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Chlorpyrifos causes histopathological changes and modulates vitellogenin levels in zebrafish *(Danio rerio)*

Ümran ÖZTÜRK[1] · Selin ÖZKAN-KOTİLOĞLU[2] · Pınar ARSLAN YÜCE[3] · Aylin SEPİCİ DİNÇEL[4] · Aysel Çağlan GÜNAL[5] · Figen ERKOÇ[6]

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Abstract

Chlorpyrifos is the most widely used insecticide worldwide for domestic and agricultural purposes. It is also an endocrine-disrupting chemical that affects non-target organisms even at low concentrations. The present study aimed to investigate sublethal concentration of chlorpyrifos in male zebrafish, focusing on its endocrine-disrupting effects on vitellogenin levels and tissue-level impact, as determined by histopathology. In our study, zebrafish were exposed to chlorpyrifos at concentrations of 10 and 100 µg/L for 24 h and 7 days, and compared to a control group. The results of vitellogenin levels revealed that exposure to sublethal concentrations caused significant increases ($p < 0.05$). Histopathological changes, including hyperplasia and epithelial detachment, were observed in the gill tissue. Hyperemia and hydropic degeneration were observed in liver tissue, hyperemia in brain tissue, and cavum glomeruli expansion and tubular degeneration in kidney tissue. In testicular tissue, degeneration and a decrease in sperm cells were detected at a 100 µg/L chlorpyrifos concentration after 24 h and 7 days. The results of this study indicate that chlorpyrifos can impact aquatic life and health even at extremely low concentrations.

Keywords Chlorpyrifos · Zebrafish · Vitellogenin · Histopathology · Endocrine disruption

Introduction

Chlorpyrifos (CLP, CAS number: 2921-88-2; o, o-Diethyl-o-(3,5,6 3,5,6-trichloro-2-pyridyl thiophosphate), an organophosphate insecticide introduced as a replacement for dichlorodiphenyltrichloroethane (DDT), has been extensively applied in agricultural and household settings due to its cost-effectiveness and broad-spectrum insecticidal activity (Ubaid ur Rahman et al. 2021). Its widespread and often unregulated use has resulted in substantial environmental contamination, particularly in aquatic environments, where CLP is among the most frequently detected pesticides

✉ Aylin SEPİCİ DİNÇEL
asepicidincel@gmail.com

Ümran ÖZTÜRK
umran88_umran@hotmail.com

Selin ÖZKAN-KOTİLOĞLU
selin.ozkan@ahievran.edu.tr

Pınar ARSLAN YÜCE
pinarslan89@gmail.com

Aysel Çağlan GÜNAL
caglangunal@gmail.com

Figen ERKOÇ
figenerkoc@baskent.edu.tr

[1] Department of Environmental Sciences, Graduate School of Natural and Applied Sciences, Gazi University, Yenimahalle, Ankara, Türkiye

[2] Department of Molecular Biology and Genetics, Faculty of Science and Art, Kırşehir Ahi Evran University, Kırşehir, Türkiye

[3] Biology Department, Faculty of Science, Çankırı Karatekin University, Çankırı, Türkiye

[4] Department of Medical Biochemistry, Faculty of Medicine, Gazi University, Ankara, Türkiye

[5] Biology Education Department, Faculty of Gazi Education, Gazi University, Ankara, Türkiye

[6] Department of Biomedical Engineering, Faculty of Engineering, Başkent University, Baglica Campus, 06790 Etimesgut, Ankara, Türkiye

(Aaron and Coly 1998; Nallapaneni and Pope 2005; Silva et al. 2015). It has been used irregularly for a long time, contributing to severe air pollution, as well as soil, groundwater, and sediment contamination (Huang et al. 2021).

It drew the public's attention due to its persistence and low solubility in water, which is frequently detected in foods, aquatic organisms, breast milk, cervical fluid, and meconium (Ostrea et al. 2002; Samarawickrema et al. 2008). Its widespread application resulted in more investigations into its toxicity and health risks. Concerns about CLP have also increased since its adverse neurotoxic effects on nervous system development, which inhibit acetylcholinesterase, have been revealed (Majumder and Kaviraj 2019). Therefore, its usage has been restricted or banned in many countries. However, it remains a threat due to its persistence in the environment (Sirisha et al. 2007; Watts 2012; Silva 2020). In addition to its neurotoxic effects, it was reported that the toxicity of CLP interferes with endocrine, cardiovascular, and reproductive systems and causes oxidative stress, eye irritation, behavioural, developmental, immunological, haematological, histopathological, and dermatological abnormalities (Ubaid ur Rahman et al. 2021).

Bioaccumulation of CLP is higher in aquatic ecosystems due to its resistance to hydrolysis. Considerable research has revealed that aquatic species are among the most affected groups by CLP exposure. Additionally, it is well established that CLP is toxic to aquatic species, particularly fish (Zhang et al. 2012; Raibeemol and Chitra 2020). Studies examining the toxic effects of CLP on various aquatic animals, including fish, prawns, mussels, and aquatic midges, have demonstrated that CLP exhibits both neurotoxic and genotoxic properties (Ubaid ur Rahman et al. 2021). It is widely used for its action on the central and peripheral nervous systems, inhibiting acetylcholinesterase activity.

Endocrine-disrupting chemicals (EDCs) are compounds that interfere with the endocrine system, leading to abnormalities in an organism's development, metabolism, reproduction, and overall health. These chemicals include synthetic hormones, personal care products, industrial substances, metals, fungicides, herbicides, pesticides, and some drugs, and affect organisms in a dose and time-dependent manner (Diamanti-Kandarakis et al. 2009; Gore 2016; Zlatnik 2016). Various researchers have studied the endocrine-disrupting potential of CLP and reported that CLP exhibits endocrine-disrupting features, including interference with the estrogen receptor beta (ERβ), decreased testosterone biosynthesis, and alterations in thyroid function (Grünfeld and Bonefeld-Jørgensen, 2004; De Angelis et al. 2009; Viswanath et al. 2011; Ventura et al. 2012).

Potential EDC effects of chlorpyrifos are interference with ERα, a change in mRNA levels, and antiandrogenic activity due to partial inhibition of testosterone biosynthesis. The estrogenic effect on zebrafish embryonic development was shown to alter estrogen marker gene expression in pattern analysis. Furthermore, chlorpyrifos-modified embryonic incubation of embryos with acridine orange staining provided evidence for cell proliferation and apoptosis in zebrafish (0.75 and 1.00 mg/L CPF) (Yu et al. 2015). Although chlorpyrifos indirectly contaminates aquatic ecosystems through household and agricultural applications, the chemical is also directly released into water to control mosquitoes (Raibeemol and Chitra 2020).

Vitellogenesis is a phase of oogenesis where yolk material is deposited by binding vitellogenin protein to the estrogen receptors. Although this process only occurs in females, it can be triggered in males with estrogen-mimicking chemicals (Tyler et al. 1999). Therefore, vitellogenin is a robust biomarker commonly preferred in research investigating EDCs. Besides its endocrine-disrupting effects, CLP has also been shown to cause tissue alterations (Marigoudar et al. 2018; Mishra and Singh 2021).

Zebrafish (*Danio rerio*, Hamilton 1822) serve as a key model in aquatic toxicology due to their genetic tractability, physiological relevance, and sensitivity to environmental pollutants. Prior studies have shown that zebrafish exhibit neurotoxic, developmental, and endocrine responses to CLP exposure (, ; Yu et al. 2015). However, data regarding sublethal, environmentally relevant exposure durations and their effects on VTG expression and tissue morphology remain limited.

This study aimed to evaluate the endocrine-disrupting effects of sublethal CLP exposure in male zebrafish by assessing the changes in vitellogenin levels and histopathological alterations in hepatic and gonadal tissues following 24-hour and 7-day exposures. We hypothesized that sublethal exposure to chlorpyrifos, even over short durations, would elicit measurable endocrine disruption in male zebrafish, as evidenced by increased vitellogenin levels and detectable histopathological alterations in liver and testis tissues.

Materials and methods

Animal maintenance

The adult male zebrafish was preferred in the current study as its features suit toxicological research. The species (3–4.5 cm) were maintained by a local supplier for fifteen days before exposure and kept in a stock aquarium at the Department of Medical Biochemistry, Faculty of Medicine, Gazi University, where all experiments were conducted, and institutional animal care guidelines were followed. A total of sixty zebrafish were used for each experiment set.

The fish were acclimated to the laboratory conditions and water temperature. During this 15-day adaptation period, they were fed an appropriate amount of dry feed, equivalent to up to 2% of their weight, once a day, and their behavior was closely monitored. Before exposure experiments, aquariums were prepared using 10 L of aerated and dechlorinated tap water, maintained under continuous aeration and at a constant temperature of 23 ± 1 °C. Zebrafish were randomly stocked in the aquariums and maintained on a 14-hour light and 10-hour dark photoperiod.

Preparation of the stock solution and sublethal concentrations

Technical-grade chlorpyrifos (CLP; 98.5% purity) was obtained from Dr. Ehrenstorfer GmbH and dissolved in analytical-grade dimethyl sulfoxide (DMSO) to prepare the stock solution. Sublethal and lethal concentrations were identified through preliminary range-finding experiments, after which 10 µg/L and 100 µg/L were selected as sublethal exposure levels. The final DMSO concentration in all treatment tanks remained within established safety limits and was identical across exposure groups, including controls.

All stock and working solutions were stored at + 4 °C and equilibrated to room temperature before use. The appropriate volumes of CLP solutions were added to the experimental tanks using calibrated automatic pipettes to ensure accurate dosing. Selected sublethal concentrations (10 µg/L and 100 µg/L) were chosen to reflect environmentally realistic exposure, considering field-measured CLP levels (3–9 µg/L in agricultural drains and up to 30 µg/L in runoff) reported in previous surveys. 100 µg/L represents a worst-case or pulse-exposure scenario (e.g., following spills or concentrated runoff). Therefore, using these doses enables us to model both typical contamination and extreme, yet plausible, field events (Hossain et al. 2015).

Experimental design

All experiments were designed according to APHA, OECD, and ISO methods (APHA, 2005; OECD 1981; ISO, 1996). Zebrafish were taken from aquarium stocks and placed in tanks containing 10 µg/L and 100 µg/L CLP, along with control groups. Zebrafish were exposed to CLP for 24 h and 7 days. For the 7 day exposure period, the water in the tanks was changed every 48-hours. Water quality was monitored daily and remained stable. At the end of the exposure time, the length and weight of the zebrafish were measured and recorded. To measure the vitellogenin level, the whole body of five (5) adult zebrafish from each group was frozen in liquid nitrogen and stored at -80 °C until homogenization. For histological analysis, five (5) zebrafish from each group were fixed in 10% buffered formalin.

Vitellogenin (Vtg) measurement

Vitellogenin levels of zebrafish from each experimental group were determined using a commercial kit designed for zebrafish (Biosense Laboratories, Prod. No. V01008402). Frozen whole bodies were homogenized, and the homogenate was analysed according to the manufacturer's protocol by enzyme-linked immunosorbent assay (ELISA). Given the wide range of Vtg levels found in experimental studies, a different series of dilutions was done on the samples, and the dilution results with absorbance values that fall within the standard curve working range were directly used. Results were given as ng/mL of diluted whole-body homogenates.

Histopathological analysis

Following CLP exposures, zebrafish were placed on ice and euthanized by decapitation. Zebrafish were then fixed using 10% buffered formalin. Following immersion in buffered formalin, tissues were put into cassettes and washed overnight. Then, tissues were dehydrated by placing them for 2 h in increasing percentages of ethyl alcohol (50%, 70%, 80%, 90%, 96%, and 100%) and xylol series. Tissues were embedded following incubating at 56–58 °C in xylol-paraffin and paraffin. All dehydration steps were conducted using a Leica tissue processor. Tissues embedded in paraffin wax were kept at + 4 °C. Paraffin-embedded tissues were cut at a thickness of 5–6 µm using a rotary microtome (Thermo Shandon, Fine-Science 325, USA), placed on a microscope slide in a water bath, and then dried at 56 °C. According to Harris's method, tissues were stained with hematoxylin and eosin (Harris 1990). The slides were covered using Canada balsam. Microslides were examined under a light microscope (Carl Zeiss Primostar). Gills were evaluated for hyperplasia, epithelium detachment, and telangiectasia. In liver tissue, hyperemia and hydropic degeneration were evaluated. In the kidney, widening of the cavity, as well as glomerular and tubular degeneration, was observed. In the brain, hyperemia was noted. Lastly, the testicle was assessed for degeneration and reduction of sperm cells.

The histopathological alterations were scored as "(-) none (no histopathological alterations), which represents normal histological structure; (+) histopathology in 20% of fields (mild); (++) histopathology in 20–60% of fields (moderate) and (+++) histopathology in 60% of fields (severe)" (Benli et al. 2008).

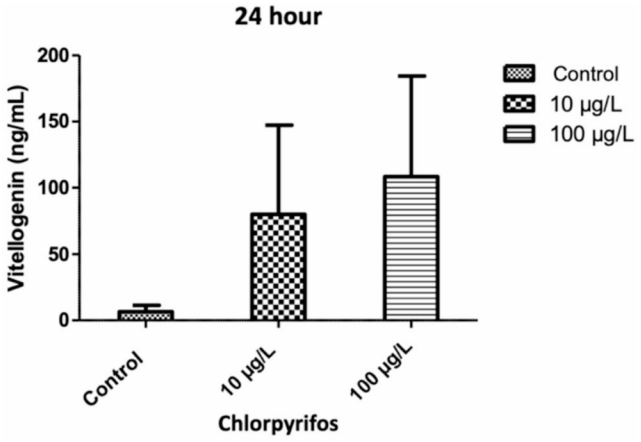

Fig. 1 Levels of vitellogenin (ng/mL) in zebrafish following 10 and 100 µg/L chlorpyrifos exposure for 24 h

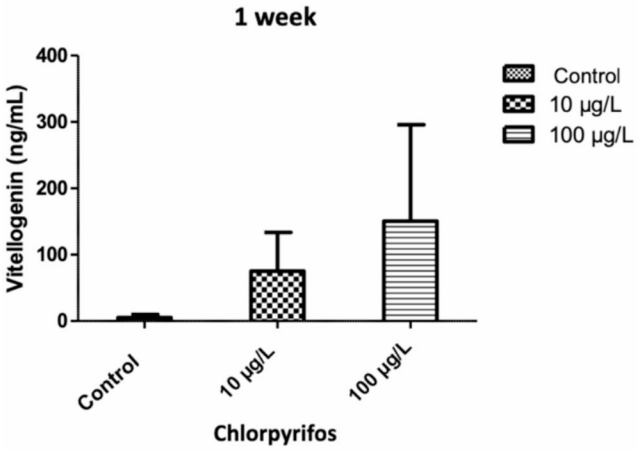

Fig. 2 Levels of vitellogenin (ng/mL) in zebrafish following 10 and 100 µg/L chlorpyrifos exposure for 7 days.

Statistical analysis

The obtained data were statistically analysed using Kruskal-Wallis and Mann-Whitney U non-parametric tests. The *P* value < 0.05 was accepted as significant.

Results

In the current study, zebrafish exposed to two different concentrations of CLP were investigated in both hormonal and histological aspects to examine the effects following 24 h and 7 days of exposure. Experiments were conducted in two replicates.

Table 1 Histopathological results in different tissues following Chlorpyrifos exposure

	10 µg/L CLP 24 h	100 µg/L CLP 24 h	10 µg/L CLP 7d	100 µg/L CLP 7d
GILLS				
Hyperplasia	++	+++	++	+++
Epithelium detachment	+	+	+	+
Telangiectasia	-	-	-	+
LIVER				
Hyperemia	-	-	+	+
Hydropic degeneration	-	+	+	+
KIDNEY				
Widening of cavum glomeruli	-	+	-	+
Tubule degeneration	-	+	-	+
BRAIN				
Hyperemia	-	+	+	+
TESTICLE				
Degeneration and reduction of sperm cells	-	-	+	+

(-) none (no histopathological alterations),

(+) histopathology in 20% of fields (mild),

(++) histopathology in 20–60% of fields (moderate),

(+++) histopathology in 60% of fields (severe)

Vitellogenin (Vtg) levels

Vitellogenin levels in each exposure group for both periods were significantly increased ($p < 0.05$). While the Vtg level of the control group was (mean ± SD) 11.47 ± 1.64 ng/mL after 24 h, 147.18 ± 12.74 ng/mL Vtg and 184.36 ± 32.57 ng/mL Vtg were measured from the zebrafish exposed to 10 µg/L and 100 µg/L chlorpyrifos, respectively (Fig. 1). Following a 1-week exposure, the Vtg levels in the 10 µg/L and 100 µg/L groups were 133.39 ± 16.89 ng/mL and 295.76 ± 5.09 ng/mL, respectively, whereas they were 9.91 ± 0.29 ng/mL in the control group (Fig. 2). The highest Vtg level was observed in zebrafish exposed to 100 µg/L for a week, which was statistically significant ($p < 0.05$).

Histopathological results

The histopathological effects of exposure to sublethal CLP on zebrafish tissues were summarized in Table 1.

Effects of Chlorpyrifos on gill histology

It was determined that hyperplasia and epithelium detachment were the most detected histopathological variations in gill tissue. 7-day exposure to CLP did not alter the severity, intensity, or frequency of epithelial detachment, which triggered the severity of hyperplasia (Table 1). Histopathological

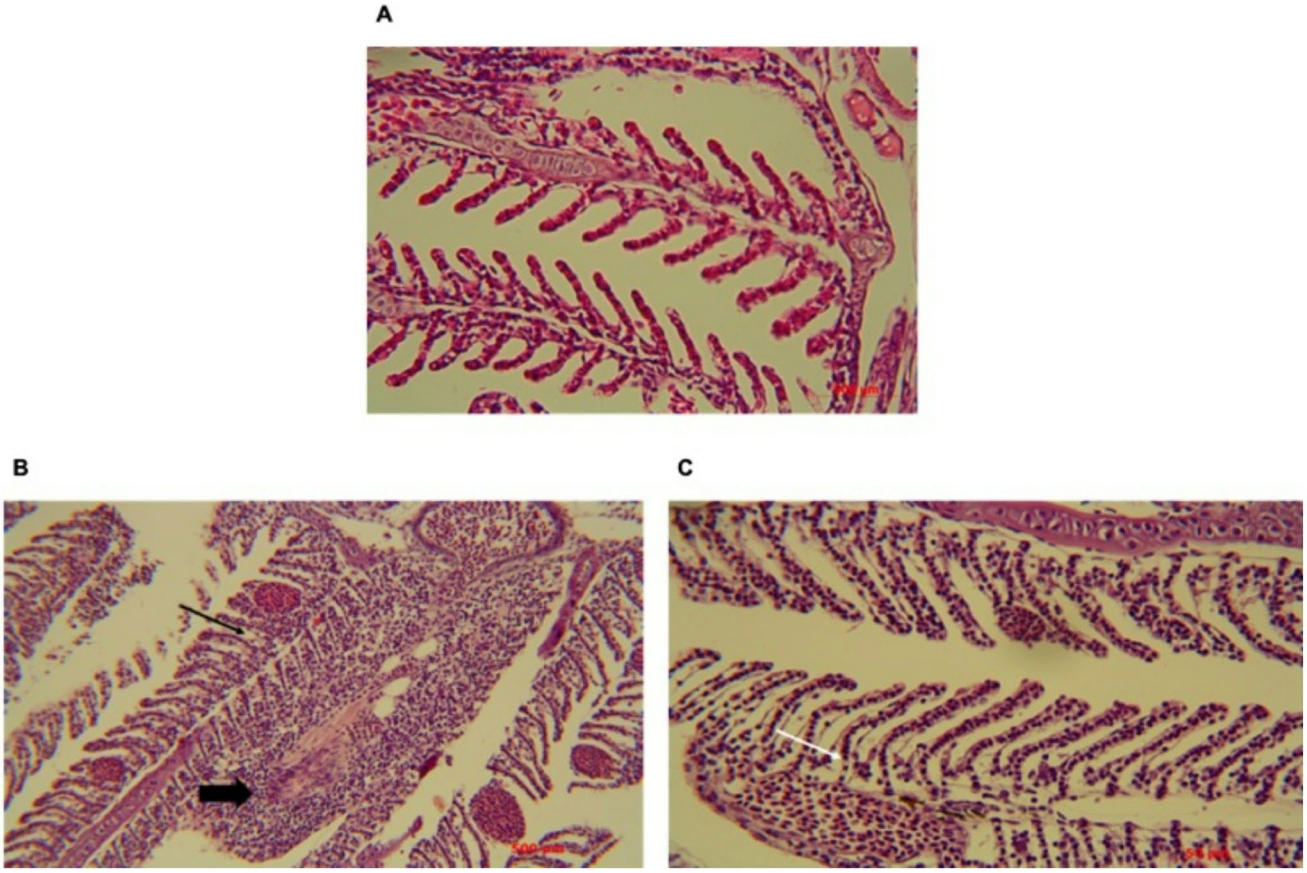

Fig. 3 Histopathological modifications in gill tissue following chlorpyrifos exposure (H & E)

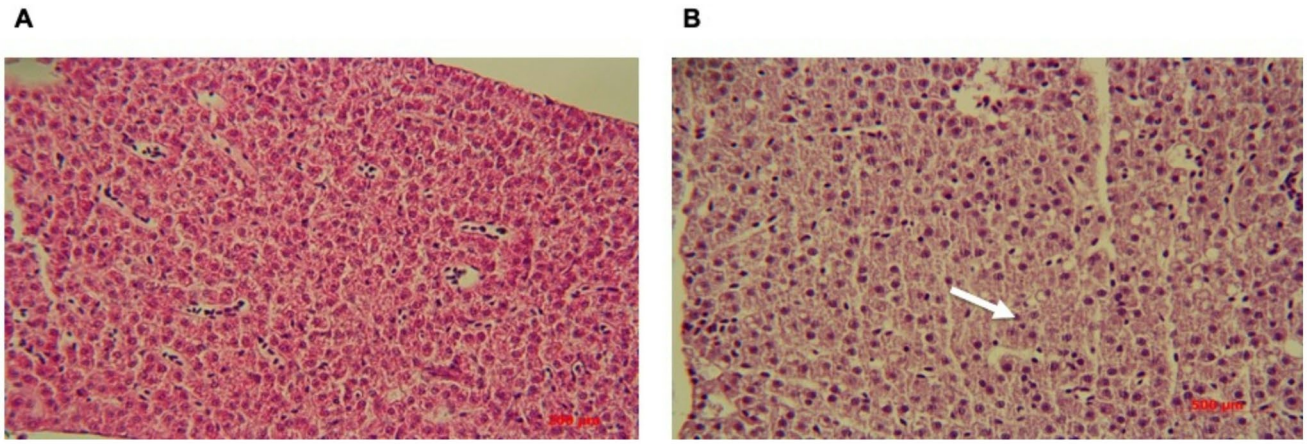

Fig. 4 Histopathological modifications in liver tissue following chlorpyrifos exposure (H& E). Figure 4A: Liver tissue of the control group; Fig. 4B: 7 days, 10 µg/L CLP group hydropic degeneration (white arrow, B)

variations observed in the gill tissues of zebrafish were demonstrated in Fig. 3.

Figure 3 A: Gill tissue of the control group; Fig. 3 B&C: 24 h 10 µg/L CLP group; hyperplasia (thick black arrow, B), telangiectasia (thin black arrow, B), and endothelium detachment (white arrow, C).

Effects of Chlorpyrifos on liver histology

Following CLP exposure, hyperemia and hydropic degeneration were observed in the liver (Fig. 4). Hyperemia was detected in the liver after 7 days of exposure, but not after 24 h, for both concentrations. Hydropic degeneration was

observed in liver tissues exposed to all concentrations and periods except 10 µg/L for 24 h (Table 1).

Effects of Chlorpyrifos on kidney histology

Bleeding, severe bleeding, widening of the Bowman's capsule, and tubular degeneration were the modifications observed in the kidney following chlorpyrifos exposure (Fig. 5).

Effects of Chlorpyrifos on brain histology

Hyperemia (Fig. 6) was detected in brain tissue for both doses and periods, except for 10 µg/L for 24 h exposure (Table 1).

Effects of Chlorpyrifos on testicle histology

In the testicles, the most frequently observed histopathological variations were a reduction in sperm cells, hyperplasia in Leydig cells, and degeneration (Fig. 7). Degeneration and reduction of sperm cells were detected following a 7-day exposure to 10 and 100 µg/L chlorpyrifos (Table 1).

Discussions

This manuscript investigates the endocrine-disrupting potential of chlorpyrifos and its histopathological effects, as observed through pathological changes in various tissues and organs, a gold standard approach in pharmacology and toxicology studies. Additionally, it presents data on toxic impacts on adult male zebrafish, with an emphasis on vitellogenin (Vtg) levels. To ensure any tissue-level effects, we studied all relevant tissues; more specifically, the testicular site was the target of the EDC effect. We were able to determine overall tissue-level damage using the main xenobiotic tissue targets. We justify our selection of CLP, which exhibits endocrine-disrupting effects that interfere with the endocrine, cardiovascular, and reproductive systems, causing oxidative stress, eye irritation, behavioral, developmental,

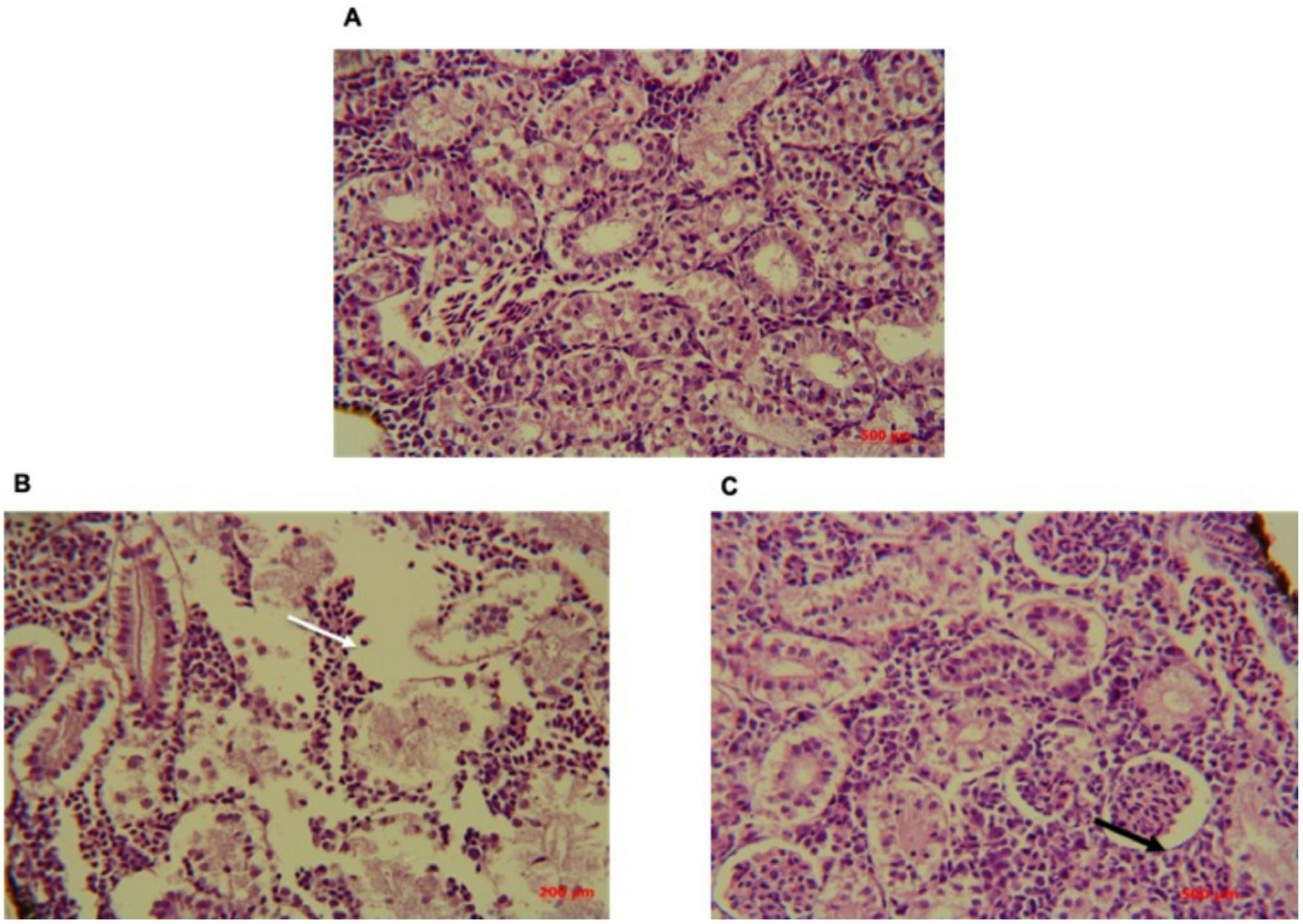

Fig. 5 Histopathological modifications in kidney tissue following chlorpyrifos exposure (H&E). Figure 5A: Kidney tissue of the control group; Fig. 5B: 24 h 100 µg/L CLP group tubule degeneration (white arrow, B); Fig. 5C: 24 h 100 µg/L CLP group widening of the cavum glomeruli (black arrow, C)

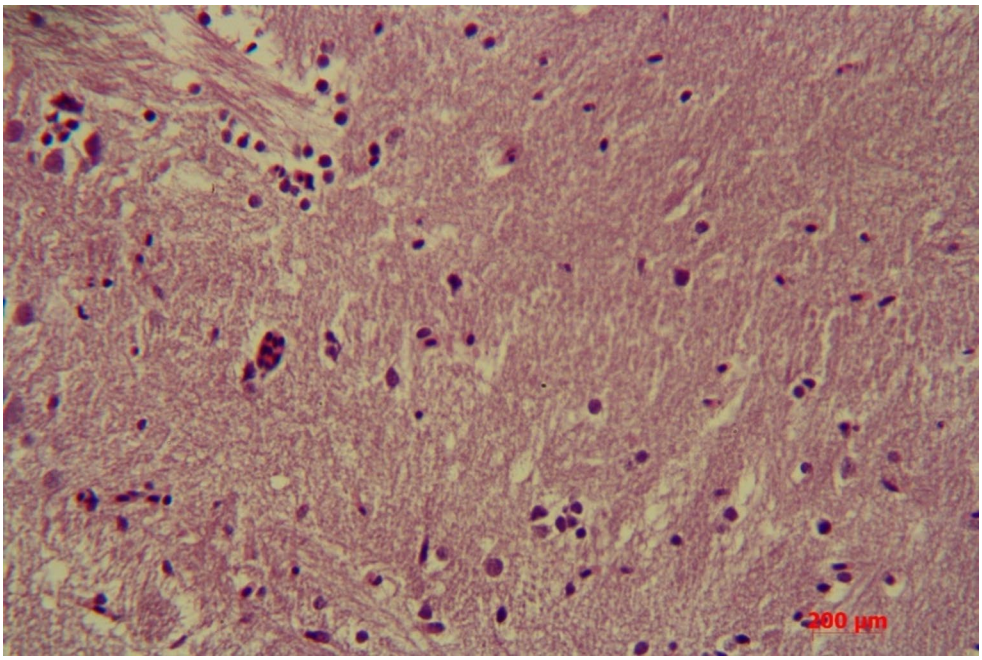

Fig. 6 24 h 100 µg/L CLP group, hyperemia in brain tissue following chlorpyrifos exposure (H & E)

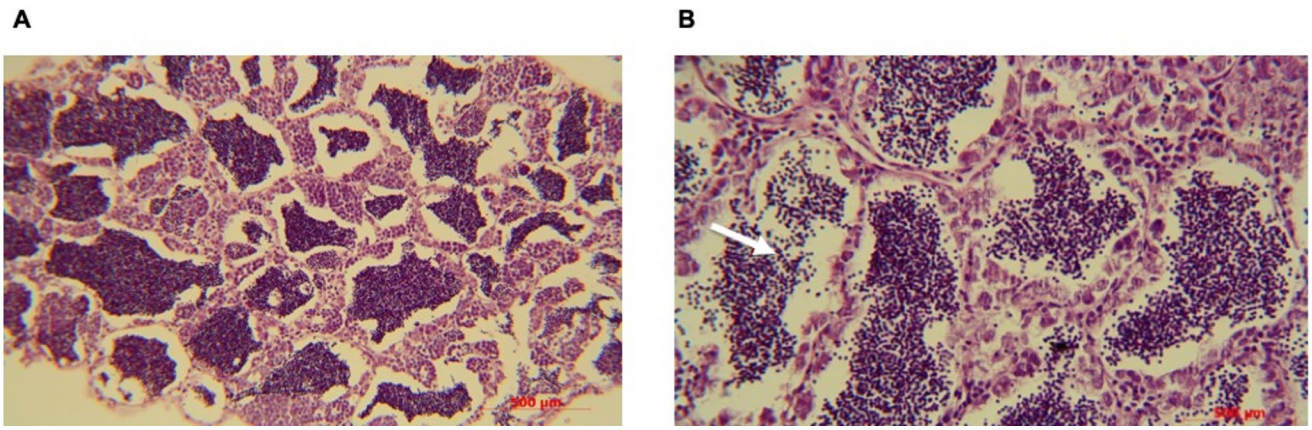

Fig. 7 Histopathological modifications in testicles following chlorpyrifos exposure (H & E). Figure 7A: Testicle tissue of the control group; Fig. 7B: 7 days 10 µg/L CLP group, degeneration and reduction of sperm cells (white arrow, B)

immunological, hematological, histopathological, and dermatological abnormalities (Ubaid ur Rahman.etal.2021).

Chlorpyrifos is a conventional pesticide whose toxicity to non-target organisms has been extensively studied (Majumder 2024a, b). In our study, the experimental design employs both 24 h and 7 days exposures at sublethal concentrations, utilizing Vtg as a biomarker of exposure. Model organisms have significantly different life spans and life cycles compared to humans and other higher mammalian species. Acute toxicity is typically assessed within 24 h in pharmacological actions and toxic effects accepted among decision-makers, academics, and researchers. While studying LC_{50} values, exposure durations are mainly 24, 48, 72, and 96 h. Tissue-level damage may not be observable within the first 24 h; therefore, we chose to extend exposure monitoring to 7 days, the standard sub-chronic duration in ecotoxicology. Unlike previous work that has focused chiefly on developmental toxicity or acute lethality, this investigation simultaneously evaluates endocrine disruption (via vitellogenin induction) and tissue-level alterations at two environmentally relevant sublethal concentrations over both 24 h and 7 days exposure periods. The combined analysis of hormonal and histological endpoints under these specific conditions fills a critical knowledge gap regarding the rapid and progressive effects of CLP on the physiology of adult fish. Besides, Manjunatha and Philip had already tested CLP for 24–96 h only in testis tissue, which makes our study distinct from theirs (Manjunatha and Philip 2016).

According to the acute results (24 h), compared to control groups, the 10 and 100 µg/L concentrations of CLP were 13.4 and 16.1 times higher Vtg values, respectively. Similar to 24-hour exposure, 7-day results showed that the

Vtg values of 10 and 100 µg/L CLP were 13.5 and 29.8 times higher than those of the control groups.

Our results are similar to those of other studies of other fish species exposed to pesticides. The female freshwater stinging catfish *Heteropneustes fossilis*, exposed to a 0.174 µM concentration of CLP for 96 h, exhibited increased Vtg levels in the liver, ovary, and serum (Mishra and Singh 2021). The male zebrafish exposed to 200 µg/L CLP for 24, 48, 72, and 96 h showed an increase in plasma Vtg levels in a time-dependent manner. However, the female zebrafish showed no changes (Manjunatha and Philip 2016).

The findings of our study demonstrate that exposure to chlorpyrifos, even at sublethal concentrations, induces significant endocrine and tissue-level disturbances in zebrafish, with important physiological and ecological implications. The marked elevation in vitellogenin levels at both exposure durations indicates a substantial estrogenic disruption, suggesting that CLP can interfere with reproductive endocrine pathways.

Apart from the increased Vtg values, abnormalities were also observed in the gills, liver, kidneys, brain, and testes tissues. In both exposure periods, hyperplasia and epithelial detachment in the gills were demonstrated in all exposed groups, which are likely to impair respiratory efficiency, ion exchange, and overall metabolic stability. Liver hydropic degeneration and kidney tubular damage reflect compromised detoxification and osmoregulatory functions, which may reduce the organism's ability to cope with environmental stressors. Hyperemia and hydropic degeneration in the liver, hyperemia in the brain, and degeneration and reduction of sperm cells in the testicular tissues were observed in both concentrations on the seventh day. The degeneration of testicular tissue observed after 7 days is of particular concern, as reductions in sperm cell integrity and abundance could suggest decreased reproductive status, ultimately affecting population dynamics. The kidney tissue abnormalities were only observed in the higher concentration exposed for 24 h and 7 days.

Overall, the histopathological results demonstrate a clear dose- and time-dependent toxic effect of chlorpyrifos on multiple organ systems in zebrafish. The cumulative physiological effects of CLP may substantially diminish long-term fish health, reproductive capacity, and survival in natural ecosystems, raising concerns about its ecological safety in aquatic environments.

In another study, the long-term negative impacts of exposure to parental zebrafish (F0) led to generational toxicity (F1, F2), which were studied using transcriptomic analysis. Exposure to 60 µg/L chlorpyrifos resulted in a significant decrease in egg production, a loose ovary structure, follicular membrane damage, and vacuolar damage or rupture compared to the control. Chronic exposure inhibited ovarian maturation and decreased egg production. It disrupted the metabolic pathways during oocyte maturation, which may interfere with oocyte development and quality, ultimately influencing the survival of zebrafish offspring. Parental exposure to CPF could induce early developmental toxicity in the F1 generation, which may be attributed to oxidative stress, inflammation, apoptosis, and metabolic reactions in larvae (Ma et al. 2023).

Vitellogenin production was determined in juvenile male tilapia (Oreochromis spp) exposed to sublethal concentrations (4, 8, and 12 µg/L) of chlorpyrifos for 28 days. Significant damage in the brain (degeneration and gliosis of the optic tectum), kidneys (vacuolar nephrosis and tubular hyaline granules), and liver (karyomegaly, binucleation, and hyaline granules in hepatocytes) was reported. Vtg synthesis in the liver and gonads indicated chlorpyrifos-induced Vtg synthesis in male fish, which was supposed to be inhibited (Taborda et al. 2012).

The histopathological alterations in our study were similar to other studies of different species showing vacuolisation and elongated seminiferous tubules (Mishra and Singh 2021) in testis; hyperplasia, degeneration, epithelial lifting, fusion, mucous accumulation, vacuoles formation, and infiltration (Marigoudar et al. 2018), congestion of afferent blood vessels, hypertrophy, and telangiectasia (Dawood et al. 2020a), deformity of cartilaginous, telangiectasia and edema (Dawood et al. 2020b) in gills, congestion of blood sinusoids, degeneration and vacuolation of hepatocytes (Dawood et al. 2020a), and degeneration and lymphocytic infiltration (Dawood et al. 2020b) in liver tissues.

Conclusion

This study has demonstrated that exposure to chlorpyrifos for 24 h and 7 days significantly elevated vitellogenin levels in a dose-dependent manner and produced measurable histopathological alterations in non-target organisms, which may adversely impact reproduction and metabolic functions. These findings demonstrate the endocrine-disrupting potential of chlorpyrifos in adult zebrafish and highlight early biomarkers of exposure that may have relevance for ecological risk assessment.

Author contributions A.S.D., A.Ç.G., F.E. wrote the main manuscript text, P.A.S prepared figures, Ü.Ö., S.Ö.K, methodology.

Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interests The authors declare no competing interests.

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