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Thyroid disrupting effects and associated mechanisms of TCPP in GH3 cell line and zebrafish (*Danio rerio*) larva and adult

GH3 세포주와 초기 발달 시기 및 성어 시기 제브라피쉬 (*Danio rerio*)를 이용한 대체 난연제 TCPP의 갑상선 교란 영향과 기전

2017년 2월

서울대학교 보건대학원 환경보건학과 환경보건 전공 이 지 은
Abstract

Thyroid disrupting effects and associated mechanisms of TCPP in GH3 cell line and zebrafish (Danio rerio) larva and adult

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Since polybrominated diphenyl ethers (PBDEs) have been phased out due to their persistence and potential adverse effect, uses of tris(1-chloro-2-propyl) phosphate (TCPP) have been increased as an alternative flame retardant. TCPP, one of the most frequently detected organophosphate flame retardants (OPFRs) in various environments and biota, has been regarded as an important contaminant because of its high potency of human exposure. However, information on toxicological effects of TCPP is limited, so far.

This study was conducted to investigate the adverse effects of TCPP on the thyroid endocrine system in zebrafish larvae and adult, and the underlying mechanisms related hypothalamic-pituitary-thyroid (HPT) axis were determined using a rat pituitary (GH3) cell line.

In the GH3 cell, down-regulated expression of Type 2 deiodinase (dio2) following TCPP exposure was verified, which was not the same manner of T3. In zebrafish larvae at 120 hours post-fertilization (hpf), significant
increased T4 concentration was observed following 3.16 or 10 mg/L TCPP exposure. Significantly decreased T3/T4 ratio was also observed. TCPP exposure significantly up-regulated the gene expressions involved in thyroid hormone synthesis (tshβ, tshr, tra and trβ) and metabolism/elimination (dio1 and ugt1ab). Increasing whole-body thyroid level following TCPP exposure can be at least part of theses altered gene expressions. In addition, thyroid hormone metabolism led to be up-regulated as a response to maintain their hormone homeostasis. In adult, however, the thyroid hormone levels were not altered following TCPP exposure for 14 days. This can be interpreted that TCPP exposure is more sensitive to disrupt thyroid hormone regulation in zebrafish at the early developmental stage than in adult.

These results showed that TCPP exposure could disrupt the thyroid hormone level in zebrafish at early developmental stage through the HPT axis. Potential adverse effects on thyroid hormone disruption of TCPP should be considered for better understanding of alternative flame retardants. This study will be important for determining which flame retardants are safer alternatives for PBDEs.

**Keywords:** GH3 cell line, Tris(1-chloro-2-propyl) phosphate (TCPP), Organophosphate flame retardants (OPFRs), Thyroid hormone, Zebrafish

**Student number:** 2015-24062
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1. Introduction

Polybrominated diphenyl ethers (PBDEs), the most widely used flame retardants, have been globally restricted to use and phased out due to their persistency and the toxicity, e.g., reproductive effects, neurodevelopment, and thyroid disrupting effects (Chen et al., 2012; McDonald, 2002; Muirhead et al., 2006). At the same time, the use of organophosphate flame retardants (OPFRs) has been increased as one of the suitable alternative flame retardants taking the place of PBDEs.

Tris(1-chloro-2-propyl) phosphate (TCPP) is one of the most highly produced OPFRs. The production of TCPP is expected to be continuously increased until 2020, whereas the production of tris(1,3-dichloro-2-propyl) phosphate (TDCPP) and tris(2-chloroethyl) phosphate (TCEP) steeply decreased after 2012 (Schreder et al., 2016). Along with this, TCPP has been frequently detected in various environment and biota, e.g., air, house dust, drinking water, and human urine (Araki et al., 2014; Lee et al., 2016; Liu et al., 2016; Van den Eede et al., 2015). Lee et al. showed that TCPP levels in several types of drinking water in Korea were the highest among the OPFRs (Lee et al., 2016). TCPP was also found in all the collected human urine samples in previous study (Van den Eede et al., 2015). In addition, TCPP was revealed that the most detected OPFR in human breast milk (Sundkvist et al., 2010). Various environmental levels of TCPP in detail are summarized in Table 1. Considering the high amount of production and uses of TCPP, human exposure to TCPP is also expected to be increased. Understanding of the adverse effect caused by TCPP exposure
needs to be examined.

Thyroid hormones (THs), i.e. triiodothyronine (T3) and thyroxine (T4), are important in regulation of development, growth, and energy balance (Meeker and Ferguson, 2011). Blood levels of THs are systemically regulated by negative feedback via hypothalamus-pituitary-thyroid (HPT) axis. Since thyroid hormones particularly play essential roles in an early life stage, this stage may be sensitive period to exposure to thyroid hormone disrupting chemicals. For this reason, it is necessary to make sure the adverse effects on thyroid system in the developing period.

Recent studies have demonstrated that several OPFRs have potential to disrupt thyroid hormone regulations in vitro and in vivo (Kim et al., 2015; Liu et al., 2012). Moreover, negative associations between the OPFRs, i.e. TDCPP and triphenyl phosphate (TPP), concentrations in the house dust and thyroid hormone level in adult men were reported (Meeker and Stapleton, 2010). In spite of this serial evidence of the possibility to disrupt thyroid hormone by OPFRs exposure, few studies have been conducted to reveal thyroid disrupting effects of TCPP. Decreased transthyretin (ttr), which binds to thyroid hormone in blood to transport to the target tissue, were reported in chicken embryo hepatocyte (Crump et al., 2012). The other study showed histological changes in thyroid in bird after TCPP exposure (Fernie et al., 2015). However, in vivo studies focusing on alterations of thyroid hormonal levels caused by TCPP exposure are still limited. In addition, the disrupting mechanisms in molecular levels are unclear.

The aim of this study is to verify the potential to thyroid disruption by TCPP exposure with hormonal level in zebrafish at two different life stages.
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*Mean value, †Metabolites
ND, not detected; NR, not reported
2. Materials and Methods

2.1 Chemicals

TCPP (tris(1-chloro-2-propyl) phosphate, CAS No. 13674-84-5) was purchased from Wako Pure Chemical Industry (Tokyo, Japan). The molecular structure and physicochemical information are shown in Table 5. In this study, dimethyl sulfoxide (DMSO, purity ≥99%, purchased from Junsei Chemical Co. (Tokyo, Japan)) was used as solvent, of which volume was not exceeded 0.01% v/v for in vivo test and 0.1% v/v for in vitro exposure test, respectively.

2.2 GH3 cell culture and exposure

The GH3 cell line was obtained from American Type Culture Collection (ATCC). GH3 cells were maintained in growth medium which contains a Dulbecco’s modified Eagle’s medium/Ham’s F-12 nutrient mixture (Sigma Aldrich) with 1.2 g of sodium bicarbonate. Additionally, 10% fetal bovine serum (FBS; Gibco®, Life Technologies, Carlsbad, CA, USA) and 1% penicillin-streptomycin (Sigma Aldrich) as antibiotics were added in the growth medium. The cells were incubated at 37 °C in a humidified atmosphere with 5% CO₂.

For the cell viability test, WST-1 cell proliferation assay (Roche Applied Science, Indianapolis, IN, USA) was performed. The GH3 cells were seeded at a density of 4.0×10⁴ cells/well in 96-well plate. After 20 h, the growth
medium was removed and replaced with serum-free medium with 1% of BD ITS+premix (BD Bioscience, 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). This step is to minimize the effects by steroid hormones and growth factors in the GH3 cells. Four hours after the media replacement, the cells were exposed to 0, 0.316, 1, 3.16, 10, 31.6, or 100 mg/L TCPP for 48 h in quadruplicates per treatment (n=4). The proliferation of cells was measured following the manufacturer’s protocol.

For the gene transcriptional analysis, GH3 cells were seeded with a density of 2.5×10^5 cells/well in 24-well plate. The medium was replaced with serum-free medium with the supplements after 20 h of incubation. After 4 h, the cells were exposed with 0, 1, 3.16, or 10 mg/L TCPP, which were determined as exposure concentrations based on the cell viability test. After 48 h incubation, the exposed cells were harvested for gene analysis.

### 2.3 Zebrafish culture and exposure

#### 2.3.1 Embryo/larval exposure

Wild-type zebrafish (*Danio rerio*) embryos within 3 hours post-fertilization (hpf) were obtained from the adult zebrafish (>6 months), which were routinely maintained in our laboratory. The embryos were exposed to 150 mL TCPP exposure solution (0, 0.316, 1, 3.16, or 10 mg/L) at the same time. All groups were 4 replicates, and 220 embryos were randomly distributed into each exposure solution. Then, the embryos were
exposed until 120 hpf.

The exposure concentrations were determined based on the preliminary test observing no significant mortality at the highest concentration. The 90% of the exposure solution was renewed every day during the exposure period. The exposure was performed at 25 ± 1 °C under 14:10 h light: dark. Conductivity, pH, temperature, and dissolved oxygen were recorded after every renewal. Number of hatched larvae and survivals were recorded in every 24 h. The dead were removed from the test solution.

On 120 hpf, wet-body weight of 200 pooled larvae from each replicate was measured for hormone analysis and 10 larvae were randomly collected for gene analysis, respectively. All samples were stored at -80 °C until analysis.

To investigate malformation rate, each of 10 embryos was placed in a 96 well plate. Three times of individual experiments were conducted with same condition so that total 30 embryos were observed for each treatment group. Then, any formation of yolk sac edema and/or heart edema were recorded at the end of exposure period using a Nikon Eclipse 80i microscope (Nikon, Japan) with imaging analysis software (iSolution, IMT Inc, Daejeon, Korea). Methylcellulose was used to observe morphological changes.

2.3.2 Adult exposure

Wild-type adult male zebrafish (>6 months old) were purchased from a commercial supplier (Greenfish, Seoul, Korea). Before exposure, zebrafish
were acclimated in dechlorinated tap water for 7 days with the same condition of exposure period.

In this study, two male zebrafish were kept in 2 L exposure solution (0, 0.316, 1, 3.16, or 10 mg/L TCPP) for 14 days. After 14 days exposure, 4 fish from two beakers were pooled as a replicate for further analysis. Each treatment has four replicates (n=4). The exposure solution was renewed at least three times in a week. Fish were fed with frozen blood worm twice daily. The exposure was performed at 25 ± 1 °C under 14:10 h light: dark. Conductivity, pH, temperature, and dissolved oxygen were recorded after every renewal. During the exposure period, no mortality by the chemical exposure was observed.

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

After the exposure, 10 zebrafish larvae were pooled as one replicate and homogenized using a tissue grinder. RNA from each sample was extracted by RNeasy mini kit (Quiagen, Hilden, Germany) and the concentration was measured using an Epoch Take 3 spectrophotometer (Biotek, Bad Friedrichshall, Germany). After that, complementary DNAs (cDNAs) were synthesized using the iScript™ cDNA synthesis kit (BioRad, Hercules, CA, USA) and diluted to 100 ng/μL. A total of 20 μL of quantitative real-time PCR (qRT-PCR) mix, including 10 μL of LightCycler 480 SYBR Green I Master mix (Roche Diagnostics Ltd., Lewes, UK), 1.8 μL of each PCR primer (10 pmol/μL), 4.4 μL of purified PCR-grade water, and 2 μL of the
cDNA sample, was used for qRT-PCR analysis. Running of qRT-PCR was conducted using a Light Cycler 480 (Roche Applied Science, Indianapolis, IN, USA). The thermal cycle profile was: pre-incubation at 95 °C for 10 min, 40 cycles of amplification at 95 °C for 10 sec, 85 °C for 20 sec and 72 °C for 20 sec. The melting curve analysis of the amplified products was conducted. The comparative Ct method ($2^{-\Delta\Delta Ct}$) was determined to calculate the relative change of gene transcription (Livak and Schmittgen, 2001). Primer sequences for the reference and target genes are shown in Table 6. The gene expression levels were normalized to cyclophilin and 18srrna for GH3 and zebrafish, respectively.

2.5 Thyroid hormone extraction and measurement

Whole-body levels of T3 and T4 in zebrafish after TCPP exposure were measured using a commercial enzyme-linked immunosorbent assay (ELISA) following the protocol by Yu et al., (2010) with minor modifications. Two hundreds zebrafish larvae per replicate were homogenized in 110 μL standard diluent ELISA buffer using a tissue grinder. Then, the experimental samples were immediately sonicated for 10 min on ice. After the centrifugation with 7000 x g for 10 min at 4 °C, the supernatant was collected and stored at -80 °C until analysis. The ELISA kit for T3 (Cat no. CEA453Ge) and T4 (Cat no. CEA452Ge) were purchased from Cloud-Clone Corp. (Wuhan, China). T3 and T4 were measured by a Tecan Infinite® 200 (Tecan Group Ltd., Mändorf,
Switzerland). The detection limits for T3 and T4 were reported as 47.2 pg/mL and 1.42 ng/mL, respectively.

2.6 Statistical analysis

The data normality and homogeneity of variance were analyzed by Shapiro-Wilk’s test and Levene’s test, respectively. The data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett’s test. For the trend analysis, the linear regression was applied. P-value less than 0.05 were considered statistically significant. IBM SPSS 23.0 for Windows (SPSS Inc., Chicago, IL, USA) was used. All data are presented as the mean ± standard error of the mean (SEM).
Table 2. Physicochemical properties of TCPP

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</tr>
</tbody>
</table>

$^a$Guo and Zhou et al., 2013; $^b$Liu et al., 2013; $^c$Yu et al., 2010; $^d$Wang et al., 2013; the other primer sequences are from Kim et al., 2015.
3. Results

3.1 GH3 cell proliferation

In GH3 cells, T3 exposure induced cell proliferation, whereas the exposure to TCPP did not show any cellular proliferation (Fig. 1). At the highest concentration of TCPP treatment, cytotoxicity was observed.
Figure 1. GH3 proliferation following exposure to (A) T3, and (B) TCPP. The cell proliferations (%) are represented as mean ± SEM of three independent experiments, each of which has at least three replicates.
3.2 Gene transcriptional changes in GH3 cells

Gene expression in GH3 cells following T3 exposure showed downregulated thyroid-stimulating hormone beta (tshβ) and thyroid hormone receptors (tra and trβ), 0.5, 0.5, and 0.6-fold at the highest concentration of T3, respectively. Deiodinase type 1 and 2 (dio1 and dio2) gene expressions were up-regulated (3.7-fold and 1.5-fold, respectively) by T3 exposure (Fig. 2). Gene expression of dio2 was decreased (0.7-fold) after TCPP exposure at all treated concentrations (Fig. 3). The other genes were not significantly changed.
Figure 2. Gene transcription in GH3 cells following exposure to T3. The results for the exposure groups are indicated as the relative fold change (solvent control = 1). All data are shown as the mean ± SEM (n=4). Asterisks (*) present a significant difference from the solvent control ($p<0.05$).
Figure 3. Gene transcription in GH3 cells following exposure to 1, 3.16, or 10 mg/L TCPP. All data are shown as the mean ± SEM of three replicates, and each replicate has at least three technical replicates. Asterisks (*) present a significant difference from the solvent control ($p<0.05$).
3.3 Zebrafish embryo/larval exposure study

3.3.1 Survival and developmental effects

Hatchability, larval survival, malformation rate, and body weight were not altered by TCPP exposure in zebrafish larvae (Table 7). However, the time to hatch was significantly delayed at 10 mg/L TCPP exposure group.
Table 4. Effects of TCPP on hatchability, larval survival, and body weight in zebrafish larvae

<table>
<thead>
<tr>
<th>TCPP concentration (mg/L)</th>
<th>0</th>
<th>0.316</th>
<th>1</th>
<th>3.16</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hatchabilitya (%)</td>
<td>96.0 ± 0.7</td>
<td>96.1 ± 1.7</td>
<td>95.7 ± 0.5</td>
<td>94.7 ± 1.8</td>
<td>96.9 ± 0.3</td>
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<tr>
<td>Larval survivalb (%)</td>
<td>99.0 ± 0.3</td>
<td>99.5 ± 0.3</td>
<td>97.8 ± 0.5</td>
<td>95.7 ± 1.3</td>
<td>97.6 ± 0.4</td>
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<tr>
<td>Body weightc (mg)</td>
<td>95±6</td>
<td>92 ± 8</td>
<td>88 ± 2</td>
<td>83 ± 3</td>
<td>79 ± 2</td>
</tr>
</tbody>
</table>

*aHatchability (%) is the percentage of hatching among the all embryos. Dead were considered to lose hatchability. bLarval survival (%) is the percentage of surviving larvae among the hatched one. cBody weight (mg) were measured of 200 larvae per each replicate. All data are shown as the mean ± SEM.
3.3.2 Malformation

Malformations caused by TCPP exposure are shown (Fig. 4). With linear regression, malformation was significantly increased ($p=0.020$, $\beta=1.438$). In this study, yolk sac edema and/or heart edema were considered as a deformity in zebrafish larvae. At the 10 mg/L TCPP exposure, mortality and malformation rate were 16.7% and 26.7%, respectively.
Figure 4. Malformation was observed following (A) solvent control (SC) and (B) 10 mg/L TCPP exposure in zebrafish larvae. (C) Cumulative number of malformation, dead, and normal zebrafish larvae at 120 hpf (n=30). Yolk sac edema (YSE) or/and heart edema were considered.
3.3.3 Whole-body thyroid hormone changes

Following exposure to TCPP, whole-body T3 level was not changed for any treatment groups, whereas whole-body T4 concentrations were significantly increased at 3.16 or 10 mg/L TCPP exposed groups (Fig. 5A and 5B). T3/T4 ratio was significantly decreased at all concentration except 0.316 mg/L TCPP (Fig. 5C).
Figure 5. Hormonal changes of (A) T3 content (B) T4 content, and (C) the T3/T4 ratio in zebrafish larvae, respectively. All data are shown as the mean ± SEM of four replicates. Asterisks (*) present significant differences (p<0.05) from the solvent control (SC).
3.3.4 Thyroid related gene expression changes

The exposure to TCPP influenced the expressions of thyroid related gene. Gene expressions of \( tsh\beta \), thyroid-stimulating hormone receptor (\( tshr \)), uridine diphosphate glucuronosyltransferase (\( ugt1ab \)), \( tra \), \( tr\beta \), and \( dio1 \) were upregulated following 1.8-fold, 1.9-fold, 1.7-fold, 1.5-fold and 1.9-fold, respectively, at the highest exposure group in zebrafish larvae (Fig. 6A and 6B). With linear regression analysis, corticotrophin-releasing hormone (\( crh \)) showed significant up-regulated trend (Fig. 6A). The other analyzed genes, \( dio2 \), \( NK2 homeobox 1 \) (\( nkx2.1 \)), \( paired box protein 8 \) (\( pax8 \)), and \( thyroglobulin \) (\( tg \)), were not shown any significant changes (Fig. 6B and 6C).
Figure 6. Gene expression of (A) *crh*, *tshβ*, *tshr*, *ugt1ab*, (B) *tra*, *trβ*, *dio1*, *dio2*, and (C) *nkx2.1*, *pax8*, and *tg* in whole-body of 120 hpf zebrafish larvae following exposure to TCPP. All data are shown as the mean ± SEM of three or four replicates, each replicate pooled 10 larvae. Asterisks (*) present significant differences from the solvent control by ANOVA analysis (*p*<0.05). Three statistical values (*p*, *β*) were obtained by simple linear regression analysis.
3.4 Zebrafish adult exposure study

3.4.1 Plasma thyroid hormone changes

The plasma T3 and T4 levels in male adult zebrafish did not show significant changes by TCPP exposure for 14 days (Fig. 7).
Figure 7. (A) T3 and (B) T4 levels in male adult zebrafish (ng/mL). SC means solvent control. All data are shown as the mean ± SEM of three or four replicates.
4. Discussion

Significantly increased concentrations of whole-body T4 and decreased T3/T4 ratio clearly indicate that TCPP can disrupt thyroid hormone regulation in zebrafish larvae (Fig. 5). So far, few studies have been conducted to figure out the thyroid disruption effects of TCPP with hormonal changes in vertebrate. The alteration of thyroid hormone levels following OPFRs has not been consistent in previous study. TDCPP, which has structural similarity with TCPP, led to increase of T3 and decrease of T4 in zebrafish larvae following 144 hours exposure (Wang et al., 2013). Another previous study suggested that TPP can disrupt thyroid hormone by increasing both T3 and T4 levels in developing zebrafish (Kim et al., 2015).

Although the exact manners of OPFRs are unclear, altered gene expressions in this study support the results of hormonal changes in zebrafish larvae after TCPP exposure. Thyroid hormone system is primarily regulated by the HPT axis in non-mammalian vertebrates. In fish, CRH stimulates Thyroid-stimulating hormone (TSH) secretion (De Groef et al., 2006). TSH encourages thyroid hormone to be secreted and synthesized in thyroid (Boas et al., 2006). Thyroid hormone receptors (TRα and TRβ) interact with thyroid hormone to regulate thyroid hormone system (Boas et al., 2006). Up-regulated expression of tshβ and tshr at the highest exposure level, 10 mg/L TCPP, can be suggested to lead to increase whole-body T4 levels (Fig. 5B and 6A). Significantly increased gene expression of crh with linearity analysis can also influence to increase tra and trβ as a response to the stimuli for the efforts to increase thyroid hormone level following TCPP.
exposure. With these serial results, whole-body thyroid hormone levels can be increased in zebrafish larvae after TCPP exposure. Meanwhile, this may be led to up-regulated ugt1ab gene expression as a compensatory effort to reduce excess thyroid hormone followed TCPP exposure. Also, up-regulated Type 1 deiodinase (dio1) can be explained by response to stimulate T4 metabolism. The gene expression changes as compensate responses in vivo have been reported in previous studies (Kim et al., 2015; Yu et al., 2010).

Thyroid hormone synthesis-related gene, ntx2.1, pax8, and tg, were not shown any significant changes. The ntx2.1 and pax8 genes are essential to the thyroidal cell differentiation and development (Elsalini and Rohr, 2003; Rohr and Concha, 2000). The effects on the zebrafish larvae show that TCPP can more likely to disrupt thyroid hormone in zebrafish at the early development stage affecting the thyroid hormone regulation and metabolism through the gene expression changes related the HPT axis, not directly having an effect on thyroid development.

In addition, down-regulated dio2 levels in GH3 cell line also supports that TCPP may directly affect to pituitary, which is one of the central organs of HPT axis. In the different manner with T3, TCPP did not draw any significant changes of tshβ, dio1, tra and trβ, but dio2 (Fig. 2 and Fig. 3). Deiodinases are involved to regulate thyroid activity. Dio1 recovers iodine and degrades thyroid hormone (Zhai et al., 2014). Dio2 has important role to metabolite T4 to T3, that is the inactive form of thyroid hormone (T4) can be transformed to the active form of thyroid hormone (T3) (Yu et al., 2013). Therefore, the significantly altered dio2 gene expression in GH3 cells
suggests that this compound may act to disrupt the conversion of thyroid hormone to be active (Fig. 3). Down-regulated deiodinase expressions by exposure to OPFRs have been reported in previous study (Porter et al., 2014). At the same time, GH3 cell proliferation results in Fig.1 is in line with the similar results with the previous study reporting no agonistic activity after treating TCPP in the presence of T3 (Zhang et al., 2016). Since anti-thyroid hormone activity was observed in human thyroid receptor β transfected CHO-K1 cell following TCPP exposure with T3 (Zhang et al., 2016), there may exist differences of sensitivities between species.

In the present study, gene expression of dio2 in GH3 cells was down-regulated following TCPP exposure, whereas no significant dio2 expression changes in zebrafish. Generally, thyroid hormone system in vivo is regulated by complicated biological feedback. Although the observed gene expression patterns were not exactly the same between in vitro and in vivo, both results from in vitro and in vivo can be interpreted that TCPP may disrupt thyroid hormone regulation by leading increase of T4.

Significantly increased malformation rate at the highest exposure group could be influenced by thyroid hormone disruption following TCPP exposure. From the result, it can be suggested that TCPP can affect development in zebrafish at early developmental stage (Fig. 4). Previous study has reported similar deformities that short tail, pericardial edema, and pericardial cavity edema was observed with 2.5% malformation rate in X. tropicalis embryos by 3.3 mg/L TCPP exposure (Zhang et al., 2016). Malformations occurred by thyroid hormone disruption have been reported in previous studies (Elsalini and Rohr, 2003; Ma et al., 2016). Methimazole, one of the well-known goitrogens disrupting thyroid regulation, caused
delayed hatching and malformation, i.e., yolk sac edema, curved body shape and no swim bladder (Elsalini and Rohr, 2003). Ma et al. also regarded that the edema and spinal curve caused by gene expression related the HPT axis after tris (2-butoxyethyl) phosphate (TBOEP) exposure.

The result that plasma T3 and T4 level in adult zebrafish were not changed indicates TCPP exposure is more sensitive to zebrafish at developing stage, which requires systemic thyroid hormone regulation for vigorous growth (Colicchia et al., 2014; Liu and Chan, 2002). A previous study supports that the alteration of thyroid hormone level in embryonic to larval transitory phase can draw mortal effects (Liu and Chan, 2002). Since the implication of thyroid regulation in adult zebrafish has been reported, however, the adverse effects of TCPP exposure lasted from early developmental stage should be concerned (Morais et al., 2013).
5. Conclusion

Thyroid hormone levels and the expressions of several genes related to thyroid synthesis and metabolism were altered in zebrafish larvae in this study. These observations support the thyroid hormone disrupting potential of TCPP, especially to organisms at early developmental stage. Considering increasing uses of TCPP, the adverse effect of TCPP should be highlighted. For further, adverse effects by long-term exposure at early development stage are needed to study.
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Appendix

Appendix A. Malformation of zebrafish larvae at 120 hpf by TCPP exposure

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<table>
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<thead>
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<table>
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<table>
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<table>
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<th>10 mg/L TCPP</th>
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* Heart edema, HE; hours post-fertilization, hpf; yolk sac edema, YSE
국문 초록

GH3 세포주와 초기 발달 시기 및 성어 시기
제브라피쉬(Danio rerio)를 이용한 대체 난연제
TCPP의 갑상선 교란 영향과 기전

이 지은

환경보건학과 환경보건 전공
서울대학교 보건대학원

기존 난연제로 가장 많이 사용되던 브롬계 난연제(polybrominated diphenyl ethers, PBDEs)는 환경 중 지속성과 잠재적인 독성영향 때문 에 일부 브롬계 난연제의 사용이 규제되거나 금지되었다. 이를 대체하여 사용하기 위해, 유기인계 난연제(organophosphate flame retardants, OPFRs) 중 하나인 tris(1-chloro-2-propyl) phosphate (TCPP)의 사용이 증가하고 있다. 이러한 사용량 증가와 함께 다양한 환경에서 TCPP가 높은 빈도로 검출되고 있으며 사람의 소변 등 생물체 내에서도 TCPP의 대사체가 검출되고 있어 TCPP의 인체 노출이 우려되고 있다.

서울대학교
그럼에도 불구하고, TCPP의 독성학적인 영향을 확인한 연구는 매우 부족하다.

따라서 본 연구에서는 제브라피쉬를 이용하여 초기 발달과정 단계의 치어에서와 성어 시기에서의 TCPP로 인한 갑상선 호르몬 교란 영향을 평가하였다. 또한, 쥐의 뇌하수체 세포인 GH3 세포주를 이용하여 시상하부-뇌하수체-갑상선 (HPT) 축과 관련한 갑상선 교란 영향 기전을 확인하였다.

GH3 세포주를 이용한 실험에서는 TCPP 노출로 인해 dio2 유전자 발현이 감소되었으며, 이는 T3를 노출시켰을 때 dio2 유전자가 증가하는 것과는 다른 방향의 결과이다. 초기 성장 단계인 수정 후 120시간 동안 3.16 mg/L TCPP와 10 mg/L TCPP에 노출된 제브라피쉬 치어에서 갑상선 호르몬 T4의 체내 농도가 유의하게 증가하였다. 또한, T3/T4 비가 유의하게 감소하는 것이 확인되었다. 유전자 분석 결과, 갑상선 호르몬 합성에 관여하는 감상선 자극 호르몬(tshβ), 갑상선 자극 호르몬 수용체(tshr), 갑상선 호르몬 수용체(trα와 trβ)의 유전자 발현이 유의하게 증가하였고, 감상선 호르몬 대사와 제거 역할을 하는 탈요오드화효소(dio1)과 글루쿠로노실전이효소(ugt1ab)의 유전자 발현이 유의하게 증가하는 경향을 보였다. 제브라피쉬 치어에서의 이러한 변화는 TCPP 노출로 인해 체내 갑상선 호르몬 호르몬이 증가하는 방향으로 변하고, 개체 내 호르몬 항상성 유지를 위해 감상선 호르몬의 대사를 촉진하도록 관련 유전자의 발현이 증가하는 것으로 추정된다. 한편, 14일 동안 TCPP에 노출된 성어 제브라피쉬는 갑상선 호르몬의 변화가 관찰되지 않았다. 따라서, TCPP의 노출은 성어 시기 보다 초기 발달 시기 제브라피쉬에 더욱 민감하게 갑상선 교란 영향을 일으키는 것으로 해석된
본 연구 결과는 TCPP는 초기 발달 단계의 제브라피쉬에서 시상하부-뇌하수체-갑상선 축에 의한 갑상선 호르몬 조절을 방해하고 호르몬 수준의 교란을 야기시킬 수 있다는 것을 보여준다. 따라서, 대체 난연제의 안전한 사용을 위해 TCPP의 잠재적인 갑상선 교란 영향이 고려되어야 한다. 본 연구는 안전한 브롬계 난연제 대체제를 모색하는 데에 있어 중요한 자료가 될 것으로 기대된다.

표제어: GH3 세포주, 트리스(1-클로로-2-프로필) 포스페이트(TCPP), 유기인계 난연제, 갑상선 호르몬, 제브라피쉬

학번: 2015-24062