였고, 제브라피쉬 수정란은 각각의 과불화화합물을 120시간 동안 노출하여 갑상선의 성숙, 자극, 호르몬 합성, 호르몬 수용체, 대사에 관여하는 유전자들의 발현 수준을 관찰하였다. 그리고 이 유전자 발현을 반영하는 결과라 할 수 있는 형태학적 변화 관찰을 통하여 세포 수준에서의 교란성과 초기 발달 개체에서 발생하는 갑상선 교란성을 파악하였다.

GH3에서 PFUnDA와 PFTrDA는 갑상선호르몬 합성을 촉진하는 반응과 관련된 *Tshβ* 유전자와 갑상선호르몬의 활성화와 관련된 *Dio2* 유전자의 발현을 증가시켰다. 하지만 PFUnDA는 제브라피쉬의 수정란/치어의 유전자발현 분석 결과에서 PFOA와 유사하게 갑상선호르몬의 대사와 관련된 *ugt1ab* 유전자를 유의하게 증가시키는 경향이 나타났다. 그리고 PFOA, PFUnDA와 달리 PFTrDA는 치어의 갑상선호르몬 합성 (*tshβ, nis, nkx2.1, tpo, mct8*), 제거 (*dio1, dio2*)와 관련된 유전자를 유의하게 증가시켰다. 제브라피쉬 치어의 형태학적 변화에서 PFOA, PFUnDA, PFTrDA에 노출된 치어들은 모두 안구 크기가 유의하게 감소하였고, 난황낭의 크기가 유의하게 증가하였다. 그리고 모든 노출 군에서 부레가 정상적으로 팽창하지 않은 개체 수가 증가하였다. 이 형태학적 변화들은 모두 갑상선호르몬 감소를 의미하는 대표적인 변화로 본 연구에서 관찰한 형태학적 변화를 통해 갑상선호르몬의 감소를 확인할 수 있었다. 또한 탄소 사슬 수가 증가하면 독성이 비례하게 증가한다는 기존의 과불화화합물 연구결과와 동일하게 본 연구에서도 GH3와 제브라피쉬 치어의 유전자 수준 변화를 통해 PFCAs의 갑상선 교란 정도가 구조 내 탄소 사슬 수와 비례하게 증가하는 경향을 확인할 수 있었다.

본 연구를 통하여 긴 사슬 과불화화합물인 PFUnDA와 PFTrDA의 초기 발달단계와 세포 수준에서의 교란성을 확인하였다. 이러한 변화가 이후 성장 단계에서 미칠 수 있는 영향을 확인할 연구가 추후 필요할 것으로 보인다.

주요어: 긴 사슬 과불화화합물, PFUnDA, PFTrDA, GH3 세포주, 제브라피쉬, 갑상선호르몬, 형태학적 변화, 내분비계교란

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