보건학석사 학위논문

Alteration of Thyroid Hormone Regulation by Acetyl Tributyl Citrate (ATBC) in Embryo-Larval and Adult Zebrafish (*Danio rerio*)

대체가소제 ATBC의 제브라피쉬 배아-치어와 성어단계에서의 갑상선 호르몬 교란 영향 연구

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Abstract

Alteration of Thyroid Hormone Regulation by Acetyl Tributyl Citrate (ATBC) in Embryo-Larval and Adult Zebrafish (*Danio rerio*)

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Acetyl tributyl citrate (ATBC) is a citrate used as a plasticizer to replace di(2-ethylhexyl) phthalate (DEHP). ATBC has been increasingly used for various purposes, including food additives, product coatings, and pharmaceutical excipients, and hence has been detected in indoor environments and products including processed foods, packaging, and medical tubing. Because of a lack of information on the major metabolites of ATBC, biomonitoring studies on ATBC have seldom been performed. In the present study, we evaluated the thyroid hormone-disrupting potential of ATBC, using both embryo-larval and adult male zebrafish. Changes in major gene transcription related to thyroid hormone homeostasis and thyroid hormones were analyzed. Zebrafish embryos were treated with a series of sublethal concentrations of 0, 0.003, 0.03, and 0.3 mg/L. DMSO 0.01% (v/v) was used as a solvent. For embryo-larval fish, four replicates of 300 embryos and 30 embryos were employed for each concentration for five hormones (TSH, TT3, FT3, TT4, and FT4) and gene analysis, respectively. In the adult fish experiment, exposure was carried out for 14 days in 4 repeated groups of 10 organisms each in a 5L beaker containing 4L

of ATBC exposure media: 0, 0.1, 0.3, 1 mg/L with DMSO 0.01% (v/v). On the last day of exposure, plasma collected from the caudal vein of the fish was diluted and TSH, TT3, and TT4 were measured. Following the exposure, larval zebrafish (5 dpf) showed a dose-dependent increase in T3 and T4 levels. Several key genes of the HPT axis showed alterations in transcription. *crhβ* and *trh* genes associated with the central regulations were upregulated. In addition, *tg* and *nis* genes showed upregulation, although statistical significance was not reached. Genes such as *ugt1ab* and *sult1st5* which are related to thyroid hormone elimination were downregulated, supporting increased TH levels in whole-body larvae. In adult zebrafish after exposure to 14 days, TSH was significantly decreased, but TT4 level was increased except for the highest exposure group. These observations suggest that ATBC has the potential to disrupt thyroid hormone balance in both life stages in zebrafish but showed different patterns of alteration. This is the first observation to show the potential thyroid-disrupting effects of ATBC in zebrafish which is comparable to reported observations for DEHP. Further studies on its consequences are warranted.

Keywords : alternative plasticizer, ATBC, thyroid hormone, zebrafish, endocrine disruption, life stages

Abbreviations: ATBC acetyl tributyl citrate; dpf days post fertilization; DEHP di-ethylhexyl phthalate; TSH thyroid stimulating hormone; TT3 total triiodothyronine; FT3 free triiodothyronine; TT4 total thyroxine; FT4 free thyroxine; ELISA enzyme-linked immunosorbent assay

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Table of Contents

1. Introduction

Phthalates have been mainly used in PVC as a plasticizer to soften plastics. Phthalate plasticizers represented by DEHP are well known for their endocrine toxicity such as anti-androgenic and thyroid-disrupting effects (Chang et al., 2021; Bustamante-Montes et al., 2013). To replace the phthalates, alternative plasticizers have been developed and the market trends and use of non-phthalate-based plasticizers are gradually increasing (Bui et al., 2016). We conducted a systematic literature review on exposure sources, biomonitoring, and epidemiological studies on five non-phthalate plasticizers (DEHA, ATBC, TOTM, DEHTP, DINCH) with high production in Europe and the United States. Even though it was discovered that ATBC, a member of the citrate class, was frequently found in the environment (indoor dust 85.3%), food (processed food 70.3%), and product (kids' product 92.3%), biomonitoring and endocrine system disturbance-related health effect studies, as well as toxicity data to support it, were insufficient when compared to the other four alternative plasticizers (Jung et al., in preparation). Despite the lack of toxicity information, according to ECHA data, ATBC is manufactured in and/or imported to the same amount as DEHP, in the European Economic Area

(ECHA, 2022). Additionally, a high level of detection in different media was also found to this high yield. In the environment, indoor dust and sediment are 5,200 μg /g (Larsson et al., 2017) and 26 μg /g (Nagorka and Koschorreck 2020), respectively, in product packaging and filter, 1,510 μg/dm^2 (Goulas et al., 2007), and in food, grain-processed food is high at 7,000 ng/g (García Ibarra et al., 2018). Especially, ATBC is used for a variety of purposes, from food additives to product and pharmaceutical coatings (Larsson et al., 2017; Nagorka and Koschorreck 2020; García Ibarra et al., 2018), in addition to conventional plasticizer usage, suggesting that there is a probability that the population could be exposed through everyday routines. In contrast to other plasticizers, ATBC has less in vivo toxicity research than these issues. In terms of reproductive, developmental, acute, and genotoxicity, ATBC was reported to be relatively safe, however, studies on other endocrine disruptions were lacking (Chiellini et al., 2013). There were just two studies describing thyroid-disrupting toxicity. Of the two, the other literature—not the review literature—suggested that using the *in silico* method, ATBC might affect thyroid hormone regulation. In the reported *in silico* study, the binding energy of ATBC to Trα was higher than that of the native ligand of Trα, suggesting the possibility of a thyroid hormone disturbance effect (Zughaibi et al.,

2022). Furthermore, given the well-known thyroid hormone-disrupting effect of the phthalate-based plasticizer DEHP (Jia et al., 2016) to be substituted, it has become critical to assess the thyroid hormone-disrupting effect of ATBC, an alternative plasticizer.

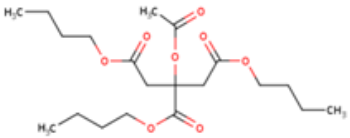
In this study, evaluating the potential mechanism of thyroid hormone disturbance caused by ATBC, this study seeks to produce knowledge about *in vivo* toxicity. Furthermore, utilizing zebrafish embryo-larval and adult fish, the effect of thyroid disruption is compared at each developmental stage.

2. Materials and methods

2.1 Chemical

Acetyl tributyl citrate (ATBC, CAS No. 77-90-7, purity: 98%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO, CAS No. 67-68-5, purity: ≥99%) used as a solvent in this study was purchased from Junsei Chemical Co. (Tokyo, Japan).

Table 1. Physico-chemical properties of Acetyl tributyl citrate

Abbreviation	Chemical structure	Formula	MW (g/mol)	Log Kow	Vapor pressure (mmHg)
ATBC (CAS No. 77-90-7)		$C_{20}H_{34}O_8$	402.5	5.07	3.0E-04 (25 ℃)

2.2 Maintenance of zebrafish and experimental design

2.2.1 Zebrafish maintenance condition

For the embryo-larvae stage experiment, wild-type zebrafish (*Danio rerio*) were cultured at the Environmental Toxicology Lab of Seoul National University (Seoul, Korea). Sexually mature adult zebrafish placed in a culture chamber at 25±1℃ with a 14:10 h light: dark cycle were mated to produce fertilized eggs. Approximately over six-month-old zebrafish were acquired from a commercial vendor (Greenfish, Seoul, Korea; Shinseong, Seoul, Korea) for the experiment on the adult stage. The fish was stabilized by acclimatization to aerated dechlorinated water for at least two weeks in an in-house culture room condition to eliminate the interference of other chemicals. The experiments were approved and performed according to the Institutional Animal Care and Use Committee (IACUC) recommendations by Seoul National University (SNU-210923-5-1).

2.2.2 Embryo-larvae exposure

Embryos within 4 hours post-fertilization (hpf) were randomly pooled, 300 embryos per 1 L beaker for hormone analysis (500 mL

of ATBC exposures), and 30 embryos per 50 mL beaker (50 mL of ATBC exposures) for genetic analysis (Fig. 1). There were 4 replicates each treatment groups. Based on the sub-lethal concentration at the embryo-larval stage confirmed through the pre-test, the following exposure concentration groups were set and exposed for 5 days post-fertilization (dpf): 0, 0.003, 0.03, 0.3 mg/L ATBC with 0.01% DMSO (v/v). ATBC exposure media were renewed more than 80% every day. Water quality measurements of exposure media, including temperature, pH, conductivity, and dissolved oxygen, were recorded every day. Also, embryo and larval survival (%), hatchability (%), coagulation of embryo (%), mortality rate (%), and time to hatch (d)were monitored every day. On the last day of 5 dpf exposure, 250 larvae for hormone analysis and 20 larvae for gene analysis were randomly collected in tubes. Through the washing step, no exposure media was left in the microtube containing the collected larvae using 1X Phosphate-Buffered Saline (PBS). In the case of hormone analysis, the weight before and after the tube was measured and used for wet weight normalization. The samples were stored at −80℃ until being used for further analysis.

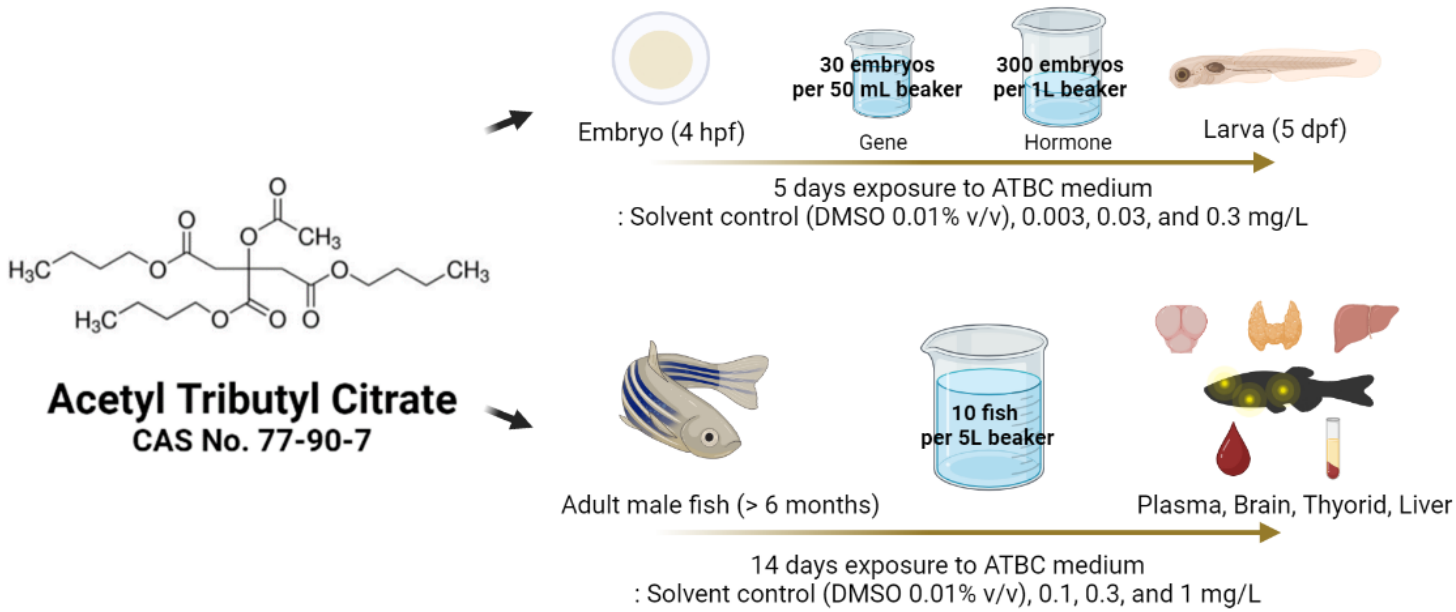


Figure 1. Experimental design of ATBC exposure of embryo-larval and adult male zebrafish.

2.2.3 Adult fish exposure

To exclude the disturbing effects of sex hormones, only male zebrafish were used in this experiment. 10 adult fish over 6 months of age, which had been acclimated for more than 2 weeks, were randomly pooled and exposed to 4L of ATBC exposure medium contained in a 5L beaker for 14 days. Based on the sub-lethal concentration confirmed through the pre-test, the exposure concentration was set as follows: 0, 0.1, 0.3, 1 mg/L ATBC with 0.01% DMSO (v/v). ATBC exposure media were renewed more than 80% every day. Water quality measurements of exposure media, including temperature, pH, conductivity, and dissolved oxygen, were recorded every day. The fish were sacrificed on the last day of exposure, plasma was separated from a blood sample and the brain, thyroid, and liver (Fig. 1), which are organs responsible for the regulation of thyroid hormone, were put in a tube and kept at −80℃ until being used for further analysis.

2.3 Thyroid hormone extraction and measurement

To evaluate thyroid hormone levels in the embryo-larval stage, the collected larvae sample was added up to 250 ㎕ PBS and 3 ceramic (zirconium oxide) beads homogenized with 3 cycles (1 cycle: 4500 rpm for 10 sec) using a Precellys® Evolution24 (Bertin technologies) leaving samples to cool on ice between repeated cycles (Fig. 2). Each sample was centrifuged at 13,000 g for an additional 20 min at 4℃ after being sonicated for 20 min at 4℃ in a water sonicator. With the volume of the hormone media obtained being recorded, the supernatant was transferred to a new microtube. Wet body weight (g) and supernatant volume (㎕) were used to normalize thyroid hormone levels. To analyze thyroid hormone changes in adult fish, samples were diluted by adding 1X PBS to plasma separated from blood was used. The supernatant from zebrafish larvae's whole body was analyzed for TSH (thyroid stimulating hormone), TT3 (total triiodothyronine), FT3 (free triiodothyronine), TT4(total thyroxine), FT4 (free thyroxine) using ELISA (enzyme-linked immunosorbent assay) following the kit protocol. Due to the limited volume of diluted adult zebrafish plasma samples, only three hormones TT3, TT4, and TSH were measured in adult fish. The test kits (CAT #TF E-2300 for TT3; CAT #TF

E-2100 for FT3; CAT # TF E-2400 for TT4; CAT #TF E-2200 for FT4) were purchased from LDN Corp (Nordhorn, Germany) and TSH ELISA kit (CAT #CSB-EQ027261FI) were purchased from Cusabio Technology LLC (Houston, USA). The thyroid hormone measurement was analyzed by Tecan spark® (Tecan Infinite 200, Tecan Group Ltd, Mändorf, Switzerland).

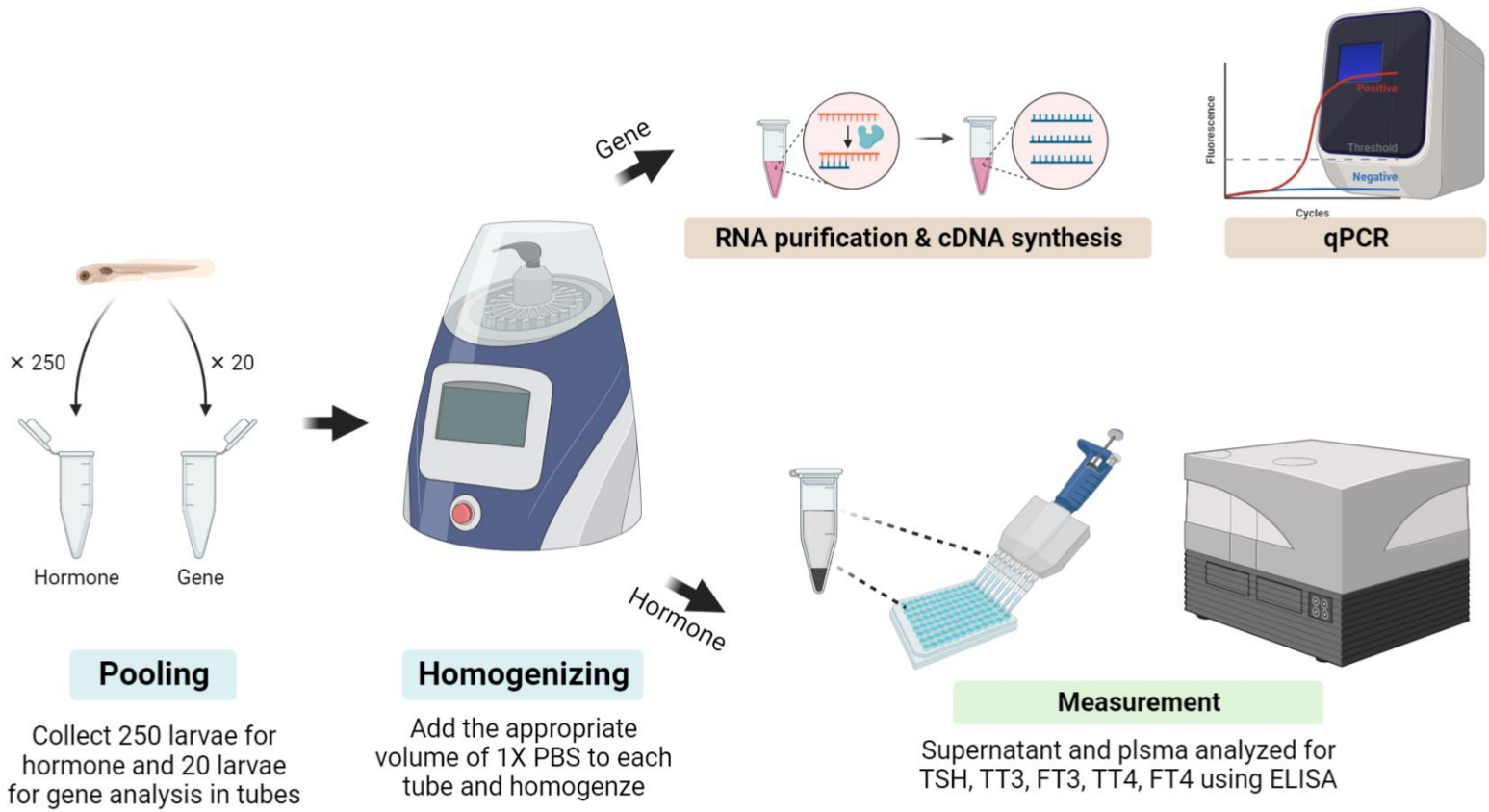


Figure 2. Protocols of measurement for thyroid hormones and related genes in embryo-larval zebrafish.

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

Following the protocol provided by the RNeasy mini kit (Qiagen, Hilden, Germany, the mRNA of 5 dpf larvae exposed to ATBC was extracted. Gen5 2.05 was used to assess the quality and concentration of RNA samples (Biotek, Winooski, USA). Using the iScript cDNA synthesis kit (BioRad Hercules, CA, USA), complementary DNAs (cDNAs) were synthesized in a total volume of 20 ㎕. The following reagents were used to make the 20㎕ of qRT-PCR reaction mix: 7.8 ㎕ of nuclease-free water, 10 ㎕ of Power SYBR Green PCR Master Mix (Applied Biosystem, Woolston Warrington, UK), 0.1 ㎕ of each PCR primer (10 ㎛M), and 2 ㎕ of cDNA samples. Table 2. shows the target gene primer sequences that were taken from the literature or obtained using an online BLAST tool. For zebrafish larvae gene assays, the reference gene ribosomal protein L13 (*rpl13*) was selected. The eleven genes related to regulating the HPT axis were analyzed (Table. 2). These genes included corticotropin-releasing hormone b (*crhβ*), thyrotropin-releasing hormone (*trh*), thyroid stimulating hormone subunit beta (*tshβ*) for central regulation; sodium/iodide symporter,

and thyroglobulin for thyroid hormone synthesis; solute carrier family 16 member 2 (mct8) and transthyretin (*ttr*) for thyroid hormone transport; iodothyronine deiodinase 1 (*dio1*), iodothyronine deiodinase 2 (*dio2*), uridine diphosphate glucuronosyltransferase 1 family a, b (*ugt1ab*), and sulfotransferase family 1, cytosolic sulfotransferase 5 (*sult1st5*) for thyroid hormone metabolism. Using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), the levels of gene transcription were standardized to housekeeping genes.

Table 2. Genes and their primer sequences of the two life stages of zebrafish investigated in the present study

<Embryo – larval zebrafish>

Gene	Primer Sequence (5'-> 3')		Accession No.
	Forward	Reverse	
rpl13	TTCAACCAGCCTGCCAGAAA	CCTTCAGCTCCTCCAAGGTG	NM_198143.1
crhβ	CAATTACGCACAGATTCTCCTCG	GAAGTACTCCTCCCCCAAGC	NM_001007379.1
trh	GCTCTCTCCGTCGGTCTGTT	GCGAGATCCGTGCTGATGA	NM_001012365.2
tshβ	GCAGATCCTCACTTCACCTACC	GCACAGGTTTGGAGCATCTCA	NM_181494.2
nis	GGTGGCATGAAGGCTGTAAT	GATACGGGATCCATTGTTGG	NM_001089391.1
tg	GTCTCTTGAGTGTTCGAATGACAAG	AAAGGCGGGCCATTAAGG	NM_001329865.1
mct8	CTTCGGATGTCGGAAAACGG	CCCAGAGTCGTGGCGAAG	NM_001258230.1
ttr	CGGGTGGAGTTTGACACTTT	GCTCAGAAGGAGAGCCAGTG	NM_001005598.2
dio1	AACTTGGAGGAGAGGCTTGCT	AGGGCATGGAGGGTCTTCTT	NM_001324404.1
dio2	CGCGAAATGGGCTTGCT	CCAGGCAAAATCTGCAAAGTTA	NM_212789.4
ugt1ab	GCCAGCTTTGATGAACTTGCC	AACTCCTCCAGTTCCTTGGTT	NM_213422.2
sult1st5	CCCATCCAACTTTTGCCTCG	GGATCCCCATACCAATTGTCCT	NM_001199903.1

<Adult zebrafish>

Gene	Primer Sequence (5'-> 3')		Accession No.
	Forward	Reverse	
rpl13	GCTGAAGGAATACCGCACCA	TCCAGTAAGCTGTGTTGCCAT	NM_198143.1
crhβ	CAATTACGCACAGATTCTCCTCG	GAAGTACTCCTCCCCCAAGC	NM_001007379.1
trh	CATGCTAGAGGACCCCACTG	GAGCAGCATCAGGTAGCGTT	NM_001012365.2
tshβ	GCAGATCCTCACTTCACCTACC	GCACAGGTTTGGAGCATCTCA	NM_181494.2
nis	CCACTGAAGATCGGCAGAAT	CAGCCAAGCCCATAGAACA	NM_001089391.1
tg	ACAATCCACTGGGTGTGTGTT	GAGAGCAAAAGACCTGCCCT	NM_001329865.1
dio1	GTTCAAACAGCTTGTCAAGGACT	AGCAAGCCTCTCCTCCAAGTT	NM_001324404.1
dio2	TGGATGCCTACAAACAGGTGA	GTCTTACCGCTGATGCTCCA	NM_212789.4
ugt1ab	GCGCGTACGGAGCTTACTTC	AGCTCGGAGATGCGGAATCC	NM_213422.2
sult1st5	GCCAGCTTTGATGAACTTGCC	AACTCCTCCAGTTCCTTGGTT	NM_001199903.1

2.5 Statistical analysis

One-way analysis of variance (ANOVA) with Dunnett's t-test was used to compare the control and treatment groups. Spearman's rank correlation was applied for trend analysis. The p-values that were less than 0.05 were considered significant. Means and a standard deviation of means are used to express data (SD). SPSS statistical software 25 was used for all analyses (IBM Corporation, New York, USA).

3. Results

3.1 Hormone and transcriptional changes in embryo-larval zebrafish

3.1.1 Alteration of thyroid hormone and TSH levels

After 5 dpf exposure to ATBC, concentration-dependent increases levels of T4 and T3 of both forms, but not for TSH in the larval zebrafish (Fig. 3). Additionally, there was a significant change in the level of FT3 in 0.03 and 0.3 mg/L exposure groups. In the highest concentration 0.3 mg/L, TT4 and FT4 levels were also significantly different.

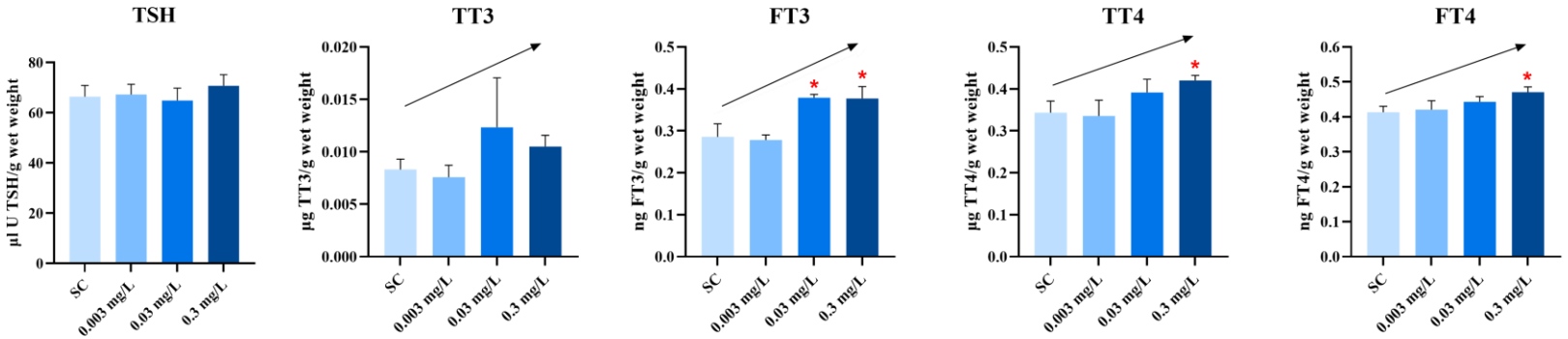


Figure 3. TSH, TT3, FT3, TT4, and FT4 concentrations in zebrafish larvae after ATBC 5 dpf exposure (0, 0.003, 0.03, 0.3 mg/L). The values are expressed as the Mean±SD of four replicates. Asterisks (*) indicate statistically significant differences ($p<0.05$) as compared to the solvent control group. Arrow indicates a significant trend.

3.1.2 Alteration of HPT axis gene transcription levels

Changes in the transcription of the genes that regulate thyroid hormones are expected to result in increased synthesis and decreased elimination of thyroid hormones after exposure to ATBC (Fig. 4). *Crhβ* and *trh* that are involved in thyroid hormone central regulation genes were upregulated. Also, *nis* and *tg* which are related to hormone synthesis genes were enhanced. The *ttr* gene related to hormone transport was also observed to be significantly increased compared to the solvent control group. The expression levels of the *ugt1ab* and *sult1st5* genes, which are involved in the elimination of hormones, were shown to be downregulated.

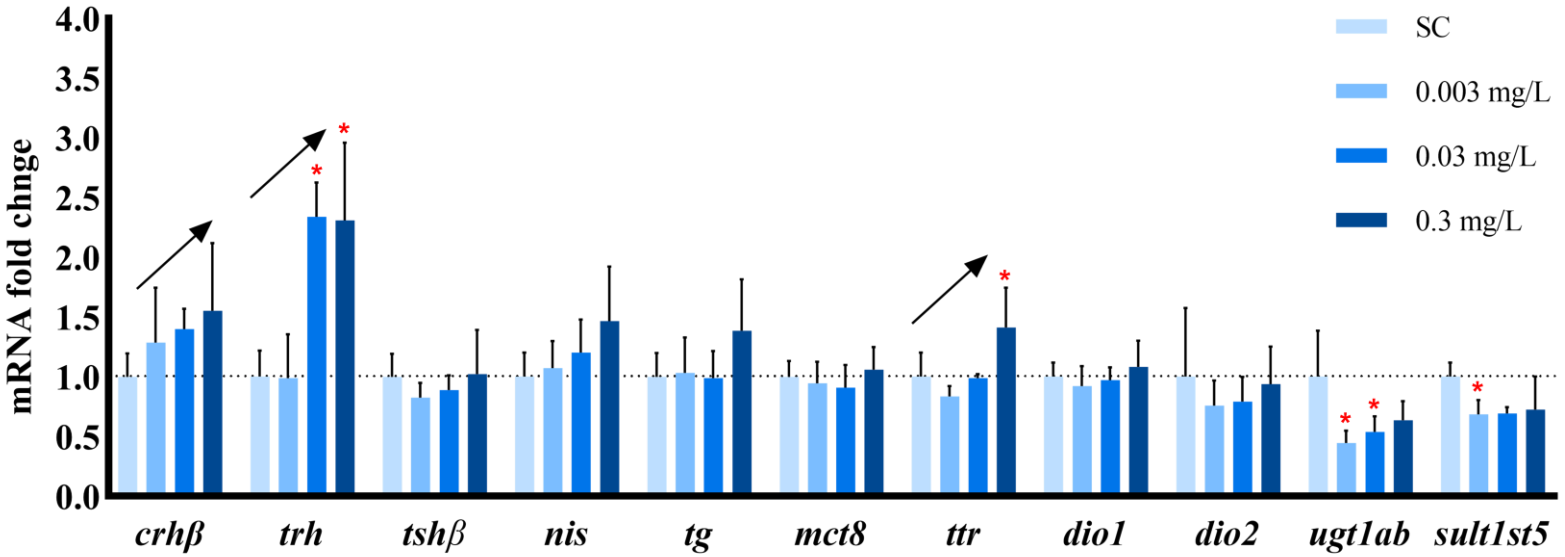


Figure 4. Transcriptional changes of thyroid hormone regulation-related genes in zebrafish larvae after ATBC 5 dpf exposure (0, 0.003, 0.03, 0.3 mg/L). The values are expressed as the Mean±SD of four replicates. Asterisks (*) indicate statistically significant differences ($p<0.05$) as compared to the solvent control group. Arrow indicates a significant trend.

3.2 Hormone change in adult zebrafish

3.2.1 Alteration of thyroid hormone levels

Among the three hormones analyzed, the TSH level showed a marked tendency to decrease in a concentration-dependent manner. Also, significant differences were observed at 0.3 and 1 mg/L compared to the solvent control group. In the case of TT3, there was a significant difference compared to the solvent control at 0.1 and 0.3 mg/L. Additionally, in TT4 there was no statistical significance, but an increasing trend was confirmed in the concentration groups except for the highest.

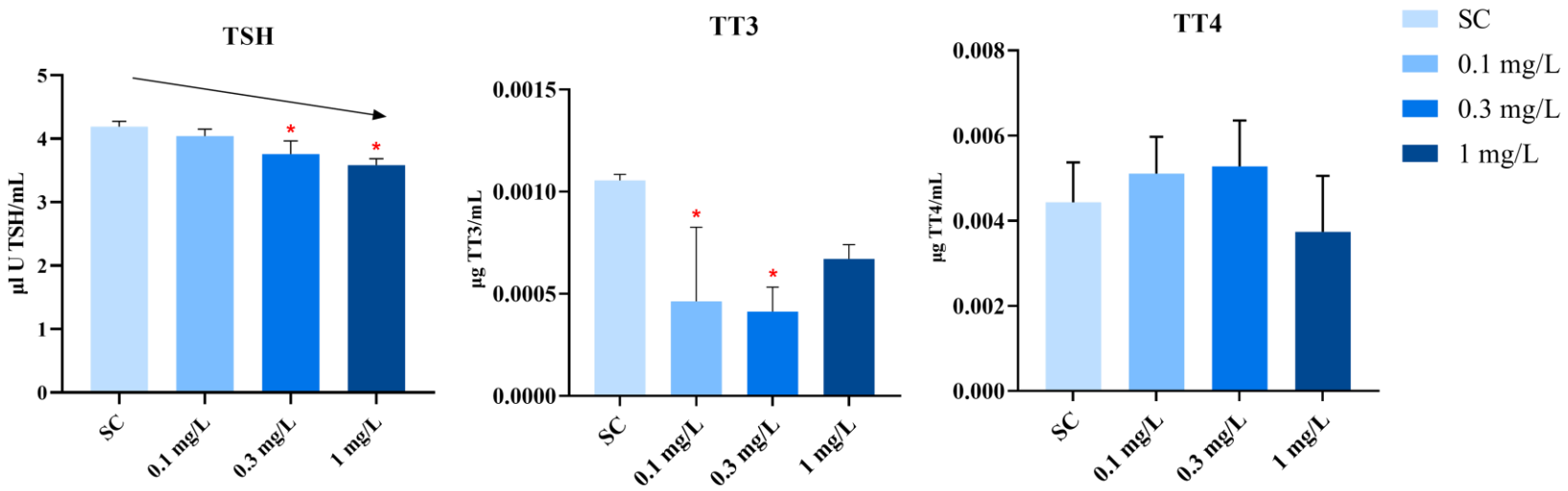


Figure 5. TSH, TT3, and TT4 concentrations in adult zebrafish after ATBC 14 days exposure (0, 0.1, 0.3, 1 mg/L). The values are expressed as the Mean±SD of four replicates. Asterisks (*) indicate statistically significant differences ($p<0.05$) as compared to the solvent control group. Arrow indicates a significant trend.

3.2.2 Alteration of HPT axis gene transcription levels in tissues

In the brain of adult male zebrafish, genes related to central regulation of thyroid hormone, i.e., *crhβ*, *tshβ*, and *trh*, were measured; in the thyroid, genes related to thyroid hormone synthesis, i.e., *nis*, *tg*, and *tpo*, and in the liver, genes related to conversion, metabolism, and elimination, *dio1*, *dio2*, *ugt1ab*, and *sult1st5* were quantified. In the adult male zebrafish brain, the *tshβ* gene showed down-regulation although statistical significance was not observed. In addition, to the thyroid gland, all three genes involved in thyroid hormone synthesis showed down-regulation trends. In the liver, both *dio1* and *dio2* genes showed down-regulation trends in a concentration-dependent manner.

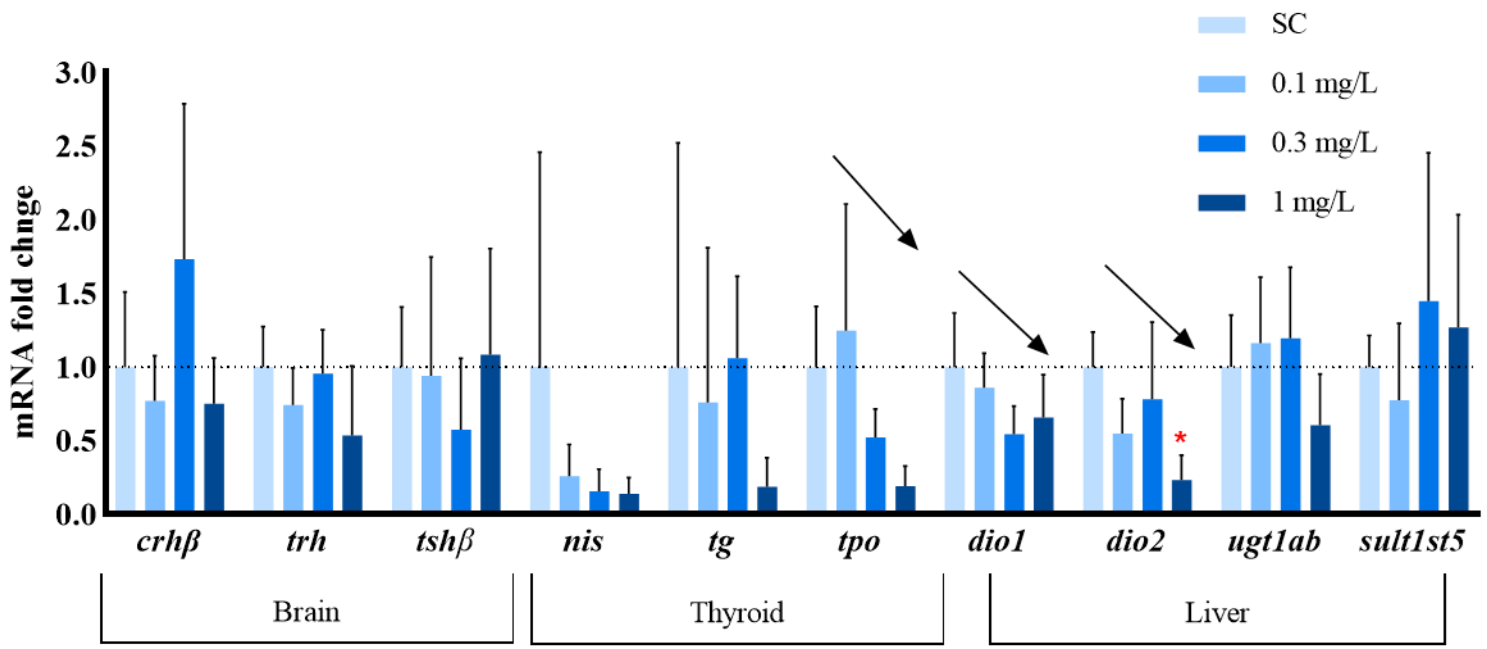


Figure 6. Transcriptional changes of thyroid hormone regulation-related genes in zebrafish brain, thyroid, and liver after ATBC 14 days exposure (0, 0.1, 0.3, 1 mg/L). The values are expressed as the Mean±SD of four replicates. Asterisks (*) indicate statistically significant differences ($p<0.05$) as compared to the solvent control group. Arrow indicates a significant trend.

4. Discussions

4.1 Thyroid disrupting effects of ATBC in embryo-larval zebrafish

Our observation clearly shows that the 5-day exposure to ATBC causes the increase of thyroid hormones in the larval zebrafish. After 5 days of exposure to ATBC, both T3 and T4 levels increased. These observations can be explained by transcriptional changes of several genes we measured. In the brain, up-regulation of *crhβ* and *trh* genes. Both genes code hormones that play a central role in regulating thyroid hormones (Fig. 4). Up-regulation of *nis* and *tg* genes in the thyroid gland can also be explained by the up-regulation of the hypothalamic and pituitary genes. Moreover, the up-regulation of *ttr* gene, which codes a plasma protein that carries thyroid hormone, supports the increase in thyroid hormones in the total form, such as TT3 and TT4. Down-regulation of *ugt1ab* and *sult1st5*, which are associated with thyroid hormone elimination, also supports the increased thyroid hormone levels in the larval fish. It is interesting to observe no significant changes in TSH levels (Fig. 3). It could be interpreted as a compensatory effort of the fish, but further confirmation is warranted.

4.2 Thyroid-disrupting effects of ATBC in adult zebrafish

In adult male fish, ATBC exposure caused thyroid hormone disruption but in a different pattern from those observed in the larval fish. A significant decreasing pattern of TSH and TT3 levels was supported by transcriptional changes of the genes related to the synthesis and conversion of thyroid hormone. First, in the case of TSH, a significant decrease was observed in the hormone level, which is presumed to be due to the down-regulation of the *tshβ* gene, among genes related to thyroid hormone regulation. It was observed that the primary response to ATBC exposure was an inhibitory effect on the thyroid hormone regulatory center. As a result, the *nis*, *tg*, and *tpo* genes analyzed in the thyroid tended to be decreased in a concentration-dependent manner compared to the solvent control. This suggests that down-regulation at the gene level may affect hormone synthesis inhibition. According to the experimental results, there was no significant change in the TT4 hormone level compared to the change at the gene level. However, in the highest experimental concentration group, 1 mg/L, the TT4 hormone level tended to decrease compared to the control group. It

is estimated that in the low-concentration group, hormone homeostasis was maintained as compensation for the effect of reduction at the gene level, but in the high-concentration group, the function became impossible. Among the four genes measured in the liver, distinct changes were observed for the *dio1* and *dio2* genes. In particular, *dio2*, which is known to be mainly expressed in the liver (Colella et al., 2020), is related to the conversion of T4 to T3, which is an active form of the thyroid hormone. Down-regulation of the *dio2* gene may hence support the observation of lowered TT3.

4.3 Difference thyroid disrupting effects depend on the zebrafish life stages

Thyroid hormone levels in both forms of T3 and T4 except for TSH were increased in zebrafish at the embryo-larval stage. However, TT3 and TSH decreased in zebrafish at the adult stage, and it was observed that the thyroid hormone disrupting effect and mechanism were different for each developmental stage of zebrafish. Differences in thyroid disturbance effects by developmental stage are presumed to have resulted from differences in age, exposure period, exposure concentration, and zebrafish fish cohort. In studies in which zebrafish were exposed to DEHP at relatively low concentrations (0, 40, 100, 200, and 400 μg/L) for 168 hpf, both T4 and T3 tended to increase (Jia et al., 2016). In Sprague-Dawley rats administered gavage with high concentrations of DEHP (0, 250, 500, and 750 mg/kg/day) for 30 days, both T3 and T4 decreased significantly (Liu et al., 2015). Also, a study conducted in the Netherlands with 1996 pregnant women, an association was found in which the concentration of FT3 and FT4 decreased in the group with higher concentrations of DEHP metabolites in urine (Derakhshan et al., 2021). Based on this, thyroid hormone levels

increased in embryo-larval zebrafish exposed to relatively low concentrations (0, 0.003, 0.03, 0.3 mg/L), but at high exposure concentrations (0, 0.1, 0.3, 1 mg/L) in adult fish. It can be predicted that in adult fish tested, the thyroid hormone level may decrease. In addition, age and exposure period differences may have resulted in differences in the effect of thyroid hormone disturbance by each developmental stage. Thyroid hormone is essential for the early development of fish (Brown et al., 2004), and since the formation of thyroid follicle cells is completed at 72 hpf (Alt et al., 2006), fish may be relatively vulnerable if exposed to chemicals at that time. In this experiment, ATBC exposure was conducted for a relatively short period of 5 days. On the other hand, the fish used in the adult fish experiment were exposed for 14 days, a rather long period, when thyroid organ differentiation and growth were completed at 6 months of age or older. In a study in which old male zebrafish were chronically exposed to DEHP for 3 months, most of the HPT axis-related genes, *tshβ*, *nis*, *tg*, *ugt1ab*, and *dio2*, were downregulated (Junaid et al., 2018). This study supports the gene expression results observed in adult zebrafish in this study. Therefore, considering the thyroid development stage and age of the zebrafish used in the two developmental stages, it is expected that different thyroid disturbance effects appeared. Lastly, it can be assumed that

this is because the cohorts of the fish used in the embryonic stage and the fish used in the adult stage are different. Due to the limitations of the culture room space, embryos grown in an in-house culture room were used, whereas adult fish were purchased from commercial vendors. Therefore, thyroid hormone disturbances due to differences in cohorts even in the same wild-type fish difference is predicted.

5. Conclusions

Alternative plasticizer ATBC has been shown to have thyroid hormone-disrupting effects in both embryo-larval and adult male zebrafish. Significant hyperthyroidism was observed in embryo-larval zebrafish and decreased TSH levels in adult fish by exposure to ATBC. The differences in the effect of thyroid hormone disturbance by each developmental stage is estimated by various factors such as age, exposure concentration group, and exposure period. This study is the first *in vivo* observation to show the potential thyroid-disrupting effects of ATBC and used two different life stages. However, the effect of thyroid disturbance in the juvenile stage and the difference in trends between embryo larvae and adult fish are still unclear, so further studies on its consequences are warranted.

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국문 초록

대체가소제 ATBC의 제브라피쉬 배아-치어와 성어단계에서의 갑상선 호르몬 교란 영향 연구

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Acetyl tributyl citrate(ATBC)는 DEHP를 대체하여 사용되는 citrate 계열의 비프탈레이트계 대체가소제이다. ATBC는 식품 첨가제부터 제품 코팅제, 그리고 의약품 코팅제 등에 이르기까지 매우 다양한 용도로 사용되고 있어 실내 환경이나 가공식품, 포장재, 의료용 튜빙 등의 제품에서 검출되고 있다. ATBC의 주요 대사체에 대한 정보가 부족하여 바이오모니터링 및 관련 건강영향에 대한 역학 연구는 거의 수행되지 않았다. 본 연구에서는 프탈레이트계 가소제의 주요한 대체물질인 ATBC의 갑상선 호르몬 및 관련 유전자 교란 영향을 제브라피쉬의 두 가지 발달단계인 배아-치아 및 수컷 성어에서 평가하였다. 갑상선 호르몬 항상성 조절과 관련된 주요 유전자의 전사 수준 변화와 갑상선 호르몬을 분석하였다. 제브라피쉬의 배아는 DMSO 0.01% (v/v)를 용매로 사용한 0, 0.003, 0.03, 0.3 mg/L의 sublethal한 농도의 ATBC 노출수에 5일간 노출하였다. 각 반복군 당 각각 배아 300개와 30개씩 4반복군으로 노출시킨 후, 다섯개의 호르몬(TSH, TT3, FT3, TT4, FT4)과 호르몬 조절과 관련된 유전자 분석을 진행하였다. 성어단계에서는 DMSO 0.01% (v/v)를 용매로 사용한 0, 0.1, 0.3, 1 mg/L 농도의 4L 노출수가 담긴 5L 비커에 각 반복군 당 10마리씩 4반복군으로 14일간 노출하였다. 노출 마지막 날, 성어에서 채취한 혈장을 희석하여 3개의 갑상선 호르몬(TSH, TT3, TT4)와 뇌, 간, 갑상선에서 호르몬 조절과 관련된 유전자를 분석하였다. ATBC에 5일 간 노출된 배아-치어 단계의 제브라피쉬에서는 T3와 T4 모두 Total form과 Free form에서 농도 의존적으로 증가하는 결과를 보였다. *Crhβ*와 *trh*와 같은 갑상선 호르몬 조절을 담당하는 유전자 발현의 유의한 증가가 나타났고 갑상선 호르몬 합성과 관

련된 *tg*와 *nis*가 증가하는 경향이 관찰되었다. 또한, 갑상선 호르몬 제거를 담당하는 *ugt1ab*와 *sult1st5*와 같은 유전자의 발현 감소 결과가 나타났다. ATBC에 14일 노출된 성어 제브라피쉬에서는 TSH가 뚜렷하게 감소하는 경향을 보였다. 이러한 결과는 ATBC에 노출되었을 때, 제브라피쉬의 두 가지 발달단계에서 갑상선 호르몬 조절을 교란할 가능성이 있다는 것을 시사하였다. 이 연구는 DEHP의 대체물질인 대체가소제 ATBC의 갑상선 교란 독성에 대한 첫 번째 *in vivo* 연구이며 해당 결과에 대해 추가적인 연구가 필요할 것이다.

주요어 : 대체가소제, ATBC, 갑상선 호르몬, 제브라피쉬, 내분비계 교란, 발달단계

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