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Effects of triphenyl phosphate on thyroid function and related mechanisms in zebrafish (*Danio rerio*) embryos/larvae and GH3 cells

유기인계 난연물질 triphenyl phosphate가 수서생물과 세포주 모델에서 갑상선 기능 및 관련기전에 미치는 영향 연구

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ABSTRACT

Effects of triphenyl phosphate on thyroid function and related mechanisms in zebrafish (*Danio rerio*) embryos/larvae and GH3 cells

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Triphenyl phosphate (TPP), one of the widely used organophosphorus flame retardants (OPFRs), has been frequently detected in environment and biota. However, knowledge on its toxicological effects is limited. The present study was conducted to investigate the thyroid hormone disruption potential of TPP and its underlying mechanisms using both *in vivo* and *in vitro* models. Zebrafish embryos/larvae were exposed to TPP until 7 days post-fertilization (dpf) to determine the changes in thyroid hormones and related gene transcriptions. After 7 d of zebrafish embryo/larval exposure, TPP significantly increased whole-body thyroid hormones, i.e., T3 and T4, concentrations. The up-regulation of the genes related to thyroid hormone synthesis (*slc5a5*, *tg*, and *tpo*) might be responsible for the increased whole-body T4 concentrations. TPP exposure also led to significant up-regulation the mRNA expression related to metabolism (*deio1*), transport (*ttr*), and elimination (*ugt1ab*) of thyroid hormones. Down-regulation of *crh* and *tshb* gene transcriptions indicates central regulatory feedback against increased

levels of thyroid hormones in zebrafish larvae. To understand the direct interaction of TPP with central regulation, GH3 cell assay using rat pituitary gland cell was performed. In GH3 cells, TPP up-regulated the transcription of *tshβ*, *trα*, and *trβ* genes. TPP may interact with pituitary cells and increase *tshb* gene transcription as shown in GH3 cells, which was different from the observation in the fish larvae *in vivo*. The expressions of both *deio1* and *deio2* mRNA were not altered. Our observations clearly showed that TPP increases the thyroid hormone concentrations in early life stages of zebrafish through affecting the thyroid hormone synthesis, metabolism, transport, and elimination pathways. Later life implications of the thyroid hormone disruption deserve further investigations.

Keywords : organophosphate flame retardants; endocrine disruption; hypothalamic-pituitary-thyroid (HPT) axis

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1. Introduction

Polybrominated diphenyl ethers (PBDEs) are most widely used flame retardants in a variety of consumer products, such as carpets, textile, polyurethane foams, plastics, electronic devices and construction materials (Darnerud, 2003). However, due to their persistence in environment and potential adverse health effects, many PBDEs have been restricted in European countries or voluntarily phased out in North America (Betts, 2008). Recently, PBDEs are newly added to the list of the Stockholm Convention on persistent organic pollutants (POPs)(UNEP, 2011). In response to the global restrictions on the use and production, many alternative flame retardants have been introduced, and organophosphorus flame retardants (OPFRs) are one such example.

Triphenyl phosphate (TPP) is one of the most frequently detected OPFRs in environment and biota. e.g., air, surface water, waste water, fish lipid, human milk (Andresen et al., 2004; Loraine and Pettigrove, 2006; Meyer and Bester, 2004; Sjödin et al., 2001; Sundkvist et al., 2010). In Canada, TPP were detected in drinking water at concentrations ranging between 0.3 and 2.6 ng/L (LeBel et al., 1981). This compound has been also detected in indoor house dust samples from Japan, Belgium, Sweden, and Philippines (Kanazawa et al., 2010; Kim et al., 2013; Marklund et al., 2003; Van den Eede et al., 2011), and the detection frequencies was up to 98% in the U.S.A.(Stapleton et al., 2009).

Most organophosphorus esters including TPP are known to exert neurotoxicity (Johnson, 1975), however its endocrine disrupting effects are less studied and hence related mechanisms are not well documented. TPP disrupts the sex hormone balance in both zebrafish and a human adrenal cell line (Liu et al., 2012; Liu et al., 2013c). In adult men, Meeker and Stapleton (2010) showed the association between TPP concentrations in the house dust

and a decrease of thyroid hormone level, implying its thyroid disrupting potentials in human males. Exposure to TPP also altered the transcriptional levels of thyroid hormone receptor α (TRα) in zebrafish larvae (Liu et al., 2013a).

Thyroid hormones (THs) play an essential role in development, growth, and energy metabolism, and they are particularly important in an early life stage of organism. The thyroid system is effectively regulated by a complex regulatory network along the hypothalamus–pituitary–thyroid (HPT) axis. Thyroid hormone balance in the body could be disrupted through many pathways, e.g., central regulation, thyroid hormone synthesis, transport, metabolism, and elimination (Murk et al., 2013; Schmutzler et al., 2007). For this reason, the mechanisms of thyroid hormone disruption could be best understood by employing both *in vivo* and *in vitro* studies (Gentilcore et al., 2012; Guo and Zhou, 2013).

The aim of this study is to understand the thyroid hormone disruption potential of TPP and its underlying mechanisms using both *in vivo* and *in vitro* models. For this purpose, zebrafish embryo/larvae and rat pituitary GH3 cell line were employed. The information obtained in the present study will help better understand the mechanisms of thyroid disrupting effects and adverse early life stage effects of TPP.

2. Materials and Methods

2.1. Chemicals

TPP (CAS No. 115-86-6) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was used as solvent in both *in vitro* and *in vivo* study. The final concentrations of solvent in the exposure media were set at 0.1% (v/v) for GH3 cell assays, and 0.005% (v/v) for zebrafish embryo/larval exposure, respectively.

2.2. Zebrafish culture and embryo/larval exposure

Zebrafish eggs were obtained from a commercial supplier (Gangnam Aqua, Suwon, Korea). On arrival in the laboratory, the embryos were examined under a stereo microscope for normal development, individually. The embryos < 3 h post-fertilization (hpf) were randomly placed into 1L glass beakers containing 800 mL of TPP exposure solution (0, 40, 200, or 500 μg/L). Each treatment of control contains six replicates. The culture medium and treatment solutions were prepared in the conditioned water which was manufactured with sodium bicarbonate (7.5 g), calcium sulfate (1.5 g), and sea salt (0.84 g) in Millipore water (100 L) (Nusslein-Volhard and Dahm, 2002). The exposure concentrations were determined by preliminary range-finding study. Throughout the 7 d exposure, circa 50% of the exposure solution was renewed daily. After hatching, the zebrafish larvae were fed with with freshly hatched *Artemia nauplii* (<24 h after hatching) and Hatchfry Encapsulon™ (Argent Chemical Laboratories, Redmond, WA, USA). *Artemia* hatched were homogenized before feeding to help easy catching. The exposure was performed in a temperature-controlled room (25±1 ℃) under a photoperiod of 14:10 h light:dark. Water quality parameters, including pH, conductivity,

temperature, and dissolved oxygen, were measured on regular basis.

On day 7 after fertilization, embryo and larval survival, hatchability, malformation rate and body weight were observed. 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. Body weight (mg) of 10 larvae was measured per replicate. For performing analysis of gene transcription and TH assays, larvae were randomly sampled at 7 day post-fertilization (dpf) and stored at -80 ℃

2.3. GH3 cell culture and exposure

Rat pituitary GH3 cells were obtained from Korean Cell Line Bank (Seoul, Korea). The cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture (DMEM/F12) (Sigma), supplemented with 10% fetal bovine serum (FBS; Gibco, Paisley, U.K.). The cells were maintained at 37 ℃ in a humidified atmosphere of 5 % CO2.

GH3 cells at a density of 2.5 x 10^5 cells/well were seeded and incubated in 24-well plates for 20 h. 4 h prior to the exposure, the growth medium was replaced with serum-free medium supplemented with 1% BD ITS+ premix, 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). The medium replacement was conducted to ensure depletion of steroid hormones and growth factors in the GH3 cells. The cells were dosed with various concentrations of TPP (0, 1, 10, 100 μg/L) and a positive control 3,5,3'-triiodothyronine (T3, 1.5 μg/L). Each treatment incubated three replicates, and exposure duration was 48 h. Quality assurance test was

performed using various concentrations of T3 (0, 0.0015, 0.015, 0.15, 1.5 μg/L) in four replicates. To avoid influence of cytotoxicity on GH3 cells, cell viability was determined by use of WST-1 reagent (Roche Applied Science, Indianaplis, IN) according to the manufacturer's protocol. Only the non-cytotoxic doses (> 80% of cell viability compared with that of solvent control) were used for evaluation of effects on gene transcription.

2.4. RNA isolation and quantitative real-time polymerase chains reaction (qRT-PCR)

20 larvae per replicate were homogenized for RNA isolation. Total RNA was extracted using RNeasy mini kit (Qiagen, Valencia, CA, USA). RNA quality and concentration were determined by the use of a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Welmington, DE, USA). Complementary DNAs (cDNAs) were synthesized from diluted RNA (a working concentration of 100 ng/μL) using iScriptTM cDNA synthesis kits (BioRad, Hercules, CA, USA), following manufacturer' s instruction. For quantitative real-time PCR (qRT-PCR), the 20 μL of qRT-PCR mix reaction mix consisted of 10 μL of LightCycler-DNA Master SYBR Green I mix (Roche Diagnostics Ltd, Lewes, UK), 1.8 μL of each PCR primer (10 pmol), 4.4 μL of purified PCR-grade water, and 2 μL cDNA diluted 1:4 with water. qRT-PCR was performed using LightCycler 480 (Roche Applied Science, Indianapolis, IN, USA). The thermal cycle profile was : preincubation at 95 ℃ for 10 min, 40 cycles of amplification at 95 ℃ for 10 s, 85 ℃ for 20 s, and 72 ℃ for 20 s. The fluorescent signals were measured at the annealing/extension step. Data were processed by Roche LightCycler 480 software version 1.5 and analyzed using Microsoft Excel 2010.

Primer sequences for selected genes and a housekeeping gene (*cyclophilin*

and *18s rrna*) are listed in Table 1. RT-PCR was performed for six replicate samples. For quantification of PCR results, the threshold cycle (Ct) was determined for each reaction. Ct values for each gene of interest were normalized to the housekeeping gene by use of the ⊿⊿Ct method (Livak and Schmittgen, 2001).

Table 1. Primer sequence for the quantitative RT-PCR analysis used in this study.

	Target gene name	Sense primer (5' -3')	Antisense primer (5' -3')
Zebrafish	*18s rrna*[a]	acgcgagatggagcaataac	cctcgttgatgggaaacagt
	crh[b]	ttcgggaagtaaccacaagc	ctgcactctattcgccttcc
	tshβ[c]	gcagatcctcacttcacctacc	gcacaggtttggagcatctca
	tshr[d]	gctccttgatgtgtccgaat	cgggcagtcaggttacaaat
	nkx2.1[e]	aggacggtaaaccgtgtcag	caccatgctgctcgtgtact
	pax8[c]	gaagatcgcggagtacaagc	ctgcactttagtgcggatga
	hhex	ctcctctttcaccagcctga	tggatgaacggagaccacag
	tg[c]	ccagccgaaaggatagagttg	atgctgccgtggaatagga
	tpo[b]	gcgcttggaacacagtatca	cttcagcaccaaacccaaat
	slc5a5	cgtctccaccagcatcaatg	cagtccagcagagaggagag
	deio1[c]	gttcaaacagcttgtcaaggact	agcaagcctctcctccaagtt
	deio2	ttctccttgcctcctcagtg	agccacctccgaacatcttt
	ttr[c]	cgggtggagtttgacacttt	gctcagaaggagagccagta
	ugt1ab[c]	ccaccaagtctttccgtgtt	gcagtccttcacaggctttc
	trα[a]	caatgtaccatttcgcgttg	gctcctgctctgtgttttcc
	trβ[b]	tgggagatgatacgggttgt	ataggtgccgatccaatgtc
GH3 cells	*cyclophilin*	tctgagcactggggagaaag	atgccaggacctgtatgctt
	tshβ	cagcattaactcgccagtgc	tggtggtgttgatggtcagg
	trα	tatcacttgtgagggctgca	cacagcgatgcacttcttga
	trβ	atgttttgtgagctgccctg	catgcccaggtcaaagatcg
	deio1[e]	gtggtggtggacacaatgcag	ttgtagttccaagggccaggtt ta
	deio2	cagctttctcctagacgcct	gcaaagtcaagaaggtggca

[a] Liu et al. (2013a); [b] Yu et al. (2010); [c] Wang et al. (2013); [d] Liu et al. (2013b); [e] Guo and Zhou (2013); other primer sequences were designed using Primer 3 online software ver. 4.0.0 (http://primer3.ut.ee/).

2.5. Thyroid hormone extractions and measurement

T3 and thyroxine (T4) levels were measured by using enzyme-linked immunosorbent assay (ELISA), following the methods of Yu et al. (2010) Wang et al. (2013). Briefly, 100 zebrafish larvae per replicate were homogenized in 300 μL ELISA buffer, using homogenizer. The samples were disrupted by sonication for 10 min on ice, and centrifuged at 5000 x g for 10 min at 4 ℃. The supernatant was collected and stored at −80 ℃ until analysis. The test kits (Cat No. E0453Ge for T3; Cat No. E0452Ge for T4) were purchased from Uscnlif (Wuhan, China) and THs assay was performed following the manufacture's recommendation.

2.6. Statistical analysis

Data normality and homogeneity of variances were analyzed by the Shapiro-Wilk's test and Levene's test, respectively. If necessary, log transformation was performed. Data were analyzed by one way analysis of variance (ANOVA) followed by a Dunnett's t-test. P-values less than 0.05 were considered significant. IBM SPSS 20.0 for Windows (SPSS Inc., Chicago,IL, USA) and Toxstat® 3.5 (Western EcoSystems Technologies, Inc., Cheyenne, WY) were used. All data were expressed as mean ± standard error of mean (SEM).

3. Results

3.1. Zebrafish embryo/larval exposure study

3.1.1. Survival and developmental changes

The effects of TPP on embryo and larval survival, hatchability, malformation rate, and body weight of zebrafish are summarized in Table 2. The malformation rate, such as yolk sac and pericardial edema, significantly increased in the 500 μg/L TPP exposure group. Embryo and larval survival, and the hatchability were not affected by the experimental concentrations, i.e., 40, 200, and 500 μg/L TPP.

Table 2. Effects of TPP on survival, hatchability, morphological rate and body weight in zebrafish until 7 dpf.

	0 μg/L	40 μg/L	200 μg/L	500 μg/L
Embryo survival (%)	94.72±1.05	96.67±0.48	93.75±1.81	92.78±0.56
Larval survival (%)	91.77±0.93	92.49±1.19	92.11±1.14	88.67±1.38
Hatchability (%)	99.70±0.19	99.42±0.43	100.00±0.00	98.07±0.81
Malformation rate (%)	2.95±0.30	4.05±0.68	4.91±0.73	11.61±0.88*
Body weight[a] (mg)	2.27±0.18	2.27±0.06	2.10±0.09	2.22±0.12

[a] Wet body weight of 10 zebrafish larvae per replicate were measured.

The results are shown as mean ± SEM of six replicates. Asterisks ($p^*<0.05$) indicate significant difference to solvent control (0.1% DMSO v/v).

3.1.2. Whole-body thyroid hormones

Following exposure to TPP, both T4 and T3 concentrations were significantly increased in all exposure groups (Fig. 1A-B).

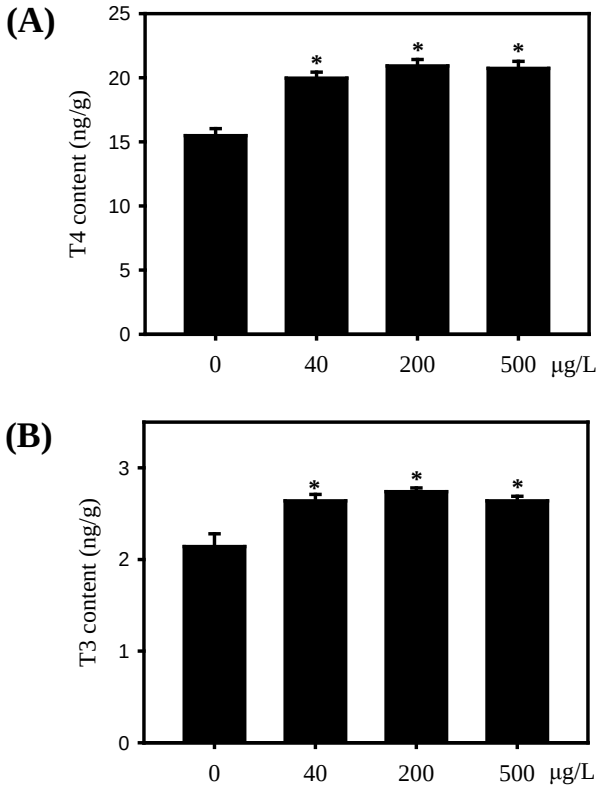
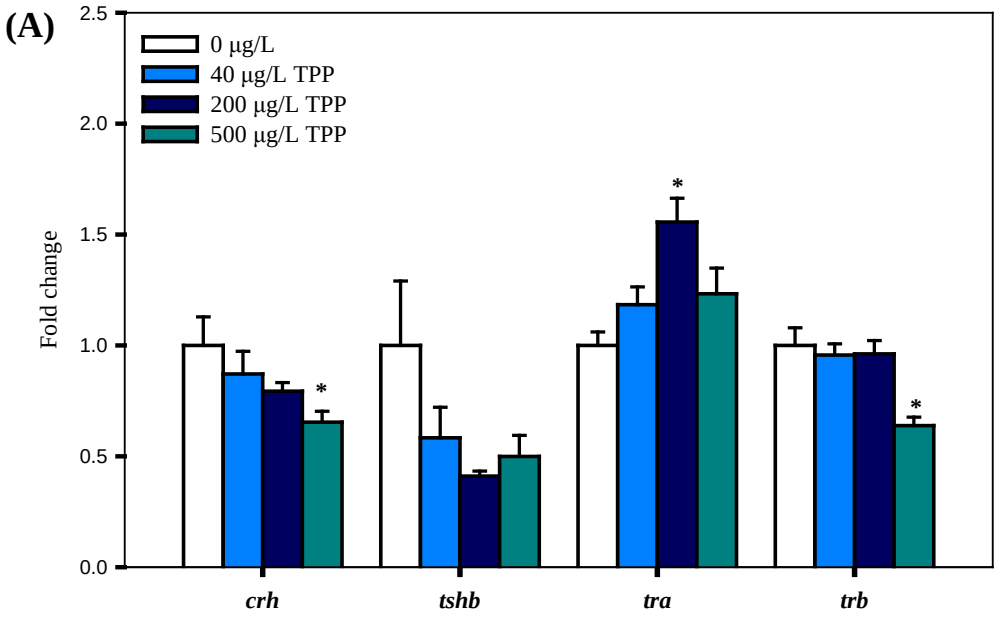
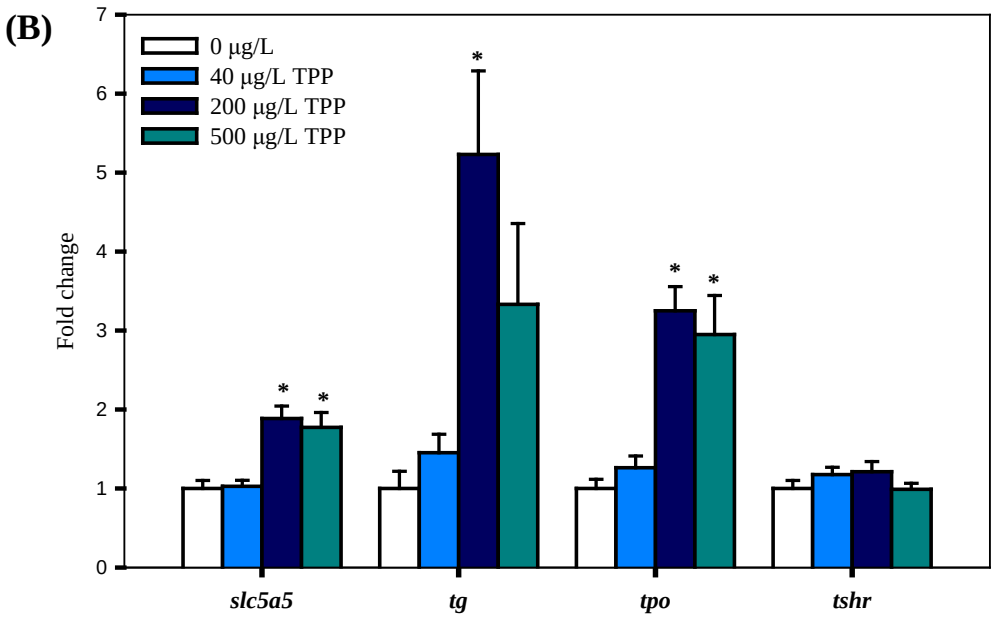


Figure 1. Concentration (ng/g wet weight) of (A) T4, and (B) T3 in whole-body of 7 dpf zebrafish larvae exposed to 0, 40, 200, or 500 μg/L TPP. The results are shown as mean ± SEM of three replicates. Asterisks ($p^{*}<0.05$) indicate significant difference to solvent control (0.005% DMSO v/v).

3.1.3. Transcriptional changes in selected genes related to thyroid hormone regulation

The exposure to TPP influenced the transcription of several genes involved in the thyroid hormone system (Fig. 2A–D). The transcription of *corticotrophin–releasing hormone* (*crh*) gene was significantly down–regulated. In addition, the *thyroid–stimulating hormone beta* (*tshβ*) gene showed decreasing trend but statistical significance was not observed (Fig. 2A). In zebrafish larvae, *trα* gene were significantly up–regulated following exposure to 200 μg/L TPP, but *trβ* gene was down–regulated at 500 μg/L TPP. Transcriptions of *sodium/iodide symporter* (*slc5a5*) and *thyroid peroxidase* (*tpo*) gene in zebrafish larvae exposed to ≥200 μg/L TPP were significantly greater while that of tsh receptor (*tshr*) was not altered (Fig. 2B). The transcription of *NK2 homeobox 1* (*nkx2.1*), *paired box protein 8* (*pax8*), and *hematopoietically expressed homeobox* (*hhex*) genes showed decreasing trend by TPP exposure in zebrafish larvae at 7 dpf, although not statistically significant (Fig. 2C). Exposure to 200 and 500 μg/L TPP significantly up–regulated transcription of *transthyretin* (*ttr*) and *uridine diphosphate glucuronosyltransferase* (*ugt1ab*) gene, respectively (Fig. 2D). Transcription of *deiodinase type 1* (*deio1*) gene was significantly up–regulated in zebrafish larvae exposed to 500 μg/L TPP but that of *deio2* gene showed a down–regulation trend, although not significant.


(A)
0 µg/L
40 µg/L TPP
200 µg/L TPP
500 µg/L TPP
Fold change
2.5
2.0
1.5
1.0
0.5
0.0
*
crh
tshb
tra
trb


(B)
0 µg/L
40 µg/L TPP
200 µg/L TPP
500 µg/L TPP
Fold change
7
6
5
4
3
2
1
0
*
slc5a5
tg
tpo
tshr

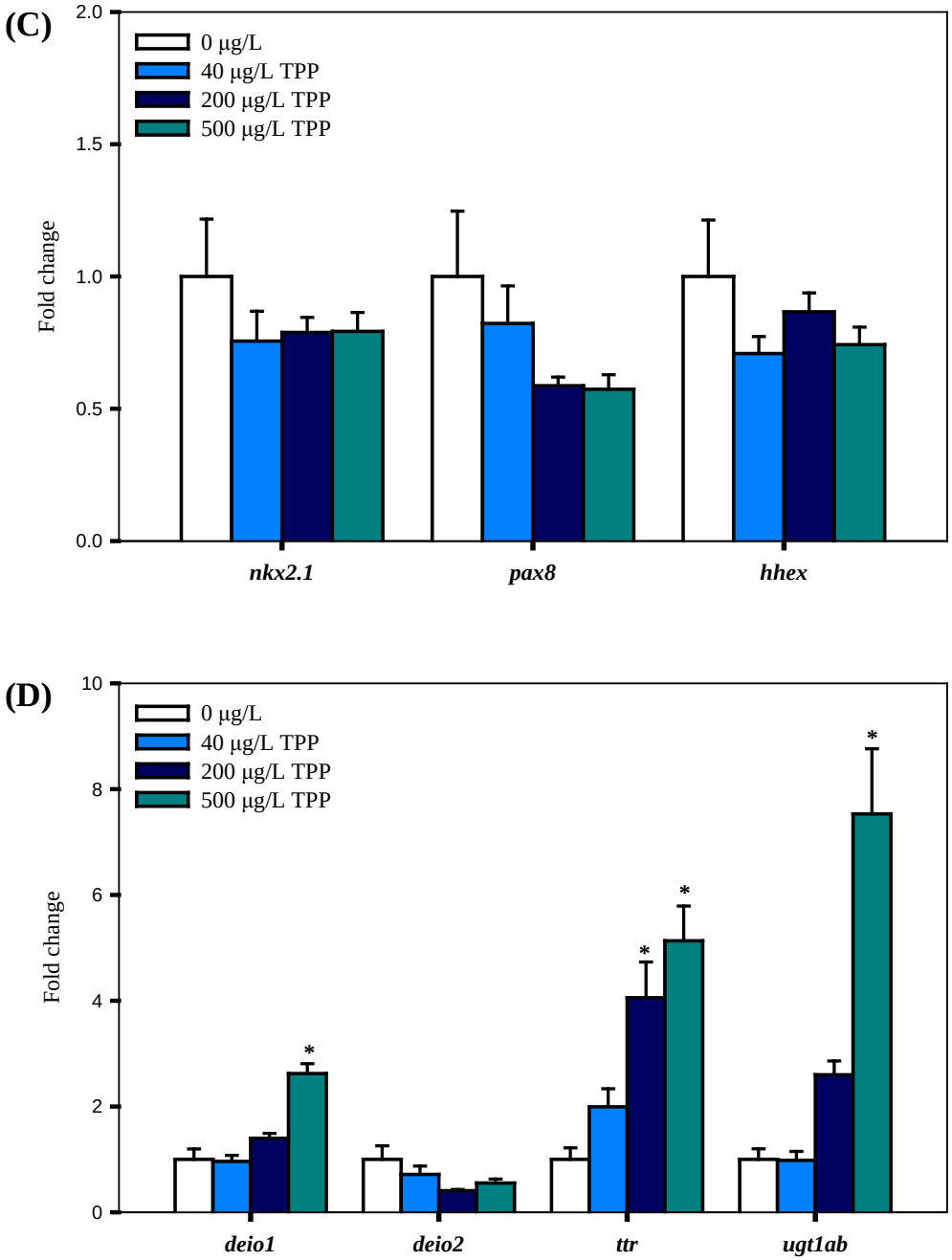


Figure 2. Gene transcription of (A) *crh*, *tshβ*, *trα*, *trβ*, (B) *slc5a5*, *tg*, *tpo*, *tshr*, (C) *nkx2.1*, *pax8*, *hhex*, (D) *deio1*, *deio2*, *ttr*, and *ugt1ab* in whole-body of 7 dpf zebrafish larvae following exposure to 0, 40, 200, or 500 μg/L TPP. The results are shown as mean ± SEM of six replicates. Asterisks ($p^{*}<0.05$) indicate significant difference to solvent control (0.005% DMSO v/v).

3.2. Alterations of gene transcription in GH3 cell

Significant up-regulations of *trα* and *trβ* gene were observed in GH3 cells following exposure to 10 and 100 μg/L TPP, respectively (Fig. 3). In contrast, exposure to T3 led to significant down-regulations of *trα* and *trβ* genes. The gene expression levels of *tshβ* was significantly up-regulated by exposure to 100 μg/L TPP, and down-regulated by exposure to T3, although not significant. Exposure to TPP did not change the transcription of both *deio1* and *deio2* in GH3 cell, while exposure to T3 led to significant up-regulation of both genes.

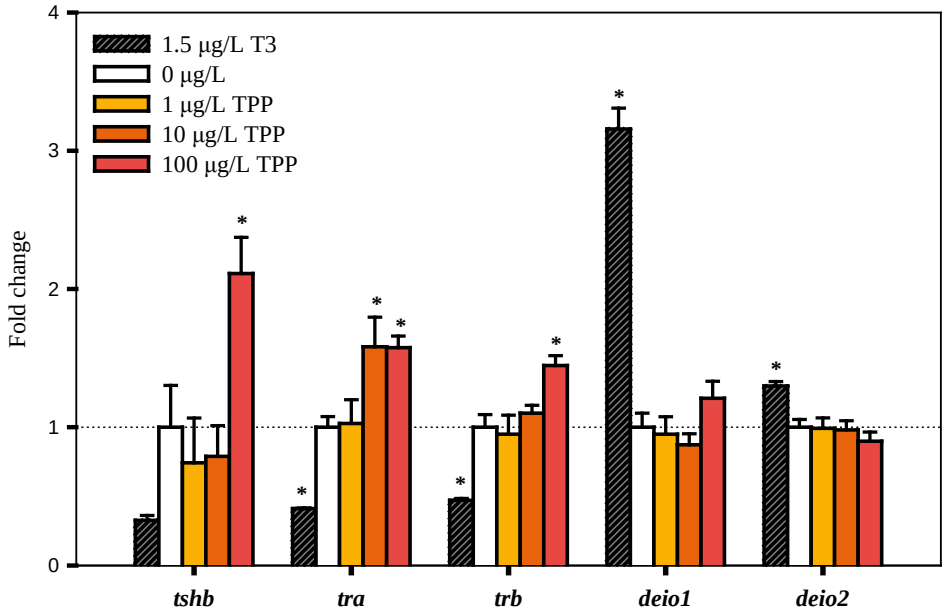


Figure 3. Gene transcriptions in GH3 cells following exposure to 1.5 μg/L T3 as a positive control and 0, 1, 10, or 100 μg/L TPP. The results are shown as mean ± SEM of three replicates. Asterisks ($p^{*}<0.05$) indicate significant difference to solvent control (0.1% DMSO v/v).

4. Discussion

The results of zebrafish embryo/larval exposure demonstrate that TPP alters whole-body thyroid hormone levels as well as gene transcription involved in the thyroid system. Responses in rat pituitary GH3 cell also show the direct interaction of TPP on pituitary gland, supporting thyroid hormone disrupting effects of this compound.

In zebrafish larvae, the significant increase of both T3 and T4 concentrations clearly indicates thyroid endocrine disruption by TPP on early developmental stage of the zebrafish. To our knowledge, this is the first study which reports the thyroid hormone changes of early life stage zebrafish by exposure to TPP. Simultaneous increase of whole-body T3 and T4 levels has been reported in F1 zebrafish after the parental exposure to PBDEs (DE-71) (Yu et al., 2011). In contrast, tris(1,3-dichloro-2-propyl) phosphate (TDCPP), another commonly used OPFR, reduced T4 levels, while T3 levels in zebrafish larvae was notably increased at 144 hpf (Wang et al., 2013). As a reason for decreased whole-body T4, the authors suggested the negative feedback taken place to compensate the increased T3 levels. Our observation of increased T4 levels by TPP exposure, which is different from that of Wang et al. (2013) for TDCPP, suggests that negative feedback mechanisms in HPT axis was not taken place for exposure to TPP. We believe that up-regulation of *slc5a5*, *tg*, and *tpo* genes (Fig. 2B) may be responsible for the increased levels of T4 in whole-body zebrafish. In addition, there is an indication that TPP possesses different modes of toxicity compared to those of TDCPP in zebrafish (Liu et al., 2013a). While exposure to TPP majorly up-regulated the transcription of *peroxisome proliferator-activated receptor alpha* (*PPARα*) and *TRα* genes, TDCPP also affected aryl hydrocarbon receptors (AhR) and estrogen receptor

alpha (ERα) centered gene networks. Although the endocrine disruption of TDCPP and TPP has been investigated and compared in several studies (Liu et al., 2013a; Liu et al., 2013c; Meeker and Stapleton, 2010), the specific mechanisms of each remains to be elucidated.

THs can be primarily regulated by control mechanisms along the HPT axis (Murk et al., 2013). In non-mammalian vertebrates, e.g., amphibians and fish, corticotropin-releasing hormone (CRH) appears to stimulate the secretion of thyroid-stimulating hormone (TSH) (De Groef et al., 2006) and is therefore suggested as a common regulator of HPT axis (Yu et al., 2011). TSH is the primary hormone to regulate growth and function of thyroid gland (MacKenzie et al., 2009). In response to TSH stimulation, thyroid gland secretes mainly T4 and to a lesser extent T3 (Murk et al., 2013). In the present study, exposure to TPP down-regulated the transcription of *crh* and *tshβ* genes (Fig. 2A), which can be interpreted as a compensatory effort of HPT axis in response to the increased whole-body thyroid hormone levels (Fig. 1A-B).

The up-reguation of *slc5a5*, *tg,* and *tpo* genes explains the increased whole-body T4 concentrations in zebrafish larvae (Fig. 2B), because those genes are involved in TH synthesis (Zoeller et al., 2007). Specifically, sodium/iodide symporter (NIS), encoded by *slc5a5*, is required for iodide uptake from the blood into the thyroid follicle cells (Alt et al., 2006). Thyroglobulin (TG) is a substrate containing tyrosyl residues, and thyroperoxidase (TPO) enzyme catalyzes the tyrosyl residues and oxidizes iodine to synthesize THs (Murk et al., 2013).

In fish, the concentration of T3 is primarily influenced by the deiodinase activity of the target organ which converts T4 to T3 in peripheral tissue

(Eales et al., 1999). To date, it has been demonstrated that type 2 deiodinase (Deio2) plays a pivotal role in producing active T3, whereas type 1 deiodinase (Deio1) is involved in both activation and inactivation of THs (Heijlen et al., 2013; Orozco and Valverde-R, 2005). Our observations that TPP exposure significantly up-regulated the transcription of the *deio1* gene, but not that of the *deio2* gene in the zebrafish larvae (Fig. 2D), are in line with the reports in zebrafish embryo/larvae exposed to TDCPP (Wang et al., 2013). In hyperthyroid state, Deio1 mainly catalyzes T3 production while Deio2 activity decreases due to ubiquitination (Maia et al., 2005). Therefore, the up-regulation of *deio1* gene may be responsible for the increased T3 levels in the zebrafish larvae in the present study. In contrast, since the Deio1 also plays a role in elimination of T4 in fish liver (Van der Geyten et al., 2005), the up-regulation of this gene might be a compensatory response against increased T4 levels.

In very early life stage of zebrafish, *nkx2.1, hhex,* and *pax8* genes are expressed in the developing thyroid gland (Rohr and Concha, 2000) and regulate growth and differentiation of thyroid (Elsalini et al., 2003). Seemingly down-regulating trend of *nkx2.1, hhex,* and *pax8* genes by TPP exposure (Fig. 2C), while statistical significance was not detected, suggests developmental retardation of thyroid in the zebrafish larvae by TPP exposure, supporting thyroid disrupting potentials of TPP.

Most of the circulating THs are bound to thyroid hormone-binding proteins (Larsson et al., 1985). Transthyretin (TTR) is one of the key TH transporters in teleost fish and amphibian, which regulates the supply of THs to the various target tissues (Kawakami et al., 2006; Power et al., 2000; Santos et al., 2002). Uridine diphosphate glucuronosyltransferases (encoded by *ugt1ab* gene) are the phase Ⅱ enzymes which play an important role in the

elimination of THs (Hood and Klaassen, 2000). Therefore, the significant up-regulations of *ttr* and *ugt1ab* genes by TPP (Fig. 2D) could be interpreted as an effort to reduce available THs and to eliminate them via bile excretion in response to the increase of TH levels.

At the cellular level of the target organs, TH signaling is generally influenced by the interaction of the active hormone T3 with the TRs. Generally, two TR isoforms, TRα and TRβ, are involved in transcriptional actions whereas TRβ does also mediate mitochondrial actions of THs (Bassett et al., 2003). In the present study, regulation of *tr* genes was different in direction by isoform. *trα* and *trβ* were up- and down-regulated, respectively (Fig. 2A). Similar results have been reported in zebrafish larvae by perfluorooctane sulfonate (PFOS) (Shi et al., 2009). In addition, the stage and tissue-dependent expression patterns of each receptor have been reported in zebrafish (Takayama et al., 2008). Although little is known about the specific functions of each TR isoforms in zebrafish, multiple lines of evidence indicate their different functions. While TRα is important for postnatal development and cardiac function, TRβ is mainly expressed in pituitary, liver, and kidney and controls liver metabolism and thyroid hormone levels (Flamant and Samarut, 2003). In zebrafish, the TR-mediated actions play crucial roles in embryonic to larval transition (Liu et al., 2000; Liu and Chan, 2002).

The pattern of transcription of many genes in GH3 cell was different from that observed in whole body larvae. While the *tshβ* gene is specifically expressed in pituitary gland, other genes, e.g., *trα, trβ, deio1,* and *deio2*, are expressed in various target organs or tissues, which may explain different transcriptional alterations between zebrafish larvae exposure and GH3 cell assays.

In GH3 cells, exposure to 1.5 μg/L T3 down-regulated the *tshβ* gene transcription (Fig. 3). Considering the role of pituitary in HPT axis as a

central regulator, the results can be explained as an compensatory effort against increased T3 levels. Similarly, our observation of decreasing *tshβ* gene transcription in zebrafish embryo/larvae (Fig. 2A) can be also interpreted as a negative feedback to increased T3 levels (Fig. 1). In contrast, TPP exposure in GH3 cell led to up-regulation of *tshβ* gene, which was different from the observation following TPP exposure in zebrafish embryo/larvae (Fig. 2A). This different direction can be interpreted as the difference between systemic *in vivo* response and *in vitro* response; while *in vivo* response incorporates both direct effect and negative feedback regulation, *in vitro* response lacks the feedback regulation. Our *in vitro* observation implies that TPP could up-regulate *tshβ* gene in pituitary gland directly. However, the findings from the zebrafish larvae show that compensatory feedback efforts of the zebrafish pituitary gland outweigh the direct interaction of TPP on the pituitary gland.

TPP did not alter the expressions of *deio1* and *deio2* mRNA in GH3 cells, whereas exposure to T3 significantly up-regulated those genes with *deio1* gene in greater extent (Fig. 3). The results show that exposure to TPP does not directly affect the transcription of *deio* in the pituitary gland, but increased concentrations of T3 do. Up-regulation of *deio1* gene in whole body zebrafish larvae by TPP exposure (Fig. 2D) that coincided by the increased THs (Fig. 1), in part corresponds with the response of GH3 cells following exposure to T3. Consistent with our observations, exposure to T3 significantly up-regulated the expression of *deio1* mRNA in GH3 cells in a previous study (Guo and Zhou, 2013).

Overall, observations of GH3 cell assay shed lights on underlying mechanisms suggested by *in vivo* zebrafish study. Further studies employing thyroid cell lines, e.g., FRTL-5 cells, would provide more insights on the actions of TPP

on thyroid related effects *in vivo*.

In summary, the results of the present study confirm the thyroid hormone disruption capacity of TPP. Further studies on the direct effects on several tissues, e.g. thyroid gland and liver, would provide clearer and more accurate understandings on its underlying mechanisms.

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국문초록

유기인계 난연물질 triphenyl phosphate가 수서생물과 세포주 모델에서 갑상선 기능 및 관련기전에 미치는 영향 연구

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유기인계 난연물질의 일종인 Triphenyl phosphate (TPP)는 최근 환경과 생체 내에서 빈번하게 검출이 보고되고 있지만, 이 물질의 잠재적인 독성 연구는 부족한 실정이다. 본 연구는 *in vitro* 와 *in vivo* model을 사용하여 TPP가 갑상선 호르몬 내분비계 교란에 미치는 영향을 확인하고 구체적인 기전을 파악하기 위하여 수행되었다. 갓 수정된 제브라피쉬를 7일 동안 TPP에 노출시킨 뒤, 갑상선 호르몬 및 관련 유전자의 전사 수준 변화를 관찰하였다. TPP에 7일 동안 노출된 제브라피쉬에서는 두 종류의 갑상선 호르몬인 whole-body T4와 T3의 농도가 유의하게 증가하였다. 유전자 분석 결과, 갑상선 호르몬의 합성에 기여하는 *slc5a5, tg, tpo* 등의 발현이 up-regulation되었다. 제브라피쉬에서 TPP 노출은 또한 갑상선 호르몬의 대사 (*deio1*), 운반(*ttr*), 제거(*ugt1ab*)에 관여하는 유전자의 전사를 up-regulation하였다. 다음으로 TPP가 조절 중추와 deidodinase 활성에 미치는 영향을 확인하기 위하여 rat의 뇌하수체 세포인 GH3 세포가 사용되었다. GH3 세포 실험 결과 TPP는 *tshβ*, *trα*, and *trβ*의 유전자 발현을 up-regulation하였다. *deio1*과 *deio2*의 유전자 전사는 TPP 노출에 의한 유의한

변화를 나타내지 않았다. 본 연구를 통해 TPP가 생체 내 갑상선 호르몬의 불균형을 초래하는 것이 확인되었다. 갑상선 호르몬의 정상적인 작용에는 호르몬의 조절, 운반, 대사 등 여러 가지 요소들이 복잡하게 관여하기 때문에, TPP가 갑상선 호르몬 합성과 운반에 미치는 영향 등 구체적인 기전을 연구할 필요가 있다.

주요어 : 유기인계 난연제; 트리페닐포스페이트; 시상하부-뇌하수체-갑상선 축; 내분비계 교란
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