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RESEARCH PAPER

Effects of Hexachlorocyclohexane (HCH-γ-Isomer, Lindane) on the Reproductive System of Zebrafish (*Danio rerio*)

Hayati Yuksel[1], Ünal Ispir[2], Aykut Ulucan[1], Cebrahil Turk[3], M Reşit Taysı[4]

[1] Bingöl University, Department of Medical Services and Technics, Vocational School of Health Services, 12000 Bingöl, Turkey.
[2] Inönü University, Faculty Fisheries, Malatya, Turkey.
[3] Bingöl University, Department of Crop and Animal Production, Genç Vocational School, 12000 Bingol, Turkey.
[4] Bingöl University, Department of Fisheries, Faculty of Agriculture 12000 Bingöl, Turkey.

* Corresponding Author: Tel.: +90.426 2160012-15; Fax: +90.426 2160028;
E-mail: hyuksel@bingol.edu.tr

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Abstract

In this study, histopathological changes induced by lindane a gamma isomer of hexachlorocyclohexane and its intoxication associated with reproductive system and hormone levels were investigated in zebrafish. In all, 320 zebrafish adults approximately 1 year in age, (*Danio rerio*) obtained from a commercial entity were used in the study. Zebrafish were divided into 8 groups, each one containing 40 zebrafish.

Groups were organized as 0 (the control group), 1 ml/L methanol, and 5, 10, 20, 40, 80, 160 µg/L/day lindane. In the study, the gamma-HCH-isomer was applied at rates of 5/10/20/40/80/160 µg/L/day as doses of immersion to each group for 21 days. Macroscopically, the drop in egg production was observed, especially in the 80 and 160 µg/L/day groups of female zebrafish. A microscopic decrease in ovulation and biochemical decreases in estradiol (E_2) levels (40/80/160 µg/L) were also observed in the female zebrafish. As a result, lindane was found to cause changes in the reproductive system, and consequently, to cause hormonal disorders and to have significant effects on ovulation and fertilization in the female zebrafish.

Keywords: Zebrafish, ovulation, fertilization, histopathology.

Hexachlorocyclohexane (HCH-γ-Isomer, Lindane)'nın Zebra Balıklarının (*Danio rerio*) Üreme Sistemi Üzerine Etkisi

Özet

Bu çalışmada, HCH-γ-Isomer olan "Lindane" intoksikasyonu sonucu zebra balıklarının reproduktif sisteminde meydana gelen histopatolojik değişimler ile hormonal düzeyleri incelendi. Çalışmada ticari bir işletmeden temin edilen 320 adet, yaklaşık 1 yaş grubu, Zebra balığı (Danio rerio) kullanıldı.

Balıklar her grupta 40 balık olacak şekilde 8 gruba ayrıldı. Gruplar 0 (Kontrol grubu), 1ml/L methanol, 5, 10, 20, 40, 80, 160 µg/L / gün lindane olarak oluşturuldu. Çalışmada deneme gruplarına 1 ml methanolde çözülen HCH-γ-Isomeri sırasıyla 5/10/20/40/80/160 µg/L / gün dozlarında her gruba 21 gün boyunca immersiyon şeklinde uygulandı. Makroskobik olarak dişi zebra balıklarının özellikle 80/160 µg/L gruplarında yumurta sayısında azalma görüldü. Mikroskobik olarak dişi zebra balıklarında ovulasyonda azalma, biyokimyasal olarak da Estradiol (E_2) seviyelerinde (40/80/160 µg/L) düşme saptandı. Çalışma sonucunda, Lindane'nın dişi zebra balıklarının reproduktif sisteminde bozukluğa ve hormonal değişimlere sebep olduğu, ovulasyon ve fertilizasyon üzerinde önemli etkilerinin bulunduğu gözlendi.

Anahtar Kelimeler: Zebra balığı, ovulasyon, fertilizasyon, histopatoloji.

Introduction

Organochlorine compounds are used as pesticides, insecticides, herbicides, and fungicides in agriculture and forestry, as well as for various purposes in humans or animals (Samanta and Chainy, 2002; Choudhary and Joshi, 2003). Among these compounds, the hexachlorocyclohexane (HCH-γ-isomer, lindane) has different isomers and is an insecticide group studied by researchers in many industrialized countries in terms of the environment and human health (Dalsenter *et al*., 1997; Walsh and Stocco, 2000; Raizada *et al*., 2001; Bretveld, *et al*., 2006; Lu *et al*., 2014).

The endocrine disrupting chemicals, which are common in nature, disrupt the normal functioning of the hormonal balance of humans and wildlife. It is reported that especially wildlife is susceptible to endocrine disrupting effects of pesticides. Most of these pathological effects are related to exposure to

organochlorine pesticides and influence the reproductive function (Mnif *et al*., 2011).

Lindane is highly toxic to fish, bees, and aquatic invertebrates. It is very stable in both fresh and salt water environments. It will disappear from the water by secondary mechanisms such as adsorption on sediment, biological breakdown by microflora and fauna, and adsorption by fish through gills, skin, and food (Lawson *et al*., 2011). Although no clinical signs have been observed in aquatic organisms subjected to lindane toxicity, lindane toxicity affects the endocrine system of fish as a result of biochemical changes, resulting in hormonal imbalance due to the breakdown of steroidogenesis in the ovary, and a significant decrease in egg production (Singh *et al*., 1993; Ensenbach and Nagel, 1997; Pesando *et al*., 2004; Consiglio *et al*., 2009; Lawson *et al*., 2011; Mnif *et al*., 2011).

Due to the similarity of its genome structure with humans and other vertebrates, and almost the same metabolism and embryonic development, the zebrafish became a test organism utilized frequently in toxicology studies (Kimmel *et al*., 1995; Lele and Krone, 1996; Belair *et al*., 2001; Andreasen *et al*., 2002; Stern and Zon, 2003; Bello *et al*., 2004; Balasubramani and Pandian, 2008; Lawson *et al*., 2011; Chang *et al*., 2013; Lu *et al*., 2014). Therefore, in the present study, the effects of lindane, which causes serious problems in the reproductive system of mammals, were examined histologically and biochemically on the reproductive system of zebrafish.

Materials and Methods

One-year-old mature male and female zebrafish (*Danio rerio*), obtained from a commercial enterprise, were used. The fish were put in continuously aerated 12-liter aquariums, each with 28 ± 1°C water temperature at pH 7. Every other day, one-third of the water was changed. Ambient temperature, pH, and oxygen levels were monitored and kept under control. To establish the photoperiod of the environment of the aquarium, 12 hours of light and 12 hours of dark was provided. Before starting experiment, the fish were fed 3 times daily for 2 weeks with a commercial aquarium fish food for adaptation to the environment.

Experimental Plan

During the study, the fish were divided into 8 groups of 40 fish each (20 male, 20 female zebrafish) in 12-liter polyethylene tanks in the Bingol University, Faculty of Agriculture, Department of Fisheries Laboratory. In the study, the doses of Lindane to be administered to groups were determined according to the previous study conducted by Ensenbach and Nagel, 1995; (for 48 hours LD_{50} 0.14 mg/L and for 96 hours LD_{50} dose of 0.11 mg/L for zebrafish). Other than the control group, 1 ml/L methanol, and 5/10/20/40/80/160 µg/L/day lindane were given to each group separately. The HCH-γ-isomer (Lindane, Aldrich, CAS Number: 58-89-9) dissolved in 1 ml of methanol was applied to the fish kept in a bath for 21 days with doses of 5/10/20/40/80/160 µg/L/day, respectively, in the study. The fish were kept under observation during the entire day, and clinical changes were recorded.

To collect eggs, 3 female and 9 male fish from each group were put into a separate aquarium. Small cages made of nylon tulle with 2-mm mesh were placed in aquariums to the prevent fish from eating their own eggs. The eggs in this aquarium were collected and counted by a siphoning method and taken to a separate medium to complete their development. The number of unfertilized and fertilized eggs, which were kept in a separate environment, the hatching rate, changes in embryonic and larval stages, and the teratogenic effects were observed through a stereo microscope with digital camera.

At the end of the experiment, systemic necropsy was performed on the surviving fish in the control and experimental groups after a benzocaine hydrochloride (benzocaine HCL) bath anesthesia (250 mg/L) (Neiffer, *et al*., 2009). The gonads were taken from 20 fish (10 males, 10 females) in each group, and homogenized in 1 ml saline. The homogenate was then centrifuged and the supernatants were stored at -20°C to be used to perform the hormone analysis. The estradiol (E_2) and testosterone (T) levels were biochemically measured using the competitive ELISA kits (abcam, ab108666, ab108667, Germany) in the collected supernatants. The gonads of other fish that underwent necropsy were evaluated macroscopically and fixed in neutral formalin solution with 10% buffer. Later, cross-sections of 5-micron thickness were taken by microtome through the paraffin blocks from the fixed tissues and stained with hematoxylin and eosin, then examined under a light microscope (Anderson, G. and Bancroft, J. 2002).

Statistical Evaluation

The t-student test was used for the obtained group averages and the determination of standard deviation. The one-way ANOVA test was performed using Minitab 14 statistical software to demonstrate the differences in the number of eggs and the hormone analysis values of fish among the groups. A difference of $P \leq 0.01$ and $P \leq 0.05$ was considered significant.

Results

In this study, the histopathological changes in the reproductive system and hormone levels of the fish were analyzed after applying 0, methanol at 5, 10, 20, 40, 80, and 160 µg/L/day lindane in a bath for 21 days to the fish separated for reproduction.

Clinically, deaths (6 zebrafish) were observed only in the 160 µg/L experimental group on the 2nd day throughout the study, and there were no other clinical findings in the control and other experimental groups. At the end of the experiment, no noticeable macroscopic findings were found in the reproductive system of the systemic necropsied Zebrafish in any experimental group.

Histologically, the 4th and 5th stages of oocytes were found common in the control group of zebrafish **(Figure 1 A)**. In most cases, the 3rd and 4th stage oocytes were identified in addition to partly atretic follicles in the groups with 5, 10, 20 µg/L/day lindane administered, whereas in the groups with 40, 80, and 160 µg/L/day lindane, the 2nd and 3rd stage oocytes and atretic follicle were common; however, the number of mature oocytes had decreased (Figures 1 B, C, D).

Throughout the experiment, counting the fish eggs collected by the siphoning method was performed under a stereo microscope. For the egg counts, the average number of eggs was 485 both in the control group and in the methanol groups. According to the egg counts, it was observed that the difference in the number of eggs was statistically significant: the number of eggs in the experimental groups was decreased significantly, especially in the 80 - and 160-µg/L groups, in line with the increasing dose of lindane (Figure 2.). After counting and storing in a separate medium, the eggs were monitored for 3 days (72 hours). In the counting larvae that was performed under a stereo microscope to determine the hatching rates, it was observed that the average numbers of larvae from eggs in the control and methanol groups were 405 (83.45%) and 377 (81.2%), respectively. And, in the experimental groups, it was observed that the average number of hatched larvae was reduced. In line with these results, examining the comparison of the experimental groups with the control and methanol groups, it was observed that the number of hatched larvae and number of eggs were decreasing gradually, and there was a significant difference in this rate ($p \leq 0.05$) in the 40, 80 and ($p \leq 0.01$) in the 160 µg/L groups .

No teratogenic changes were observed in the fish, either in the eggs or in the larval period or afterward.

In the biochemical and hormonal evaluation of the female zebrafish, it was observed that the levels of estradiol (E_2) were decreased in the experimental groups compared with the control and methanol groups, and that these decreases were statistically significant, especially in the 40, 80, and 160 µg/L/day groups ($p \leq 0.05$) (Figure 3.). No significant change was observed in the testosterone (T) levels of female zebrafish in the experimental groups as compared to the control and methanol groups. Further, in the biochemical and hormonal evaluation of male zebrafish, it was observed that the levels of estradiol (E_2) were decreased in the experimental groups compared with the control and methanol groups, and that these decreases were statistically significant, especially in the 80–160 µg/L/day Lindane groups ($P \leq 0.05$). However, no significant difference was observed in the testosterone (T) levels of male zebrafish between the experimental groups and the control and methanol groups.

Discussion

Several endocrine-disrupting chemicals, such as pesticides, can interact with the female reproductive system and lead to endocrine disruption (Bretveld *et al*., 2006; Costa *et al*., 2014). It has been reported that, depending on the lindane toxicity in fish, inhibition of ovarian steroidogenesis was found together with the affected reproductive system, and consequently, hormonal imbalances and significant decreases in egg production were observed (Singh *et al*., 1993; Ensenbach and Nagel, 1997; Mnif *et al*., 2011).

Mills and Chichester (2005) have pointed out that alterations in plasma sex steroid concentrations may have resulted from several different mechanisms of action, including direct effects on steroidogenic enzymes or through indirect modifications associated with altered feedback loops. Mechanisms of action of organochlorine compounds can trigger two types of response: mimicking hormonal action, as an agonistic effect, or leading to a lack of response and preventing the binding of the natural hormone, an antagonistic effect (Mnif *et al*., 2011; Costa *et al*., 2014). It was reported in other studies that organochlorine compounds did not lead to a significant change in the testosterone and estradiol (E_2) levels in male zebrafish, whereas the levels of estradiol and testosterone in female zebrafish had decreased (Singh *et al*., 1993; Chang *et al*., 2013). In the present study, a statistically significant decrease was observed in the comparison of estradiol (E_2) levels between the control+methanol groups and the 40, 80, 160 µg/L/day groups in female zebrafish, and the 80 and 160 µg/L/day groups in male zebrafish. No significant difference was observed in the comparison of testosterone (T) levels with the control and methanol groups between male and female zebrafish.

At the same time, it was reported that the estradiol and testosterone affected the amount of eggs during gametogenesis in female zebrafish (Chang, 2013). In that study, effects of the organochlorine chemicals on the reproductive system in female zebrafish were investigated; it was reported that organochlorine chemicals significantly decreased the egg production. (Chang, 2013). Histopathologically, in the present study, we observed a reduced number of mature oocytes and decreased number of eggs in the groups with 40, 80 and 160 µg/L/day of lindane.

In a study where lindane was administered to male rats orally in gradually increasing doses, it was observed that the proliferation in spermatogonia and

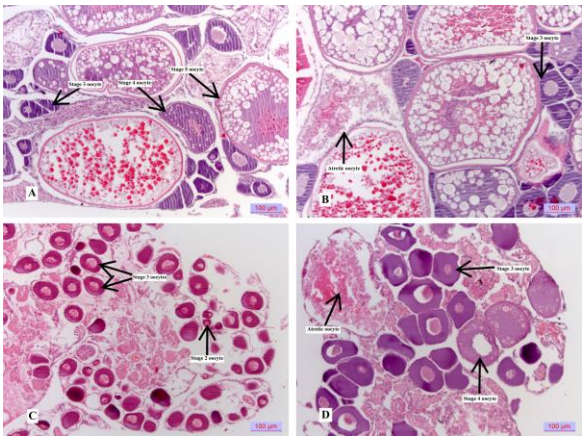

Figure 1. Histologic appearance of the ovaries. A. Control B. 40 µg/L dose C. 80 µg/L dose D. 160 µg/L dose. HE x200.

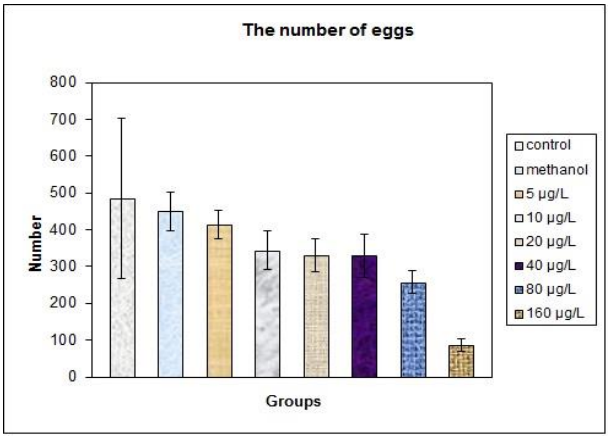

Figure 2. Control and experimental groups, showing the number of eggs retrieved from zebrafish according to dose.

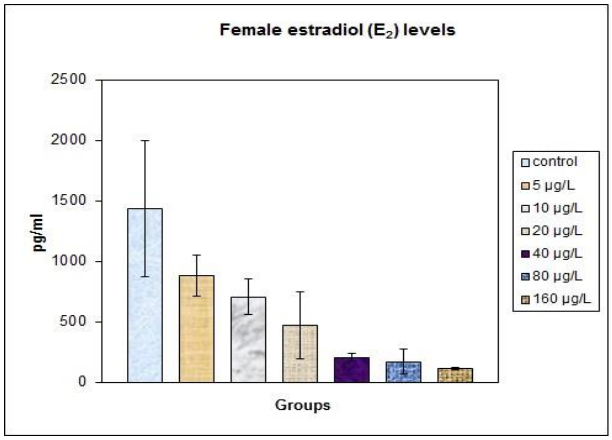

Figure 3. Control and experimental groups by dose, showing the levels of estradiol in female zebrafish.

spermatocytes (PCNA positivity) had decreased significantly, depending on the dose of the toxic substance (Yuksel *et al*., 2009). In the present study, although there were no histopathologically significant changes in the testes of male zebrafish, there were significant differences between the control group and the experimental groups in terms of the number of fertilized eggs and the number of hatched larvae. Similar to a study carried out with male rats (Yuksel *et al*., 2009), this decrease in the rate of fertilization was thought to be associated with a decrease in proliferation in the germ cells of the zebrafish .

As a result, it was observed that lindane does not lead to a histopathological change in the testes of male zebrafish. Further, the decrease in the number of eggs and ovulation and the decrease in estradiol (E_2)

levels in female zebrafish reveal that hormonal changes take place in the reproductive system of female zebrafish. Based on these results, it was determined that there was a significant decrease in the ovulation and the number of eggs caused by the adverse effects of lindane on the reproductive system and hormones of the female zebrafish. These results emphasize that toxic substances have major impacts on the ovulation and fertilization in female zebrafish.

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