Di-(2-ethylhexyl)-phthalate'e Maruz Kalan Alburnus tarichi (Güldenstädt, 1814) Erken Larvalarında Östrojen Reseptör ve P450 Aromataz mRNA Seviyeleri

Levels of Aromatase and Estrogen Receptors mRNA in Early Larvae of Alburnus tarichi (Güldenstädt, 1814) Exposed to Di-(2-ethylhexyl)-phthalate

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ÖZ

Amaç: Bu çalışmanın amacı Alburnus tarichi'nin erken larval gelişimi üzerine fitalatın etkisini belirlemektir.

Yöntem: Erken larval dönemde (yumurtadan çıktıktan 2 gün sonra) 0.1, 1, ve 10 µg/L fitalat'a, 6 gün, maruz bırakılarak larvalarda östrojen reseptörleri alfa, beta-1 ve beta-2 ve aromataz B ve A'nın mRNA seviyeleri ölçüldü.

Bulgular: Fitalat'ın her üç konsantrasyonunda östrojen reseptör alfa, beta1, beta2 ve aromataz B'nin mRNA seviyelerinde belirgin bir değişiklik olmadığı belirlendi. Bununla birlikte aromataz A mRNA seviyesi fitalat'ın 0.1 µg/L uygulamasında belirgin olarak artarken 1 ve 10 µg/L uygulamalarda değişiklik gözlenmedi.

Sonuç: Bu sonuçlar, fitalat'ın çevresel konsantrasyonlarının kısa sureli uygulamalarda, Alburnus tarichi erken larval gelişimi sırasında, steroid sentezindeki genleri önemli seviyede etkilemediğini göstermiştir. Bununla birlikte fitalat'ın gen ekspresyonu üzerindeki etkisini belirlemek için, dış beslenmeyi de içine alan uzun süreli uygulamalı çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: Alburnus tarichi, Di-(2-Etilhekzil)- fitalat, Östrojen Reseptör, Gen Ekspresyonu, P450 Aromataz.

ABSTRACT

Objective: The aim of this study is to determine the effect of phthalate on the early larval development of Alburnus tarichi.

Methods: The larva (2 days after hatching) of Alburnus tarichi were exposed to 0.1, 1, and 10 µg/L phthalate for 6 days (during yolk sac nutrition), and the levels of estrogen receptor alpha, beta-1 and beta-2, and aromatase B and A mRNA levels were measured.

Results: No difference was found in the estrogen receptor alpha, beta-1, beta-2, and aromatase-B mRNA levels with all 3 concentrations of phthalate. However, the aromatase-A mRNA level was significantly increased with 0.1 µg/L of phthalate, while no changes were observed with 1 and 10 µg/L of phthalate.

Conclusion: These results suggest that short-time expose to environmentally relative concentrations of phthalate do not significantly affect genes in steroid synthesis during the early larval development of Alburnus tarichi. However, long-term applied studies including external nutrition are need to determination the effect of phthalate on steroid gene expression.

Key words: Alburnus tarichi, Di-(2-ethylhexyl)-phthalate, Estrogen Receptor, Gene Expression, P450 Aromatase.

1. INTRODUCTION

Endocrine disrupters are a large group of man-made chemicals that enter the environment in different ways, such as industry, wastewater treatment plants, and agricultural and human waste, and they can have a serious impact on humans and wildlife by interfering with their endocrine functions.

Di-(2-ethylhexyl)-phthalate (DEHP) is widely used as a plasticizer in flexible vinyl products. Plastics may contain from 1% to 40% DEHP by weight and are used in consumer products, such as food packaging materials, children's toys, container for blood products and transfusion, insecticide carries, liquid detergents, component of cosmetic products, clothing and car products, and so on. Hence, DEHP is the most comment pollutant chemical of our general environment, and it has the potential to accumulate in soil, sediments, underground water, and air due to its low soluble and vaporization abilities. Domestic and industrial wastewater treatment plants (1) in landfill leachates and the effluent from leachate treatment facilities (2) are the major source of DEHP contaminants in fresh water such as rivers and lakes. Most studies have shown that DEHP has been found to exist in freshwater and sediments (3,4), as well as in agricultural soils (5).

Studies have shown that DEHP causes adverse effects on human reproduction and health (6,7). Di-(2-ethylhexyl)-phthalate has an anti-androgenic activity and leads to reproductive abnormalities by decreasing the testosterone synthesis in fetal testis (8). In fish, mixed results have been reported regarding the effects of DEHP on reproduction. Di-(2-ethylhexyl)-phthalate has been shown to weaken estrogenic activity in fish cell culture (9). In Japanese medaka (Oryzias latipes), DEHP has shown anti-estrogenic activity in female fish, while no adverse effects have been observed in male fish (10,11). However, some studies have shown that DEHP has adverse effects on the reproductive system of male fish (12,13). In general, these effects depend on the concentration of the DEHP and exposure time. However, fish are more sensitive to DEHP during early development than in the adult stage (14-17).

Estrogen receptors (ERα, ERβ1, and ERβ2) and P450 aromatase (P450arom) isomers (CYP19A and CYP19B) have a more important role in gonadal development during early embryonic life in fish (18-20). Endocrine disrupting chemicals (EDCs) can alter gene expression via endogen receptors. Wildlife and laboratory studies have shown that EDCs are response for reproductive abnormalities (21- 24). CYP19 is a key enzyme in the steroidogenesis pathway, which is catalyzed by the conversion of androgen into estrogens. In all of the studied fish, there are 2 different P450arom isomers (brain-derived CYP19B and ovary-derived CYP19A), and they have different structures and functions (25,26). Changes in the ERα, Vtg, and P450 aromatase (especially Cyp19b) mRNA levels are an accepted indicator of environment xenoestrogens (27,28). In our previous study, DEHP was indicated in sediment from Lake Van (29).

The aim of this study was to indicate the effects of ERα, ERβ1, ERβ2, CYP19B, and CYP19A on gene expression during the larval development (during yolk sac feeding) of Alburnus tarichi. A. tarichi is an endemic cyprinid species to Lake Van Basin, in Turkey, and only this fish has adapted to Lake Van which is an alkaline lake with a Ph of 9.4–9.8.

2. MATERIALS AND METHOD

Chemicals

The DEHP (purity ≥99.5%) used in the experiments was purchased from Sigma Aldrich, St. Louis, MO, USA). A stock solution of 1 mg/mL in dimethylsulfoxide was prepared. This solution was further diluted with rested tap water to nominal concentrations of 0.1, 1, and 10 µg/L of DEHP prepared immediately before use. Reproductively mature female and male A. tarichi were collected from Karasu river (May 12, 2013) and transferred to the lab. Fish were keep in a big tank with airing using dechlorided water (60 L) at room temperature (17 °C) and at natural a light/dark photoperiod of 13:11 for 1 day to be relaxed. The fertilizing and embryonic developing were carried out in a small aquarium, as previously described (30). The hatching larvae were transferred to in glass beakers containing 250 mL water. A total of 100 larvae were put into each beaker and they were exposed to nominal concentrations of 0.1, 1, and 10 µg/L of DEHP in aired conditions at room temperature. In addition to the water control, a solvent vehicle control (2.5 µL) was also added. The treatment of 1 and 2 and days old larvae was canceled because one larva was died 1 day on third day. So, it was used 2 days old larvae. The treatments of each concentration and the controls were performed in duplicate with replacement of the media every 24 h. When the yolk proteins were almost completely absorbed and before starting external nutrition, the treatment was stopped (after 6 days). For each sample, 18 larvae were pooled and treated with RNAlater (Sigma), frozen at –80 °C, and shipped to Boston University, Boston, MA, USA on dry ice and stored at –80 °C until RNA extraction was performed.

RNA Extraction and Gene Expression Analysis

The total RNA was extracted from frozen larvae using Trizol (Sigma Aldrich St. Lous, MO) according to the manufacturer's instructions. RNAs were run on a 1% agarose gel to assess the quality, and the total RNA was quantified using a Nanodrop (Thermo Fisher Scientific). cDNAs were obtained with 5 µg of total RNA using SuperScript II transcriptase and oligo (dT)18 primer according to the manufacturer's instructions (Invitrogen).

Real-time quantitative PCR (qPCR) primers, which were previously designed, were used (29). An amplification efficiency value was obtained for each primer using serial dilutions of the sample. The reverse transcribed mRNA was measured by the qPCR using target-specific assays. The qPCR was performed on an ABI Prism 7900HT sequence detection system (Applied Biosystems) with a SYBR® green fluorescent label. β-actin, the reference gene, was used as an internal control to normalize the mRNA expression values.

Data Analysis

Data deduced by the qPCR were first analyzed using the Applied Biosystems Sequence Detection System 2.2.1. Analyses were conducted with qGene to normalize the data obtained. Relative quantification was performed by a modified comparative critical threshold method that corrects for different PCR amplification efficiencies among primer pairs (31). The normalized gene expression is given as the mean normalized expression (MNE) = (EPPlmeanCTPP1)/E ERαmeanCTERα), where E = the PCR efficiency (E = 10-(1/slope)) and the mean threshold cycle (CT) is the average CT from the 3 replicates (32). Data were rejected if the SEM

percentage was greater than 20%. The average MNE was determined for each set of replicates obtained from an individual animal, and the standard deviation was calculated for each SEM of the MNE. The MNEs were then averaged for each group analyzed. Statistical analyses (Student's t test and 1-way ANOVA) were performed using the SAS 9.2 package. Significance was set at $P < 0.05$.

3. RESULTS

We measured the ERα, ERβ1, ERβ2, CYP19B, and CYP19A mRNA levels of larval of A. tarichi exposed to concentrations of 0.1, 1, and 10 µg/L of DEHP. No differences were found between just the water and with the solvent controls. The ERβ1 mRNA transcript did not change at all with the treated concentrations ($P>0.05$). The levels of ERα and ERβ2 mRNA increased with 0.1 and 1 µg/L of DEHP, but they were not significantly important (Figure 1; $P>0.05$). Moreover, no differences were found in the expression of CYP19B with any of the treated concentrations. However, the level of CYP19A mRNA significantly increased with 0.1 µg/L of DEHP, while no difference was found with 1 and 10 µg/L of DEHP (Figure 2; $P>0.05$).

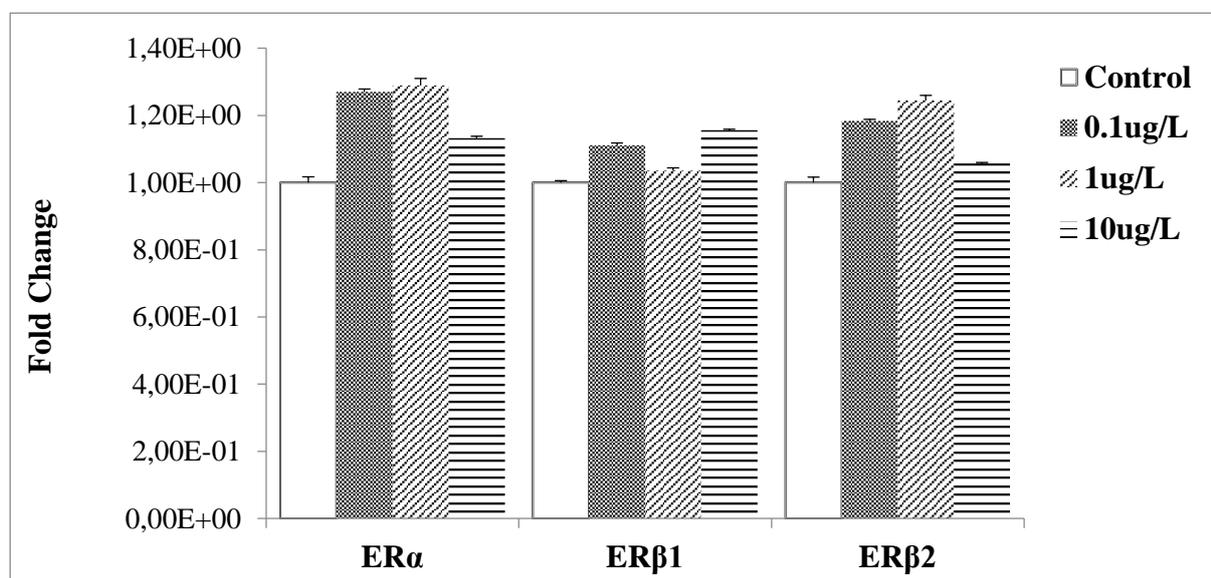**Figure 1.** Relative mRNA transcript expressions of the ER of early larval *A. tarichi* exposed to 0.1, 1, and 10 µg/L for 6 days. Gene transcription changes are expressed as the fold change relative to the corresponding control. The results are the mean ± standard error of the mean of 4 samples. $P < 0.05$ indicates a significant difference between the DEHP exposure groups and the corresponding control.

4. DISCUSSION

The present study used early larval A. tarichi to assess the impacts of environmentally relevant concentrations of DEHP. For this, the gene transcriptions of the ERs and aromatase isomers were measured. We found that DEHP at low concentrations with short-time exposure did not affect the gene expression of the ERs and CYP19B in A. tarichi larvae. However, the CYP19A gene was sensitive with 0.1 µg/L of DEHP. DEHP was first accepted as an EDC with weak estrogenic activity using an in vivo assay by Jobling et al. (9). Phthalate ester plasticizers are accepted as anti-androgenic in mammals (8). There are mixed results in fish. However, the
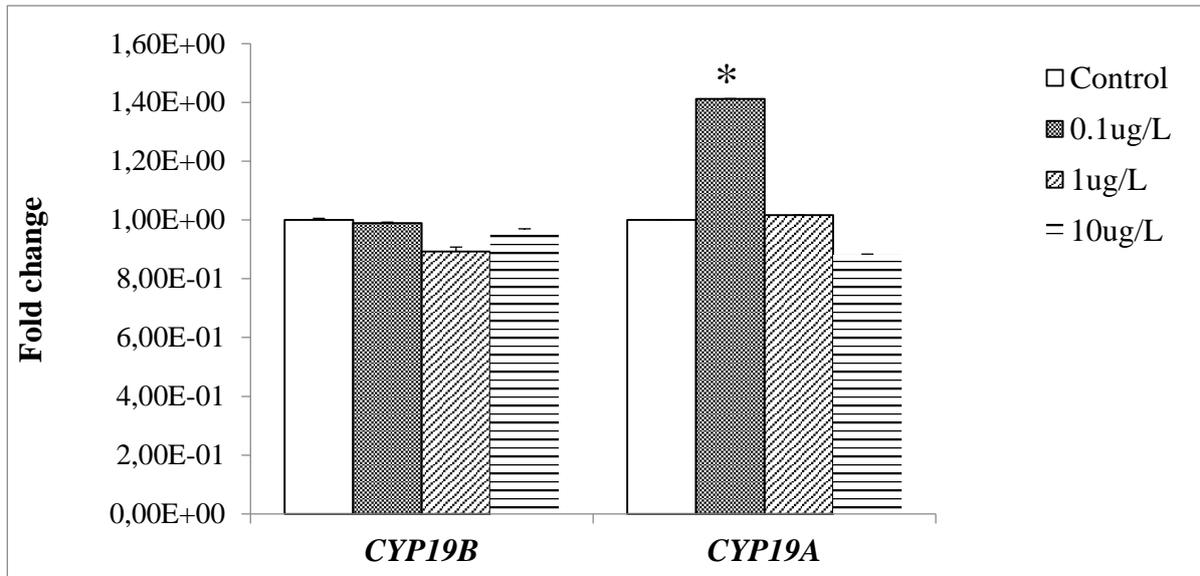
Figure 2. Relative mRNA transcript expressions of the CYP19-arom of early larval *A. tarichi* exposed to 0.1, 1, and 10 µg/L for 6 days. Gene transcription changes are expressed as the fold change relative to the corresponding control. The results are the mean ± standard error of the mean of 4 samples. $P < 0.05$ indicates a significant difference between the DEHP exposure groups and the corresponding control.

results in the studied fish have shown that DEHP has had adverse effects on embryos, growth, behavior, and reproductive ability depending on the concentration and exposure time (11,12,15,16,33,34). We used 2-day larvae in the experiment because one of the one day larvae died in 10 µg/L concentration. Fish are more sensitive to the effects of chemicals during early developmental stages compared to maturity. However, in male zebrafish (35), male fathead minnow (36), and Chinese rare minnow (37), the expressions of ERs and aromatase mRNA transcripts did not change with environmentally relative concentrations (12 and 12.8 µg/L) of DEHP. Similar to these results, no difference was found in the mRNA transcript levels of ERα, ERβ1, ERβ2, and CYP19B in early larval A. tarichi. However, a significant increase in CYP19A of 0.1 µg/L was observed. This increase may be related gonadal development. Generally, CYP19B plays a role in sex differentiation in early embryonic development in fish (38), while CYP19A is involved in ovarian differentiation (39,40) and gametogenesis (41). Moreover, CYP19B expression (not CYP19A) is up-regulated by E2 and environment xenoestrogens (25,27,42) because of CYP19B has estrogen-responsive elements (43). Hence, laboratory and wildlife studies have shown that the changes in Cyp19b indicate estrogenic activity or contaminations. In the present study, the CYP19A mRNA transcript increased in larval A. tarichi exposed to 0.1 µg/L DEHP. However, the CYP19B mRNA transcript remained unchanged in A. tarichi. Our results support the Wang et all. (37)'s hypothesis that DEHP changes plasma sex hormone levels at higher concentrations (>39.4 mg/L) than environmental concentration. We can also say that short-term exposure to environmental concentrations of DEHP did not directly affect estrogenic or anti-estrogenic activity during the early larval life of A. tarichi. However, it may induce gonadal development during early development life in A. tarichi. Therefore, further research is required to indicate the long-term effects on the life and gonadal development in fish exposed to DEHP.

5. CONCLUSION

Gene transcription measurements of the ERs and CYP19arom isomers indicated that 6 days of exposure to environmentally relevant concentrations of DEHP did not result in altered estrogenic or anti-estrogenic effects during the early larval life of A. tarichi.

Acknowledgments

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