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Reproduction and endocrine related effects of life-cycle exposure to 2-ethylhexyl 4-methoxycinnamate (EHMC), a sunscreen agent, in Japanese medaka (Oryzias latipes)
Abstract

Reproduction and endocrine related effects of life-cycle exposure to 2-ethylhexyl 4-methoxycinnamate (EHMC), a sunscreen agent, in Japanese medaka (*Oryzias latipes*)

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2-Ethylhexyl 4-methoxycinnamate (EHMC) is one of the most frequently used UV-filters in personal care products (PCPs). Due to its widespread use, EHMC has been detected in aquatic biota as well as in surface waters. Despite its presence in aquatic environment, limited information on its toxicity in aquatic biota is available, especially in fish. In the present study, reproductive toxicity of EHMC and its underlying mechanisms were evaluated using...
Japanese medaka (*Oryzias latipes*), especially focusing on sex and thyroid hormone disruption. To fully understand the toxicity, a life-cycle test was conducted. Eggs were exposed to 0, 0.05, 0.158, 0.5, 1.58, or 5 mg/L of EHMC. At juvenile stages of F0 generation, transcription of genes related to sex and thyroid hormone disruption was quantified. At adult stages, the fish were mated and the eggs were counted every day. At 5 months post-hatch (mph), genes related to sex and thyroid hormone disruption were analyzed in the liver and gonad of the F0 fish. Spawned eggs (F1 fish) were collected and used for early-life stage (ELS) toxicity test. In adult stages, plasma 17β-estradiol (E2) levels were not altered in both adult male and female. Decreasing patterns of transcript levels for steroidogenesis-related genes in ovary were observed. At juvenile stages of F1 generation, thyroid hormones and transcription of genes related to sex and thyroid hormone disruption was quantified. Significant decreased reproductive performances were observed in all treatment groups at as low as 0.05 mg/L, i.e., environmentally relevant concentration. Significant down-regulations of *dio2* transcripts were observed at juvenile stages (31 day post-hatch (dph)) in both F0 and F1 generations. Whole-body triiodothyronine (T3) concentrations were significantly decreased and decreasing patterns of whole-body thyroxine (T4) concentrations, not between-group differences, were observed at juvenile stages of F1 generation. Our observations indicate that long-term exposure to EHMC can disrupt overall reproductive health outcomes and thyroid
homeostasis during juvenile stages.

Keywords: EHMC; personal care products; reproductive toxicity; long-term exposure

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Abbreviations

ar: androgen receptor
dio: deiodinase
dph: day post-hatch
E2: 17β-estradiol
EHMC: 2-ethylhexyl 4-methoxycinnamate
er: estrogen receptor
gth: gonadotropin
hpf: hour post-fertilization
mph: month post-hatch
PCPs: personal care products
T3: triiodothyronine
T4: thyroxine
thr: thyroid hormone receptor
trh: thyrotropin-releasing hormone
qRT-PCR: quantitative real-time polymerase chain reaction
vtg: vitellogenin
WWTPs: wastewater treatment plants
1. Introduction

2-Ethylhexyl 4-methoxycinnamate (EHMC) is one of the most commonly used UV-filters in a variety of personal care products (PCPs). Its frequency as ingredients in PCPs on the market was high in several countries (Poiger et al., 2004; Manová et al., 2013; Kim et al., 2011). Especially in the Kim et al. (2011) study, EHMC was the most frequently used ingredient in commercial suncare products among analyzed eight UV-filters. Due to its widespread use, EHMC can be frequently detected in the aquatic environment and biota. EHMC was detected up to 3 µg/L levels in Lake Cospuden for recreational purposes (Rodil et al., 2009). EHMC was detected up to 67 and 0.5 µg/L levels in wastewater treatment plants (WWTPs) influents and effluents, respectively (Hernández Leal et al., 2010; Tsui et al., 2014). EHMC was detected up to 3 µg/g dw level in sewage sludge (Gago-Ferrero et al., 2011). Widespread presence of EHMC and its lipophilic characteristics (logKow 5.80) have led to its frequent detection in aquatic biota (Gago-Ferrero et al., 2011; Fent et al., 2010) as well as human breast milk (Schlumpf et al., 2010). Furthermore, EHMC was detected in the greatest frequency in human breast milk among the measured UV-filters such as BP-3, 4-MBC, HMS, OCT, OD-PABA, and 3-BC (Schlumpf et al., 2010). In aquatic biota such as fish, mussel, and crustacean, EHMC was reported for bioaccumulation (Fent et al., 2010). Despite its widespread use and presence in biota and the environment, limited information about adverse effects of EHMC is available.
Heightened concern on UV-filters is driven by studies reporting their endocrine disrupting potential. Many UV-filters were previously reported for their endocrine disrupting effects in *in vitro* and *in vivo* studies (Krause et al., 2012). One of the concerns on these endocrine disrupting effects may be associated with reduced reproductive health outcomes, which potentially leads to population-level impacts (Fent et al., 2008; Kim et al., 2014; Krause et al., 2012). Endocrine disrupting potential of EHMC has been suggested by several studies (Kunz et al., 2006; Kunz and Fent, 2006; Schreurs et al., 2002). In recombinant yeast systems, EHMC showed both anti-estrogenic and anti-androgenic activities, combined with weak androgenic activity (Kunz and Fent, 2006). *In vivo* studies of EHMC may suggest that EHMC has estrogenic effects on rats. Short-term and long-term oral exposure of EHMC to rats was observed for increased uterine weight (Schlumpf et al., 2001; Klammer et al., 2005; Seidlová-Wuttke et al., 2006). EHMC induced histological changes in gonads of fathead minnow (Christen et al., 2011). EHMC also induced thyroid endocrine disruption in rodents (Klammer et al., 2007; Schmutzler et al., 2004; Seidlová-Wuttke et al., 2006; Axelstad et al. 2011). However, these endocrine disrupting effects and reproductive toxicity posed by EHMC in fish have yet to be clearly understood. Especially, adverse effects of EHMC on fecundity in fish remain to be elucidated.

An overall aim of this study is to find out potential toxicity of EHMC, focusing on reproduction and its underlying mechanisms, e.g. sex and thyroid endocrine effects. To fully understand the potential toxicity of EHMC, life-
cycle test including two generations was conducted. For this purpose, Japanese medaka, a proposed model for reproductive toxicity, was employed because it has short generation time and spawns eggs on a daily basis (Arcand-Hoy and Benson, 1998).
2. Materials and Methods

2.1 Chemicals

EHMC (CAS No. 5466-77-3) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). DMSO was used as a solvent and concentration of the solvent in the exposure media was 0.01% (v/v).

2.2 Maintenance of fish and experimental design

Eggs within 24 h post-fertilization (hpf) were collected from adult Japanese medaka (>3 months old). The eggs were randomly distributed into glass beakers containing 50 mL of EHMC exposure solution (0, 0.05, 0.158, 0.5, 1.58, or 5 mg/L). There were four replicates with 20 eggs per replicate. Hatchability and mortality were recorded daily. Newly hatched fry were fed with Topmeal® (Jae-il Food, Korea) for the first 5 d after hatching, and after that, newly hatched Artemia nauplii twice a day. Test solutions were prepared in the conditioned water containing sodium bicarbonate (75 mg/L), calcium sulfate (15 mg/L), and sea salt (8.4 mg/L) in deionized water (Nusslein-Volhard and Dahm, 2002). During the experiment period, fish were maintained at 27±1 °C and under 15:9 h light:dark photoperiod. Over nine-tenth of the water was renewed three times per week, and water quality parameters (DO, pH, temperature, and conductivity) was measured at renewal.
At 10 and 31 dph, three fish in a beaker were anesthetized with ice-cold water. The length and body weight were recorded and stored at -80 °C until analyses. The remaining fish were raised until the fish were sexually matured. The fish were subsequently mated with a ratio of 2:2 male:female (n=6) and the eggs were counted every day. Spawned eggs were tested for the F₁ generation early-life stage (ELS) toxicity test. At about 5 months post-hatch (mph) of F₀ generation, adult fish were anesthetized with ice-cold water, and the tissue and plasma were stored at -80 °C until analyses (Overall schematic of the experiment is shown in Fig. 1).
Figure 1. Overall schematic of the experiment (dph = day post-hatch, mph = month post-hatch).
2.3 RNA isolation and quantitative real-time polymerase chains reaction (qRT-PCR)

Total RNA in tissue or whole body samples was extracted using Maxwell® 16 LEV simplyRNA Purification Kit (Promega, Madison, WI, USA) according to manufacturer’s instruction. After quality and quantity of RNA are checked with an EPOCH microplate spectrophotometer (BioTek, Winooski, VT, USA), complementary DNA was synthesized from total RNA by iScript cDNA Synthesis Kit (BIORAD, Hercules, CA, USA). Quantitative real-time PCR (RT-PCR) was performed with LightCycler-DNA Master SYBR Green I mix (Roche Diagnostics Ltd, Lewes, UK) using LightCycler 480 (Roche Applied Science, Indianapolis, IN, USA). The thermal cycle profile consisted of pre-incubation at 95°C for 10 min, followed by 45 cycles of amplification at 95°C for 10 s, 55°C for 20s, and 72°C for 20s. For quantification of PCR results, the threshold cycle (Ct) was determined for each reaction. Ct values for each gene of interest are normalized to the housekeeping gene by the use of the ΔΔCt method (Livak and Schmittgen, 2001). In this study, β-actin was selected as housekeeping gene.
2.4 Hormone measurement

Plasma was immediately separated from the collected blood samples through centrifugation at $5000 \times g$ for 10 min. For one replicate, 3 separated plasma samples were pooled. Plasma 17β-estradiol (E2) was measured by enzyme-linked immunosorbent assay (ELISA) using commercial kits (Cat # 582251), following the manufacture’s instruction (Cayman Chemical, Ann Arbor, MI, USA).

Triiodothyronine (T3) and thyroxine (T4) were extracted from whole-body at 31 dph of F1 generation. Concentrations of whole-body T3 and T4 were measured by an ELISA using commercial kits (Cat # CEA453GE for T3 and Cat # CEA452Ge for T4) following the manufacturer’s protocol (Uscnlife, Wuhan, China). 7 to 10 medaka fish per replicate were homogenized in 120 μL ELISA buffer, using a motor-driven tissue grinder (Ginbko Bioscience, China). The samples were then sonicated for 10 minutes on ice and centrifuged at $5000 \times g$ for 10 minutes at 4 °C. The supernatant was collected and stored at −80 °C until analysis.
2.5 Statistical analysis

All statistical analyses were conducted using SPSS 20.0 K for Windows (SPSS, Chicago, IL, USA). Normality of data and homogeneity of variances were assessed by Shapiro–Wilk test and Levene’s test, respectively. Group differences were conducted by a one-way analysis of variance (ANOVA) followed by Dunnett’s test. Linear regression analysis was conducted to evaluate concentration dependant trends in data. For comparisons between control and solvent control group, *t*-test was carried out. In the all statistical analysis, *p* values less than 0.05 were considered to be statistically significant. All data are shows as mean ± standard error of mean (SE).
3. Results

3.1 Growth factors

Alterations in growth factors after exposure to EHMC were not observed during juvenile and adult stages of both F0 and F1 generations in this study (Tables 1, 2, and 3). At 10 and 31 dph of F0 generation, we found no significant difference between solvent control and exposure groups in growth including body length, wet weight, and condition factor (Table 1). Growth factors were not significantly altered at 5 mph (Table 2). At 31 dph of F1 generation, we also found no significant alterations in growth factors (Table 3).
Table 1. Effects of EHMC on body length, wet weight, and condition factor in 10 and 31 dph medaka fish of F0 generation

<table>
<thead>
<tr>
<th>Concentration (mg/L)</th>
<th>10 dph</th>
<th></th>
<th>31 dph</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length (mm)</td>
<td>Wet weight (mg)</td>
<td>Condition factor</td>
<td>Length (mm)</td>
<td>Wet weight (mg)</td>
</tr>
<tr>
<td>0</td>
<td>6.50±0.15</td>
<td>2.58±0.16</td>
<td>0.94±0.03</td>
<td>12.33±0.36</td>
<td>12.31±1.13</td>
</tr>
<tr>
<td>0.05</td>
<td>6.29±0.09</td>
<td>2.50±0.14</td>
<td>0.99±0.03</td>
<td>12.50±0.35</td>
<td>15.75±1.20</td>
</tr>
<tr>
<td>0.158</td>
<td>6.25±0.13</td>
<td>2.62±0.26</td>
<td>1.06±0.09</td>
<td>11.62±0.27</td>
<td>12.81±1.12</td>
</tr>
<tr>
<td>0.5</td>
<td>6.20±0.09</td>
<td>2.55±0.13</td>
<td>1.06±0.05</td>
<td>11.12±0.34</td>
<td>12.92±0.86</td>
</tr>
<tr>
<td>1.58</td>
<td>6.31±0.13</td>
<td>2.72±0.21</td>
<td>1.09±0.09</td>
<td>11.08±0.36</td>
<td>11.67±1.04</td>
</tr>
<tr>
<td>5</td>
<td>5.90±0.12</td>
<td>2.22±0.21</td>
<td>1.05±0.06</td>
<td>11.25±0.26</td>
<td>12.95±0.62</td>
</tr>
</tbody>
</table>

The results represent mean ± SE. There was no significant difference or trend among groups for all the observed end points.
Table 2. Effects of EHMC on body length, wet weight, and condition factor in 5 mph medaka fish of F0 generation

<table>
<thead>
<tr>
<th>Concentration (mg/L)</th>
<th>Male</th>
<th></th>
<th></th>
<th>Female</th>
<th>Wet weight (mg)</th>
<th>Condition factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length (mm)</td>
<td>Wet weight</td>
<td>Condition factor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>28.68±0.44</td>
<td>200.55±0.36</td>
<td>0.90±0.02</td>
<td>25.86±0.38</td>
<td>183.24±5.96</td>
<td>1.04±0.03</td>
</tr>
<tr>
<td>0.05</td>
<td>28.42±0.32</td>
<td>206.68±6.96</td>
<td>0.94±0.02</td>
<td>26.54±0.40</td>
<td>195.06±8.50</td>
<td>1.20±0.06</td>
</tr>
<tr>
<td>0.158</td>
<td>28.79±0.59</td>
<td>228.06±16.88</td>
<td>0.95±0.02</td>
<td>25.6±0.42</td>
<td>201.23±9.06</td>
<td>1.01±0.03</td>
</tr>
<tr>
<td>0.5</td>
<td>28.23±0.48</td>
<td>216.81±11.20</td>
<td>0.93±0.02</td>
<td>25.72±0.45</td>
<td>178.41±28.27</td>
<td>1.11±0.05</td>
</tr>
<tr>
<td>1.58</td>
<td>28.58±0.51</td>
<td>221.60±16.80</td>
<td>0.89±0.01</td>
<td>25.41±0.41</td>
<td>182.38±8.45</td>
<td>1.13±0.05</td>
</tr>
<tr>
<td>5</td>
<td>27.96±0.40</td>
<td>196.28±7.07</td>
<td>0.99±0.02</td>
<td>22.5±0.49</td>
<td>179.73±5.40</td>
<td>1.13±0.03</td>
</tr>
</tbody>
</table>

The results represent mean ± SE. There was no significant difference or trend among groups for all the observed end points.
Table 3. Effects of EHMC on body length, wet weight, and condition factor in 31 dph medaka fish of F1 generation

<table>
<thead>
<tr>
<th>Concentration (mg/L)</th>
<th>Length (mm)</th>
<th>Wet weight (mg)</th>
<th>Condition factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>13.79±0.28</td>
<td>19.38±1.18</td>
<td>0.73±0.01</td>
</tr>
<tr>
<td>0.05</td>
<td>13.91±0.39</td>
<td>21.82±1.75</td>
<td>0.78±0.02</td>
</tr>
<tr>
<td>0.158</td>
<td>14.20±0.21</td>
<td>24.57±1.56</td>
<td>0.84±0.03</td>
</tr>
<tr>
<td>0.5</td>
<td>14.37±0.28</td>
<td>25.43±1.91</td>
<td>0.83±0.02</td>
</tr>
<tr>
<td>1.58</td>
<td>13.66±0.36</td>
<td>21.66±1.75</td>
<td>0.82±0.02</td>
</tr>
<tr>
<td>5</td>
<td>14.2±0.33</td>
<td>25.64±1.93</td>
<td>0.87±0.02</td>
</tr>
</tbody>
</table>

The results represent mean ± SE. There was no significant difference or trend among groups for all the observed end points.
3.2 Thyroid hormones and transcriptional alterations in genes related to thyroid hormone

At juvenile stages in both F0 and F1 generations, thyroid disruption was observed (Fig. 2 and Fig. 3). EHMC induced similar gene transcription patterns at 31 dph in both F0 and F1 generations. EHMC significantly down-regulated transcription levels for \textit{dio2} at 31 dph in both F0 and F1 generations, but not at 10 dph (Fig. 2A). Transcription levels for \textit{trh} gene were up-regulated at 31 dph of F0 and F1 generations (Fig. 2B and C). EHMC significantly down-regulated whole-body T3 concentrations at 31 dph in F1 generation (Fig. 3). Decreasing trends of T4 levels were also observed, but between-group differences were not significant. At adult stages, overall increasing trends of gene transcripts for \textit{dio2}, \textit{thra}, and \textit{thrβ} in the liver were observed in both male and female medaka fish, but there were no significant between-group differences (Fig. 6).
Figure 2. Effects of EHMC on transcription on *era*, *ara*, *vtg1*, *dio2*, and *trh* gene at (A) 10 dph of F0, (B) 31 dph of F0, and (C) 31 dph of F1 medaka fish. The results are shown as mean ± standard error (SE) of four replicates. Asterisk indicates significant difference from control (*p*<0.05 and **p**<0.01). The *p* value of linear regression analysis is shown when significance was observed (*p*<0.1).
Figure 3. Concentrations of (A) triiodothyronine (T3) and (B) thyroxine (T4) in F1 medaka fish at 31 dph. The results are shown as mean ± SE of four replicates except for 3 replicates in 5 mg/L treatment group. Seven to ten fish were pooled to one replicate. Asterisk indicates significant difference from control (*p<0.05 and **p<0.01). The p value of linear regression analysis is shown when significance was observed (p<0.1).
3.3 Sex hormone and transcriptional alterations in genes related to sex hormone and steroidogenesis

Transcriptional levels for *era, ara*, and *vtg1* were not altered at juvenile stages of F0 and F1 generations. Plasma E2 levels were not altered in both adult male and female (Fig. 4). In the liver, transcription levels for *era* and *vtg1* genes were not altered in both adult male and female (Fig. 5). Though increasing trends of *ara* gene transcription were observed in male, there are no between-group differences. Transcriptional levels for *ara* were not altered in female. In ovary, genes involved in steroidogenesis showed down-regulated trends, but between-group differences were not observed (Fig. 5). Decreasing trends of transcription levels for *star, hsd3b*, and *cyp19a* were observed in ovary, but no significant alterations in transcription levels were observed in testis (Fig. 5).
**Figure 4.** Effects on plasma E2 levels in medaka fish at 5 mph. The results represent mean ± SE of 4 replicates. Three medaka fish were pooled to one replicate.
Figure 5. Effects of EHMC on transcription on (A) star, (B) hsd3b, (C) hsd17b3, and (D) cyp19a gene in the gonad of male and female medaka fish at 5 mph. The results are shown as mean ± SE of 5 replicates. Asterisk indicates significant difference from control (*p<0.05 and **p<0.01). The p value of linear regression analysis is shown when significance was observed (p<0.1).
Figure 6. Effects of EHMC on transcription on (A) era, (B) ara, (C) vtg1, (D) dio2, (E) thra, and (F) thrb gene in liver of male and female medaka fish at 5 mph. The results are shown as mean ± SE of 5 replicates. Asterisk indicates significant difference from control (*p<0.05 and **p<0.01). The p value of linear regression analysis is shown when significance was observed (p<0.1).
3.4 Effects of EHMC on fecundity

Concentration dependent reduction in egg reproduction was observed in seven week mating experiment. Significant decrease in the number of eggs per day was observed in all treatment groups at as low as 0.05 mg/L (Fig. 7). For seven weeks, we found no significant differences in number of eggs between control and solvent control group. Mean of number of eggs per day was 16.3 ± 0.5 (± SE) in the control group and 16.3 ± 0.7 (± SE) in the solvent control group. Number of eggs per day in the fish exposed to 5 mg/L of EHMC was decreased by 2-fold compared to the solvent control group.
Figure 7. Effects of EHMC on the (A) number of eggs per brood per day (B) cumulative egg number. The results are shown as mean ± SE of six replicates. One mating pair includes two male and two female medaka fish. Asterisk indicates significant difference from control (*p<0.05 and **p<0.01).
4. Discussion

The results of this study suggest that life-cycle exposure to EHMC reduce reproductive performances in Japanese medaka. Especially, significant reduction of reproduction was observed at as low as 0.05 mg/L (Fig. 7) which is about 10-fold higher than environmental concentrations detected in Lake Cospuden (Rodil et al., 2009). In other aquatic biota such as Scenedesmus vacuolatus and Daphnia magna, reproduction toxicity was observed (Rodil et al., 2009; Sieratowicz et al., 2011). Parameters related to reproductive toxicity of fish including alterations in transcription levels related to sex hormone and histological changes in reproductive organs support our results (Christen et al., 2011; Inui et al., 2003; Christen et al., 2011). Studies on histological changes posed by EHMC in fish support that EHMC has reproductive toxicity potential in fish. EHMC induced histological changes in testes and ovaries of fathead minnow (Pimephales promelas) at 394 µg/L (Nominal concentration was 3 mg/L) (Christen et al., 2011).

EHMC induced thyroid hormone disruption during juvenile stages of Japanese medaka. Down-regulations of thyroid hormones, i.e., whole-body T3 and T4 concentrations, and dio2 transcription levels were observed at this stage (Fig. 2 and 3). T4 is converted to T3, more active form of the thyroid hormone, by dio2. Decrease in T3 levels was followed by decrease in T4 levels and dio2 transcription levels posed by EHMC. These results are in line with previous studies reporting down-regulations of plasma T4 levels and
hepatic DIO1 activity in other vertebrates, e.g. rodents (Axelstad et al., 2011; Klammer et al., 2007). Transcription levels for trh were increased at 31 dph of both F0 and F1 generations (Fig. 2). Up-regulations of the trh gene might be possibly due to compensatory mechanisms for decreased thyroid hormone levels, suggesting that the biological responses of EHMC could be different during later life stages by feedback mechanisms. In adult stages, transcription of dio2 gene showed increasing patterns, though between-group difference was not significant. The patterns were different from those during juvenile stages. It might be due to compensatory mechanisms or different life stages. Thyroid hormones play crucial roles in development and maintenance of physiological functions. Especially, availability of thyroid hormones is important in organ development during early life stages. However, growth parameters including body length, body weight, and condition factor were not altered in this study, though T3 concentrations were significantly decreased. Previous study has shown that thyroid hormone deficiency in medaka (Oryzais latipes) eggs did not influence body length, body weight, and condition factor at 16 dph (Tagawa and Hirano, 1991). Therefore, this species, Japanese medaka, might not necessarily need thyroid hormones for growth development during larvae stages. Though decreased thyroid hormone levels were not associated with growth in this study, we could not rule out that EHMC has influences on other development functions such as organ maturation. Thyroid hormones as well as sex hormones play key roles in sexual maturation, especially during early life stages. Previous studies
indicate that thyroid hormones are involved in gonadal development of fish (Cyr and Eagles, 1996; Castañeda Cortés et al., 2014). T3 is involved in synergizing with gonadotropin (GTH) to enhance ovarian development in female fish during early stages of oocyte maturation (Cyr and Eales, 1996), and influence the development of tests in male fish (Castañeda Cortés et al., 2014). Decreased availability of thyroid hormones might contribute to retardation in sexual maturation, which could partly explain decreased reproductive performances at adult stages. The expressions of \textit{dio2} were reported for decrease with gonadal maturation, suggesting its important roles in gonadal development in vertebrates (Castañeda Cortés et al., 2014). Therefore, down-regulations of thyroid hormones and \textit{dio2} gene support the idea that thyroid endocrine disrupting effects of EHMC during early life stages could influence gonadal development, which leads to reducing reproductive performances. Thyroid differentiation of the medaka fish occurs at least partially before hatching, and uptake of iodine at 10 dph (Egawa et al., 1980). The number of TSH cells and thyroid follicles increased during this stage (Egawa et al., 1980), which means that these rapidly developing phases can be vulnerable to chemicals. Considering thyroid hormone functions in gonadal development and rapidly developing phases during early life stages, thyroid hormone disruption of EHMC might have influences on reproduction in later life stages. We could not measure thyroid hormone concentrations during adult stages, but continuous exposure to EHMC can influence reproduction system. Therefore, thyroid disruption during adult stages can be
also associated with reduced reproduction, and it needs further investigations.

In the present study, plasma E2 levels were not altered in both male and female medaka (Fig. 4), but slightly decreasing trends of genes related to steroidogenesis in ovary were observed (Fig. 5). Down-regulated trends of star, hsd3b, and cyp19a genes in ovary were observed, but not in testis (Fig. 5). This is in line with the previous study showing a down-regulated tendency of 3β-hsd transcription in ovary of fathead minnow (Christen et al., 2011). We observed no alterations in vtg1 gene transcription in the liver of female and male. However, previous studies have shown that 7 d exposure to 9.87 mg/L of EHMC induced significant up-regulations of transcription levels for vtg1 in male medaka after (Inui et al., 2003), and overall increasing patterns were observed in both male and female fathead minnow after 14 d exposure (Christen et al., 2011). 14 d exposure to EHMC induced increase in transcription levels for vtg1 in male zebrafish (Zucchi et al., 2011). This might be partly explained by different experimental species, exposure duration and concentrations. Though slightly decreasing trends in genes related to steroidogenesis were observed in ovary, there are some limitations to support reduced reproductive performances even at the lowest concentration. Therefore, we cannot rule out the possibility that other endocrine axes such as thyroid axis may also play crucial roles.

We observed the toxicity of EHMC on thyroid function and reproduction of Japanese medaka after life-cycle exposure. To our knowledge, this is the first study to identify reproductive toxicity and its underlying mechanisms of
EHMC after life-cycle exposure in fish. Reduced reproductive performances were observed even at the lowest concentration, environmentally relevant concentration. While disruption of sex hormones in adult fish was not noted, EHMC exposure could alter the thyroid hormone balance at juvenile stages of both F0 and F1 fish. Thyroid hormones which play crucial roles in early gonadal development were decreased during juvenile stages, and endocrine axes interact with each other and directly or indirectly influences reproductive health outcomes. However, the link between thyroid hormone disruption and reproduction damages is not clear. Therefore, ecological risks of this popular sunscreen chemical and relationship between thyroid disruption and reproductive toxicity warrant further investigations.
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국문초록

EHMC의 장기 노출에 따른 일본산 송사리의 생식 및 내분비계 교란 영향

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Ethylhexyl methoxycinnamate(EHMC)는 화장품 등 일상 생활용품에 자외선 차단 성분으로 사용되는 물질로, 사용하는 빈도가 높아 환경과 생활에 비해히 교란된다. 자외선 차단 성분의 내분비계 교란 영향은 선행 연구들 통해 밝혀져 왔으며, 이로 인한 보건적 우려가 제기되다 가 있다. 이에 본 연구에서는 EHMC의 생식 및 이와 관련한 내분비계 교란 영향을 심화적으로 도출하기 위하여 알아보고자 한다. 이를 위해 일본산 송사리를 EHMC에 장기 노출 시켜 성호르몬 및 갑상선 호르몬 관련 영향을 알아보고자 하였다.

수정된 지 약 1주가 지나지 않은 송사리 알 20개를 50 mL 한 비커에 담아 0, 0.05, 0.158, 0.5, 1.58, 5 mg/L 농도로 EHMC를 노출시켰다. 부화한 지 10일, 31일 되는 시점에서 성호르몬 및 갑상선 호르몬 관련 영향을 알아보기 위하여 원자 수를 수정하였다. 유전자 분석 후 남은 알들을 성어가 될 때까지 보호하여, 부화한 지 99일이 되는 시점에서 암컷과 수컷 각 2마리씩을 한 반복군으로 두어 자극을 지었다. 이후 수정된 알을 EHMC의 생식 및 이와 관련한 영향을 알아보기 위해 7주에 걸쳐 매일 세대화하였다. 수정후 147일째(5개월째) 되는 날을 세대 송사리의 간과 생식선에 성호르몬 및 갑상선 호르몬 관련 유전자들을 분석하였으며, 이 세대에서 남은 알을 이용하여 2세대 early-life stage (ELS) 독성 실험을 수행하였다.

부화한 지 31일 되는 시점에서 유전자 분석을 수행한 결과 dio2 유전자의 통계적으로 유의한 감소를 확인할 수 있었다. 이러한 결과는 적절한 간은 감소가 관찰되지 않았다. 성어의 간에서 dio2, thra, thrβ
유전자 증가하는 경향성을 관찰되었으나, 집단간 유의미한 차이는 관찰되지 않았다. EHMC 노출로 인해 부화한 지 31일 된 송사리의 갑상선 호르몬 T3와 T4를 감지하였으나, ea, ara, vtg1 유전자의 유의미한 수준의 변화는 이 시기에 관찰되지 않았다. 또한 성모 단계에서 암, 수 송사리의 갑상선에서 분석한 ea, ara 유전자의 변화는 관찰되지 않았다. 생식선에서 스테로이드 함성에 관여하는 유전자 수준의 변화는 양액에서 감소하는 경향성이 관찰되었으나, 집단간 유의미한 차이는 발생되지 않았으며, 수액에서는 이러한 경향성이 관찰되지 않았다. 성모에서 성호르몬 E2의 통계학적 유의미한 차이는 암, 수 모두에서 확인되지 않았으나, 생식 독성 지표 사용되는 알 수 농도의존적으로 감소한 것이 확인되었다. 이는 본 연구에서 가장 낮은 농도인 0.05 mg/L 수준에서도 확인되었다.

일본산 송사리에 EHMC를 장기 노출시킨 결과 실험 중 가장 낮은 농도인 0.05 mg/L 수준에서도 알 수가 감소하는 것을 확인할 수 있었다. 성모와 수액에 관한 지표에서 통계적으로 유의미한 결과를 확인할 수 없었으나, EHMC의 갑상선 과할 영향, 특히 여리 시기에 미치는 결과는 확인할 수 있었다. 본 연구를 통해 EHMC의 장기 노출에 따른 생식 및 갑상선 과할 영향을 확인할 수 있었다. 갑상선 호르몬 과할 생식 독성의 관계를 단지로 두 번째로 알 수 없으며, 갑상선 호르몬이 생식선 발달에 미치는 영향을 더욱 뚜렷하게, 갑상선 과할 영향으로 인한 생식 독성을 배제할 수 없다. 따라서 EHMC의 이러한 내분비계 과할 영향 및 생식에 관한 연구에 관한 후속 연구가 필요할 것으로 사료된다.

주요어: EHMC, 여성 생식, 갑상선, 장기 노출
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