Waterborne exposure to avobenzone and octinoxate induces thyroid endocrine disruption in wild-type and thrα−/− zebrafish larvae

Yujin Ka1 · Kyunghee Ji1

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Abstract
Avobenzone and octinoxate are frequently used as organic ultraviolet filters, and these chemicals are widely detected in water. This study evaluated the potential of avobenzone and octinoxate to disrupt thyroid endocrine system in wild-type and thyroid hormone receptor alpha knockout (thrα−/−) zebra fish larvae. Following a 120 h exposure to various concentrations of avobenzone and octinoxate, larvae mortality and developmental toxicity in wild-type and thrα−/− fish were assessed. Triiodothyronine (T3) and thyroxine (T4) levels as well as transcriptional levels of ten genes associated with the hypothalamus-pituitary-thyroid (HPT) axis were measured in wild-type fish. Significantly lower larvae survival rate in thrα−/− fish exposed to ≥3 μM avobenzone and octinoxate suggests that the thyroid hormone receptor plays a crucial role in the toxic effects of avobenzone and octinoxate. A significant increase in the deio2 gene level in avobenzone-exposed zebrafish supports the result of an increased ratio of T3 to T4. Significant decrease of T4 level with upregulation of trh, tshβ, and tshr genes indicates feedback in the hypothalamus and pituitary gland to maintain hormonal homeostasis. Our observation indicates that exposure to avobenzone and octinoxate affects the thyroid hormone receptor and the feedback mechanisms of the HPT axis.

Clinical trials registration
Not applicable.

Keywords Avobenzone · Development · Knockout · Octinoxate · Thyroid endocrine disruption · Zebrafish

Introduction
Organic and inorganic ultraviolet (UV) filters are a group of substances that either absorb or reflect UV light, preventing it from penetrating the skin (Serpone et al. 2007). The United States Food and Drug Administration has classified 22 UV filter compounds, used in sunscreen products, as Generally Recognized As Safe and Effective (GRASE) (category I), those that are not GRASE (category II), and those that do not have sufficient data to support a positive GRASE determination (category III) (US Food and Drug Administration 2019). Avobenzone (also known as butyl methoxydibenzoylmethane) and octinoxate (also known as octyl methoxycinnamate or ethylhexyl methoxycinnamate) are representative components of organic UV filters (Bratkovics et al. 2015), and are classified as category III GRASE (US Food and Drug Administration 2019). As they are often used in sunscreen products, avobenzone and octinoxate are frequently introduced into water (da Silva et al. 2022). Direct release into the marine environment may occur via recreational water activities during swimming and bathing (Labille et al. 2020). Indirect release may occur via wastewater treatment plants (WWTPs) as a result of showering and washing (Poiger et al. 2004).

Avobenzone and octinoxate are frequently detected in the aqueous environment (Ekpeghere et al. 2016; Kameda et al. 2011; Tsui et al. 2014, 2019), sediment (Sun et al. 2021), and biota (Fent et al. 2010; Peng et al. 2017). In
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Measurement of thyroid hormone level

Additional experiments were performed to measure thyroid hormone levels. Three replicate groups of 250 wild-type embryos per concentration group were exposed to the test substance for 120 h, and 150 larvae per each replicate were collected after the exposure was terminated. Two independent experiments were performed to obtain data from biological replicates. Homogenized larvae samples were used for hormone measurement. The levels of T3 (Cat No. OKCA00348) and T4 (Cat No. OKCA00349) were analyzed using enzyme-linked immunosorbent assay kits (Aviva System Biology, San Diego, CA, USA) according to the manufacturer’s recommendations. To assess the hormonal balance, the ratio of T3/T4 was calculated and normalized to the solvent control group.

Gene transcription analysis

Wild-type zebrafish larvae collected at 120 h of experiment (ten fish in triplicate per each treatment group) were used for gene transcription analysis. Ten genes associated with the HPT axis (Table S1 in supplementary data) were analyzed using quantitative real-time polymerase chain reaction (qRT-PCR). Larvae samples were homogenized and messenger RNA (mRNA) was extracted from the supernatant. Extraction of mRNA and synthesis of complementary DNA (cDNA) were conducted using the RNeasy mini kit (QIAGEN, Hilden, Germany) and iScript™ cDNA Synthesis kit (BIORAD, Hercules, CA, USA), respectively. qRT-PCR was performed using the ABI 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) after adding the reaction mixture consisting of SYBR PCR master mix (Applied Biosystems), primer, and diluted cDNA to each well of a 96-well plate. The PCR program was 50 °C for 1 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The procedure permitted the relative quantification of transcriptional changes (Livak and Schmittgen 2001). Threshold cycle (Ct) of each gene was normalized based on the two reference genes (tuba1 and 18sRNA), and then these values from exposure group were normalized to the solvent control group.

Statistical analyses

Independent t-test compared the survival of wild-type and thraa−/− zebrafish. For the endpoints observed at the organism, hormonal, and genetic level from wild-type zebrafish embryo/larvae and the significance of differences between solvent control and treatment groups was assessed by one-way analysis of variance using SPSS software (version 27, IBM Corp., Armonk, NY, USA). Correlation between various endpoints were assessed using Spearman correlation analysis from the SAS program (SAS Institute, Cary, NC, USA). P-values less than 0.05 were considered statistically significant.

Results

Organism level toxicity in wild-type zebrafish

In wild-type fish, the coagulation of embryos exposed to 3 μM or more of avobenzone was significantly increased compared to the solvent control group (Fig. 1A). Fish exposed to ≥3 μM avobenzone displayed significantly decreased hatchability, which in turn led to decreased larvae survival (Fig. 1A). In addition, increased malformation and decreased larvae weight were observed in wild-type fish exposed to ≥10 μM avobenzone (Fig. 1A). In wild-type fish exposed to 30 μM avobenzone, hatching time was significantly delayed (Fig. 1A). Avobenzone did not remarkably affect larvae length compared to the solvent control group (Fig. 1A).

Octinoxate induced noteworthy effects on embryo coagulation, hatchability, larvae survival, and malformation in wild-type fish exposed to ≥10 μM (Fig. 1B). Delayed hatching time was also observed in wild-type fish exposed to 30 μM octinoxate (Fig. 1B). Larvae length and weight were decreased in a dose-response manner, but the effects were not statistically significant (Fig. 1B).

Hormone level toxicity in wild-type zebrafish

In wild-type larvae fish exposed to avobenzone, a significant increase of T3 and significant decrease of T4 was observed at a concentration of 30 μM and ≥10 μM, respectively (Fig. 2A). T3 and T4 concentrations of zebrafish larvae exposed to 30 μM octinoxate for 120 h were significantly decreased compared to the solvent control groups (Fig. 2B). The ratio of T3/T4 was significantly increased in wild-type fish exposed to 30 μM avobenzone (Fig. 2A). However, no significant differences between groups were observed in fish exposed to octinoxate (Fig. 2B).

Transcription level toxicity in wild-type zebrafish

Following the exposure to avobenzone, significant upregulation of trh, tshβ, tshr, tg, and deio2 genes and downregulation of ttaa, trβ, and tpo genes were observed in wild-type zebrafish larvae (Fig. 3A). In relation to octinoxate, transcriptions of trh, tshβ, tshr, tg, nis, and deio2 genes were significantly upregulated, while transcriptions of ttaa, trβ, and tpo genes were significantly downregulated (Fig. 3B).

Comparison of survival between wild-type and thraa−/− zebrafish

Toxicities of avobenzone and octinoxate were greater in thraa−/− fish than in wild-type fish (Fig. 1A, B). The extent of increase in embryo coagulation and decrease in hatchability and larval survival were greater in thraa−/− fish than...
Fig. 1 Effects of (A) avobenzone and (B) octinoxate on embryo coagulation, time to hatch, hatchability, larvae survival, malformation rate, larvae length, and larvae weight in wild-type (black circle) and thrαa−/− (white circle) zebrafish embryo/larvae. The results are shown as mean ± standard deviation of three replicates. Asterisk (*) indicates significant difference between solvent control and treatment groups, and # indicates significant difference between wild-type and thrαa−/− groups (p < 0.05).
in wild-type fish at ≥3 μM avobenzone (Fig. 1A). For octinoxate, embryo coagulation, time to hatch, hatchability, and larvae survival were significantly different between wild-type and thyroα−/− fish (Fig. 1B).

Relationship between endpoints

Results of correlation analysis between organism level endpoints of survival, length, and weight; hormone level endpoints of T3, T4, and T3/T4 ratio; and gene level endpoints of ten genes related to the HPT axis are shown in Table S2 and S3 (Supplementary data). In larvae fish exposed to avobenzone, weight was positively related to survival, T4 content, and transcriptional changes of trαα, trβ, tpo, and nis genes, while weight was negatively related to the T3 content, T3/T4 ratio, and transcriptional changes of trh, tshr, tg, and deio2 genes. After exposure to octinoxate in zebrafish larvae, length was positively related to survival, production of T3 and T4, and transcriptional changes of trαα and trβ genes, while length was negatively related to the trh, tshβ, tshr, tg, nis, and deio2 genes.

Discussion

Avobenzone and octinoxate have received much attention due to their environmental abundance and potential toxicity. In the present study, avobenzone and octinoxate altered thyroid hormone levels and changed the transcription levels of genes associated with the HPT axis. This is the first study using thyroα−/− zebrafish to elucidate that these two UV filters interfere with the binding of thyroid hormone receptors, resulting in thyroid endocrine disruption. More importantly, thyroid endocrine disruption induced by avobenzone and octinoxate ultimately delayed hatching and decreased larval weight. Results of correlation analysis between organism, hormone, and gene level endpoints would provide the greatest power for avobenzone and octinoxate exposures to precisely derive the flow for endocrine disrupting indicators.

The ratio of the T3 and T4 thyroid hormones is important to maintain homeostasis (Eales 2019). In the present study, avobenzone exposure ultimately increased the T3/T4 ratio. This was attributed to a decrease in T4 and an increase in T3 levels. Avobenzone belongs to the BP group along with...
Deoxybenzone, sulisobenzone (also known as BP-4), and oxybenzone (also known as BP-3) (Kullavanijaya and Lim 2005). The changes in the levels of thyroid hormones, such as the decrease in T4 level, are consistent with the effects of other BPs, including BP-1, BP-3, and BP-8 (Lee et al. 2018). Upregulation of the deio2 gene in larvae exposed to avobenzone may also contribute to the increase in T3/T4 ratio. T4 can be converted to T3 through outer ring deiodination of deiodinase type I (deio1) and II (deio2) (Walpita et al. 2009) or the glucuronidation enzymes ugt1ab (Parsons et al. 2020). Based on the results of the relationship between endpoints, upregulated deio2 transcription due to avobenzone exposure may enhance secretion of T3 and consequently increase the T3/T4 ratio. Although no significant difference in the T3/T4 ratio was observed in larvae exposed to octinoxate, the decrease in T4 level could be supported in part by a significant upregulation of the deio2 gene. Previous studies also reported that octinoxate upregulates transcription of the deio2 gene and decreases T4 level, which supports our findings (Chu et al. 2021; Lee et al. 2019).

TRH secreted from the hypothalamus stimulates the secretion of TSH from the pituitary, which subsequently stimulates the synthesis and secretion of thyroid hormones from the thyroid gland (Zhang et al. 2018). In the present study, a
significant increase in the transcription levels of trh, tshβ, and tshr genes was observed in fish exposed to avobenzone and octinoxate. These data suggest that avobenzone and octinoxate may affect TRH and TSH directly or indirectly by negative feedback responses to produce more T4. The results of correlation analysis also support the essential roles of the transcription of the trh, tshβ, and tshr genes in thyroid hormone regulation. The upregulation of tshβ and tshr genes by exposure to avobenzone and octinoxate observed in this study is similar to that of previous studies that reported elevation of TSH-related genes by BPs and octinoxate (Chu et al. 2021; Lee et al. 2018).

Thyroid hormones function by binding to their corresponding receptors (trα or trβ) (Deal and Volkoff 2020). Especially, the highly bioactive T3 hormone binds to the corresponding receptor, moves to the target tissue, and induces the intended effects. Therefore, downregulation of the trαα and trββ genes in fish larvae exposed to avobenzone and octinoxate may also suggest that these compounds can affect the expression of thyroid hormone receptors. The observation of higher mortality in trhα−/− than in wild-type fish is interesting, given that BPs and octinoxate have been reported to inhibit receptor binding capacity in previous studies (Chu et al. 2021; Lee et al. 2018). The results of correlation analysis also support the central role of the thyroid hormone receptor in the toxicity of avobenzone and octinoxate.

Thyroid peroxidase (tpo) is an enzyme that attaches iodine to thyroglobulin (tg), an important protein in the production of thyroid hormones (Nishihara et al. 2017). Decreased transcription of the tpo gene after avobenzone and octinoxate exposure indicates that these substances inhibit tpo activity in a manner similar to BP-2, thereby reducing thyroid hormone production (Lee et al. 2018). The sodium iodide symporter (nis) transports iodide (essential for thyroid hormone production) across the thyroid epithelium (Holloway et al. 2021). The results of upregulation of the nis gene in zebrafish larvae exposed to octinoxate are consistent with the results of other studies (Chu et al. 2021).

Overall, decrease in T4 contents induced by avobenzone and octinoxate exposure is potentially associated with genes along with the HPT axis, which eventually affects development. In addition, these two UV filters could affect survival or development of zebrafish larvae by interfering with the binding to trαα, which provides clues to the contribution of both substances to the binding of thyroid hormone receptors. Our observation of possible thyroid hormone perturbation in larvae shows that some UV filters may have detrimental consequences for aquatic organisms. Although developmental delay and thyroid endocrine disturbance were observed in fish exposed to avobenzone and octinoxate, levels of detection in the environment were relatively lower than those associated with effects measured in this study. Given the importance of thyroid hormone homeostasis in early development, and the results based on a short-term exposure of 120 h, the effects of long-term exposure on thyroid function should be further investigated.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary data files.

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Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis, and writing the first draft of the manuscript were performed by YK. Editing on previous version of the manuscript was conducted by KJ. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Animal research This study was approved by the Institutional Animal Care and Use Committee of Yongin University, Korea (YUIACUC-2021-7).

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