Iranian Journal of Fisheries Sciences 21(2) 316-330 2022
DOI: 10.22092/ijfs.2022.126127

Research Article

Pathological and genotoxic effects of the herbicide oxadiargyl on common carp (*Cyprinus carpio*) fingerlings

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Received: October 2018 Accepted: May 2019

Abstract

Acute toxicity and effects of sublethal concentrations of oxadiargyl herbicide (3% EC) were examined on DNA damage (Comet assay) and histopathological changes in common carp (*Cyprinus carpio*) fingerlings with average weight and length of 19.15±1.05 g and 10.09±0.47 cm, respectively. The fish were exposed to 0.1, 0.3 and 0.5 mg/L of the herbicide for 30 days. Estimated 96-h LC_{50} value for oxadiargyl in common carp was 0.6 mg/L. Histopathologically, no change occurred in different tissues of the control group, while marked lesions were induced in vital organs of fish that their severity was increased with enhancement of the herbicide concentration. Sublethal exposure to different concentrations of oxadiargyl induced: hyperplasia of lamellar epithelium, hyperemia, inflammatory cells infiltration, aneurysm and rod-like structures of secondary lamellae in gill tissues, as well as change in size and number of melanomacrophage centers in kidney and spleen tissues. Necrosis of tubular epithelium, hyperemia, and protein casts were also observed in kidney tissue. Focal necrosis, fragmentation, vaculization and shrinkage of myofibrils, and eosinophilic cytoplasm were observed in muscle tissues of exposed fish. Erythrocyte cells of fish exposed to sublethal concentrations of 0.1, 0.3 and 0.5 mg/L, showed 18.3%, 19.1%, and 31.5% tailed DNA, respectively, significantly higher than the control group ($p<0.05$). Moreover, exposure to oxadiargyl significantly decreased WBC, RBC, Hb, Hct compared with the control group ($p<0.05$). In conclusion, these results revealed that oxadiargyl is highly toxic to common carp with genotoxic and hematotoxic effects, as well as adverse effects on histopathology of vital organs.

Keywords: Oxadiargyl, Common carp, Histopathology, Hematology, DNA damage

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Introduction

Herbicides are the most potentially harmful chemicals used in agriculture that can enter into aquatic environments and affect fauna, especially fish (Wany *et al.*, 1992; Arshad *et al.*, 2006).

Exposure of fish even to low environmentally-relevant concentrations of herbicides may result in abnormal behaviors (Steinberg *et al*., 1995), retarding growth performance (Sweilum, 2006), various physiological disorders (Blahova *et al*., 2014; Ahmadivand *et al*., 2016), and devastating deaths (Bálint *et al*., 1997), as well as adverse reproductive and immune effects (Ahmadivand *et al*., 2015; Xing *et al*., 2015).

Oxadiargyl ($C_{15}H_{14}C_{l2}N_2O_3$) belongs to the group of oxadiazole, and is a broad-leaf herbicide extensively applied in rice fields in North Europa and Asia, characterized by its inhibition to protoporphyrinogen IX oxidase enzyme (Hwang *et al*., 2004). Its half-life in soil is 20-30 days and its residues can affect soil as well as water fauna (Mahmoudi *et al*., 2011).

Oxadiazole chemical family have been found to be highly toxic to fish with adverse effects on growth and biochemical parameters, as well as chemical structure of DNA (Ajani *et al*., 2015; Zanjani *et al*., 2017). However, there are a limited data concerning toxic effects of oxadiargyl on aquatic animals, and most of studies focused on phytotoxic effects of the herbicide (Nethra and Jagannath, 2011; Monjezi *et al*., 2015).

Toxic effects of herbicides on fish are investigated using many biomarkers, including histopathological studies of vital organs and haematological parameters due to association between external environment and the circulatory system, as well as changes in response to toxic substances (Wendelaar-Bonga, 1997; Ahmadivand *et al*., 2014; Blahova *et al*., 2014). Comet assay, another sensitive technique for detection of a wide variety of DNA damage, is widely used to determine genotoxic potential of pesticides on aquatic organisms (Jin *et al*., 2004; Mitkovska *et al*., 2017).

Common carp (*Cyprinus carpio*) is one of the main ichthyofauna in rivers of southern Caspian Sea, as well as commercial fish species that mostly farmed in littoral provinces of Iran (Golestan, Mazandaran, and Gilan), the area of high oxadiargyl use in rice fields (Mahmoudi *et al*., 2011). Hence, this study was aimed to investigate acute toxicity and effects of sublethal concentration of oxadiargyl herbicide on common carp (*C. carpio*) fingerlings.

Materials and methods

Herbicide

Technical grade oxadiargyl herbicide (3% EC) manufactured by Saveh Herbicide Company (Arak, Iran) was used to evaluate its toxicity to carp. Stock solutions were prepared in acetone and tap water.

Fish

Fingerlings of Caspian common carp with weight of 19.15±1.05 *g* and fork length of 10.09±0.47 cm were obtained

from Shahid Beheshti fish breeding center (Rasht, Iran) and were acclimated to laboratory condition in 1000 liter tanks with dechlorinated tap water for 10 days. The fish were fed using commercial FFC-extruded fish feed (Faradaneh Company; ShahreKord, Iran) twice a day, and maintained under a natural photoperiod (approximately 12h light/ 12h dark). During the experiment, physicochemical characteristics of water, including temperature, oxygen, pH, and total hardness were measured daily based on OECD guidelines (2016).

Determination of 96h-LC_{50} value

For acute toxicity, different concentrations of herbicides (0, 0.1, 0.3, 0.5, 0.7, 1, 2, 4, 6 and 8 mg/L) were prepared and fish in duplicated groups (n=20) were exposed to them in 100 L tanks. The 96-h LC50 was determined by Probit analysis. A control group with oxadiargyl free water was also maintained. Dead fish were counted and removed.

Sublethal exposure to oxadiargyl

For sublethal toxicity tests, 120 fish were selected and introduced into four duplicate 100 liter tanks (n=15) and were exposed to concentrations of 0.1, 0.3 and 0.5 mg/L of oxadiargyl to investigate DNA damage of erythrocyte cells and histopathological changes of vital organs in 30 days. Two control tanks (n=15) with oxadiargyl free water were also maintained. Water was completely renewed daily and its characteristics were monitored before and after water exchange, as well as herbicide concentrations were adjusted.

Hematology

On day 30 of sublethal exposure, five fish from each replicate were anesthetized with clove essence (200 mg/L), and blood was collected from caudal vein puncture using a heparinized syringe and transferred to a 2 mg/L heparinized tube containing 0.01 mg/L of sodium heparin solution (5000 I.U). Hematological parameters, including white blood cells (WBCs), red blood cells (RBCs), hemoglobin (Hb), and hematocrit (Hct) were measured (Ahmadivand *et al*., 2014).

Histopathology

For histological examinations at day 30 after sublethal exposure, three fish from each replicate were taken and a piece of kidney, gill, spleen, and muscle tissues were removed. Tissues were then fixed in 10% neutral buffered formalin (NBF), and after serial dehydration in ethanol, embedded in paraffin and sectioned at 5 µm thickness. Tissue sections were stained with hematoxylin and eosin (Hewitson *et al*., 2010), and examined under a light microscope (E600; Nikon).

Comet assay

After exposure periods, DNA damage of erythrocyte cells was determined by alkaline single cell gel electrophoresis (Singh *et al*., 1988). A mixture of a blood sample (5µL) with 0.5% (w/v) low-melting agarose (95 µL) was spread over degreased microscope slides, previously covered with 1% normal melting agarose,

and then allowed to solidify at 4°C for 20 min. The embedded cells were lysed in fresh cold lysing buffer (2.5M NaCl, 100mM Na_2EDTA, 1% Triton X-100, 10% DMSO, 10mM Tris–HCl, and pH: 10) at 4°C overnight.

After 30 min incubation in electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH ≥ 13) electrophoresis was carried out at 20 V and 300 mA for 30 min. Unwound DNA in the slides was neutralized by three washing steps in 0.4 M Tris-HCl (pH 7.5). The slides were stained with ethidium bromide (15 µg/mg/L) to visualize DNA strand breaks, and examined using a fluorescence microscope (E600; Nikon). Two slides per specimen (25 cells per slide) were analyzed, and DNA damage was quantified as percent of tailed ones.

Statistical analysis

The obtained data were analyzed using the statistical package SPSS23 software (Chicago, IL, USA) by one-way analysis of variance (ANOVA) followed by Tukey's pairwise multiple comparison test. The data were provided as mean±standard deviation and differences were considered statistically significant when $p<0.05$.

Results

Determination of 96-hLC_{50} value

Mortality rate of acute toxicity test of oxadiargyl on common carp is shown in Figure 1. Measured 96-h LC_{50} value by probit analysis was 0.6 mg/L. During the experiment, none of unexposed control fish died and showed abnormal behavior. Abnormal behaviors observed in those exposed to oxadiargyl were erratic swimming, accelerated respiration, hanging vertically, and staying motionless on the bottom of tank.

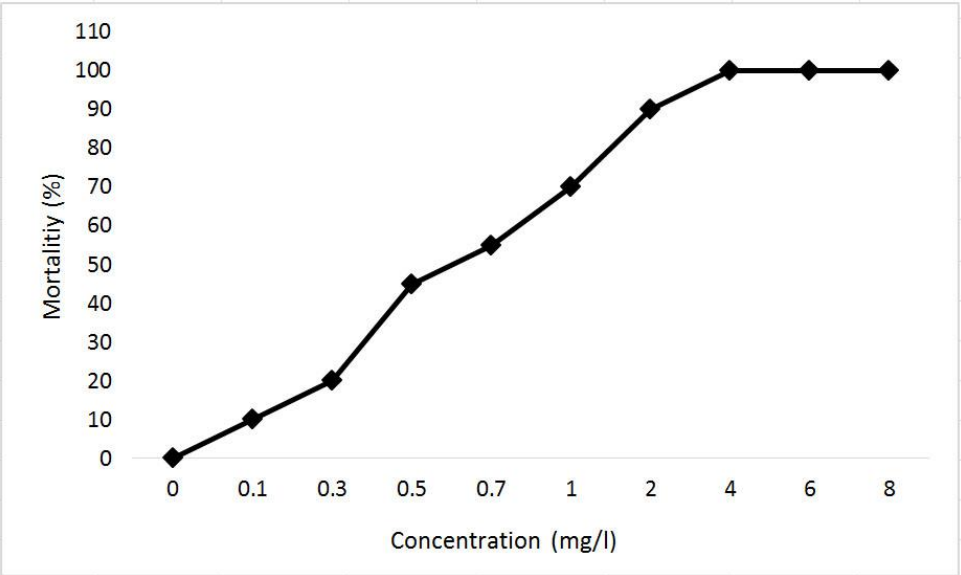

Figure 1: Mortality of common carp (*C. carpio*) at 96h after acute exposure to oxadiargyl.

Sublethal exposure

No mortality was observed in control and treatment groups during sublethal exposure (0, 0.1, 0.3 and 0.5 mg/L) period. However, some abnormal behavioral and swimming patterns were observed in 0.5 mg/L oxadiargyl exposed fish groups.

Hematology

The results of measured hematological parameters are shown in Figure 2. There was a significant increase in WBC, decrease in RBC, Hb and Hct levels of fish exposed to 0.3 and 0.5 mg/L, as well as RBC level in 0.1 mg/L trial group when compared with the control group (p<0.05). However, exposure to 0.1 mg/L did not show significant change in WBC, Hct and Hb levels (p>0.05).

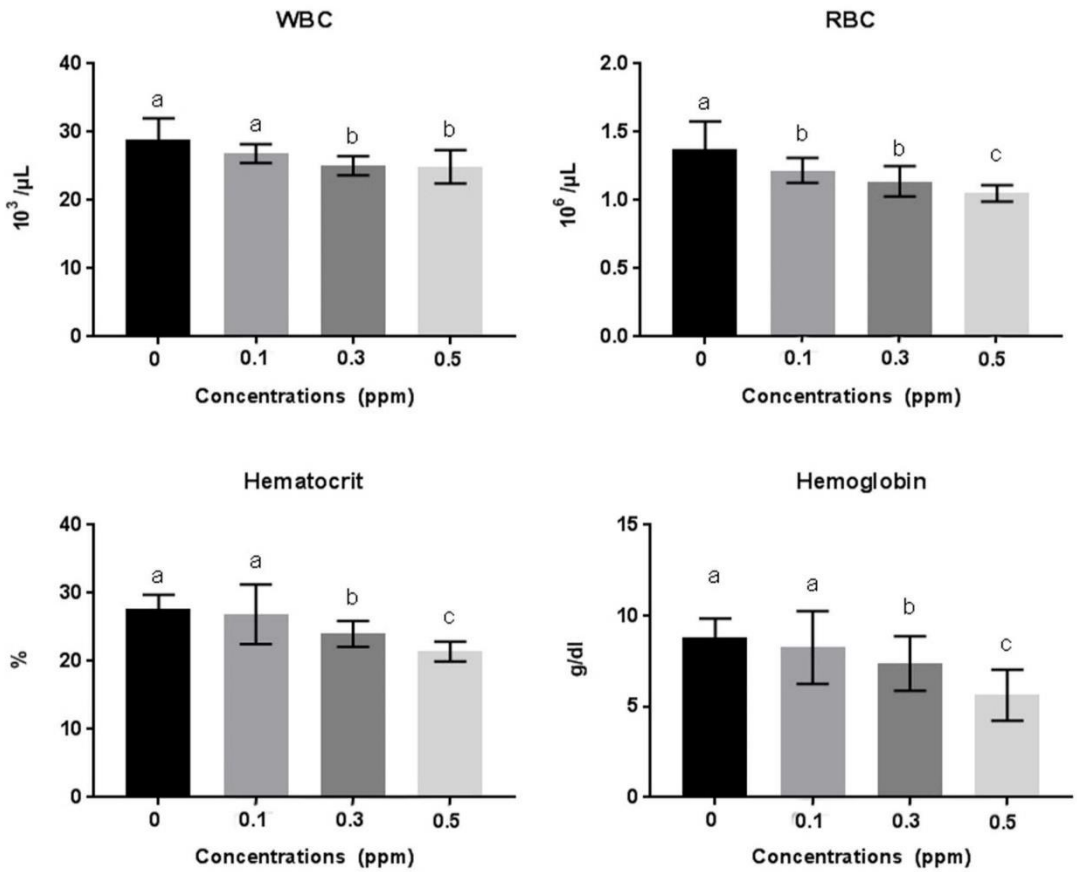

Figure 2: Hematological parameters of common carp (*C. carpio*) after 30 days exposure to different concentrations (0, 0.1, 0.3 and 0.5 mg/L) of oxadiargyl. Different letters indicate significant differences among groups at p<0.05. Error bars show standard deviation.

Histopathology

No histopathological change was observed in gills, kidney, spleen, and muscle of the control group. However, marked histological alterations were observed in examined organs of treatment groups depending on herbicide concentration (Table 1).

Table 1: Histological lesions in gills, kidney, spleen, and muscle tissues of common carp exposed to oxadiargyl.

Tissue	*Lesions*
Gills	Hyperemia, Hyperplasia, Inflammatory cells infiltration, Aneurysm, Necrosis, Rod-like structures
Kidney	Proteinuria, Melanomacrophage centers, Necrosis, Hyperemia
Muscle	Fragmentation, Vacuolization and shrinkage, Necrosis, Inflammatory cells infiltration
Spleen	Melanomacrophage centers

Gills

Sublethal exposure to different concentrations of oxadiargyl, induced hyperplasia of lamellar epithelium, hyperemia, inflammatory cells infiltration, aneurysm and rod-like structures of secondary gill lamellae in gills tissues. Severity of lesions increased with enhancing oxadiargyl concentrations (Fig. 3B-D).

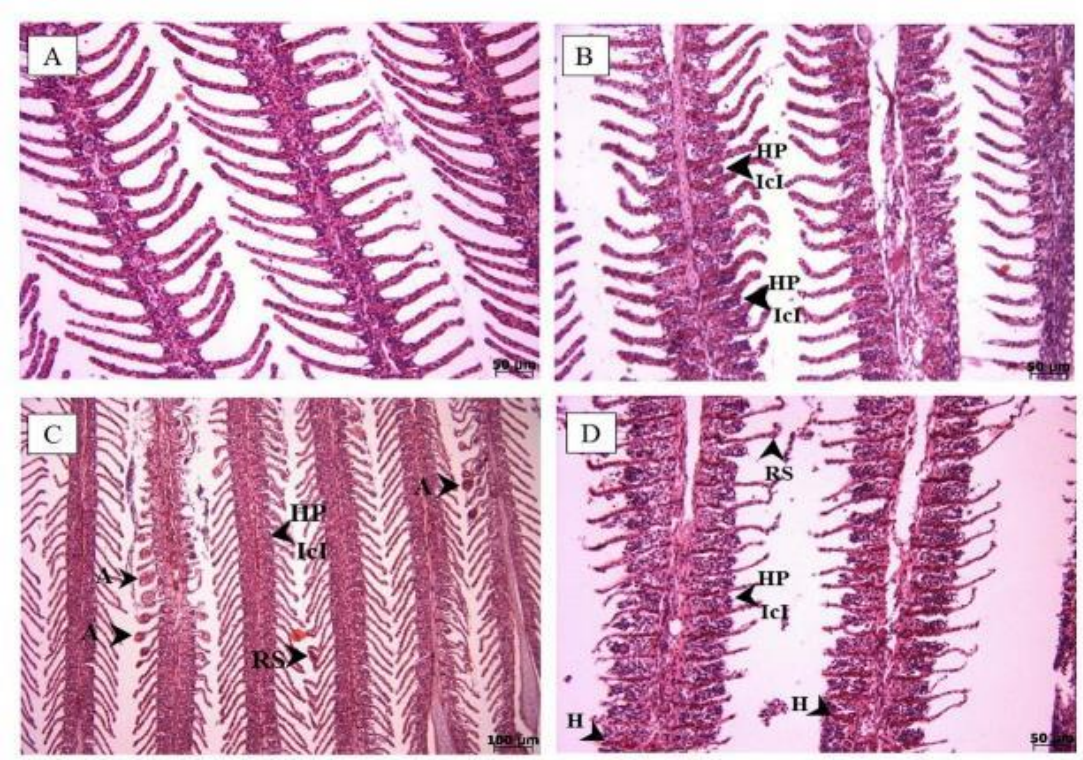

Figure 3: Histopathological changes of gill tissue of common carp (*C. carpio*) after 30 days sublethal exposure to oxadiargyl. (A) gill tissue of control treatment, (B) fish exposed to 0.1 mg/L, (C) 0.3 mg/L, and (D) 0.5 mg/L of oxadiargyl; A: aneurysm, H: hyperemia, HP: hyperplasia, I: inflammatory cells infiltration, RS: rod-like structures.

Kidney

Kidney tissue of common carp exposed to oxadiargyl, showed histopathological lesions, including necrosis of tubular epithelium, hyperemia, and protein casts in tubules (proteinuria). Moreover, change in size and number of melanomacrophage centers were seen. Severity of tissue alterations increased as herbicide concentration increased (Fig. 4B-F).

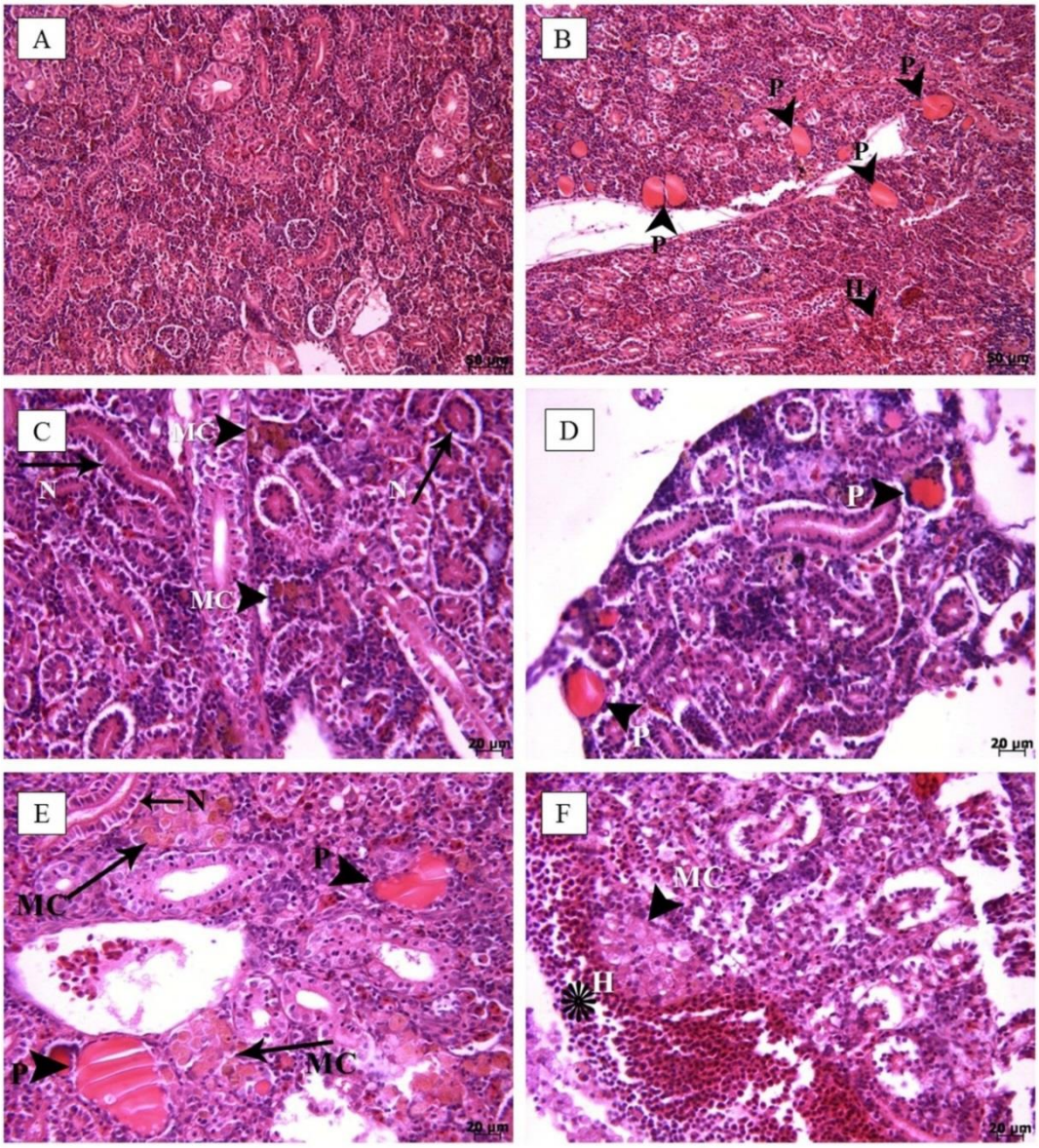

Figure 4: Histopathological changes of kidney tissue of the common carp (*C. carpio*) after 30 days sublethal exposure to oxadiargyl. (A) kidney tissue of control treatment, (B) fish exposed to 0.1 mg/L, (C) 0.3 mg/L, and (D-F) 0.5 mg/L of oxadiargyl; H: hyperemia, MC: melano-macrophage centers, N: necrosis, P: proteinuria, Asterisk: cellular infiltration.

Spleen

Increase in size and number of melanomacrophage centers in spleen tissue of exposed common carp to different concentrations of oxadiargyl was frequently seen (Fig. 5B-D).

Muscles

Structural details of muscle tissue of control treatment are shown in Figure 6(A). In muscle tissue of exposed fish, histopathological lesions, including focal necrosis, fragmentation, vacuolization and shrinkage of myofibrils, and eosinophilic cytoplasm were evident (Fig. 6B-D).

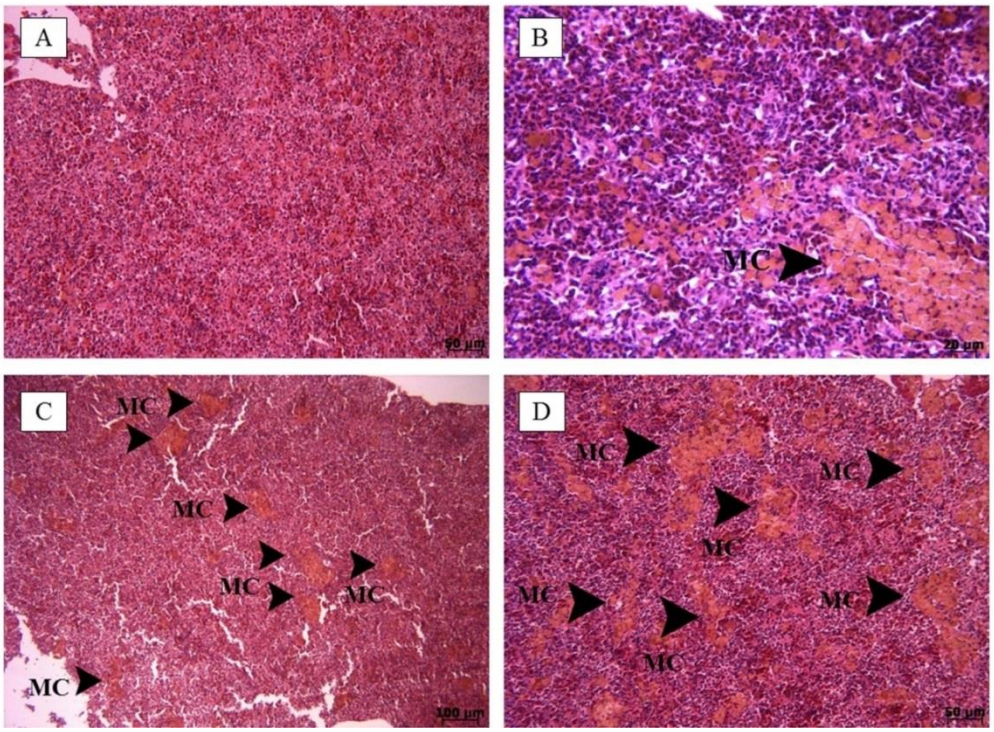

Figure 5: Histopathological changes of spleen tissue of common carp (*C. carpio*) after 30 days sublethal exposure to oxadiargyl. (A) control fish spleen, (B) spleen tissue of fish exposed to 0.1 mg/L, (C) 0.3 mg/L, and (D) 0.5 mg/L of oxadiargyl; MC: melano-macrophage centers.

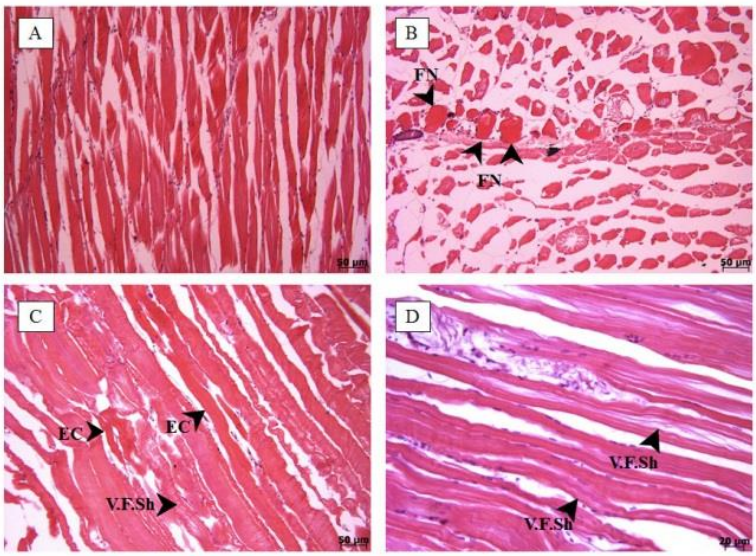

Figure 6: Histopathological changes of muscle tissue of common carp (*C. carpio*) after 30 days sublethal exposure to oxadiargyl. (A) control treatment muscle, (B) muscle tissue of fish exposed to 0.1 mg/L, (C) 0.3 mg/L, and (D) 0.5 mg/L of oxadiargyl; EC: eosinophilic cytoplasm, FN: focal necrosis, V.F.Sh: vacuolization, fragmentation, and shrinkage.

DNA damage

Results of DNA damage (% tailed DNA) induced by oxadiargyl in blood samples of *C. carpio* are shown in Figure 7. Cell viability was above 90% in the treatments, allowing comet assay to be performed. Oxadiargyl had a genotoxic effect, and treatments with 0.1, 0.3 and 0.5 mg/L resulted in 2-3 folds increases in percent of tailed DNA compared to the control (7%). Among the treatments, the highest damage (31.5%) was recorded in blood samples of 0.5 mg/L trial group followed by 0.3 mg/L (19.1%) and 0.1 mg/L (18.3%). However, there was no statistically significant difference in percentage of tail DNA in cells of fish exposed to 0.3 mg/L when compared with the 0.1 mg/L treatment.

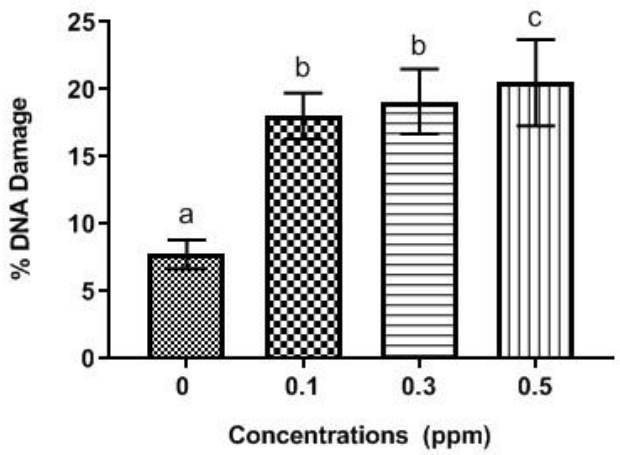

Figure 7: DNA damage (% tail DNA) in erythrocyte cells of common carp (*C. carpio*) on day 30 of exposure to different concentrations (0, 0.1, 0.3 and 0.5 mg/L) of oxadiargyl. Different letters indicate significant difference among groups ($p<0.05$). Error bars show standard deviation.

Discussion

Despite adverse effects of herbicide exposure to aquatic fauna, there is little information about toxicity effects of oxadiargyl, which is main herbicide used in rice farming in Gilan, Mazandaran and Golestan provinces. Residues of this herbicide and other kinds enter into rivers and may be transferred into Caspian Sea, where it may contaminate aquatic fauna and then human consumers. In this research, toxicity effects of oxadiargyl on Caspian common carp fingerlings wes studied. Obtained results for 96-h LC_{50} value for common carp was 0.6 mg/L which is lower than that reported by Sadeghi and Imanpoor (2015) for another member of oxadiazole group (oxadiazon) for platyfish (*Xiphophorus maculatus*; 7.59 mg/L) which may be due to difference in fish species, herbicide and also water quality (Gupta *et al*., 1981; Farah *et al*., 2004).

In response to a stressor, such as pesticide exposure, fish undergo series of hematological changes in attempt to compensate the challenge imposed on

them and thus cope with the stress (Wendelaar-Bonga, 1997). According to the results of this study, hematological parameters showed significant (p<0.05) reduction in levels of WBC, RBC, Hb, and Hct in the fish. These could be due to oxadiargyl herbicide effects on hematopoietic tissue, which together with observed changes in levels of erythrocyte and leukocytes can lead to impairment in process of hematopoiesis and reduce the fish innate immune system.

Saravanan *et al*. (2017), assessing the acute toxicity effects of 0.5, 5 and 50 μg/L concentrations of oxadiazon on carp for 96 h, found that this herbicide causes a significant decrease in RBC, Hb, and Hct. Decrease in RBC, Hct, and Hb content in this study could be explained as compensatory response that reduces oxygen-carrying capacity to maintain gas transfers and indicates a change in water blood barrier for gas exchange in gill lamellae (Jee *et al*., 2005). Obtained results indicated that exposure to sublethal concentrations of oxadiargyl, severely affected hematological parameters in common carp which was in agreement with those obtained after exposure to other herbicides (Ahmadivand *et al*., 2014; Blahova *et al*., 2014), indicating disruption of hematopoiesis, as well as induction of cellular apoptosis.

The same results of leucocytes, erythrocyte, hemoglobin, and hematocrit decrease were reported in *C. carpio* after sublethal exposure to oxadiazon (Zanjani *et al*., 2017). Decrease in RBC, Hb, and Hct also indicates hypoxic condition and/or respiration dysfunction affecting circulating system, may be due to impaired gas exchange in gill lamellae (Jee *et al*., 2005). Moreover, change in WBC levels suggests immunotoxic potential of the herbicide (Ahmadivand *et al*., 2015).

Histopathological studies of vital organs and serum biochemical parameters due to the association between external environment and the circulatory system, as well as their changes in response to toxic substances were widely used to determine the effects of pollutants on fishes (Wendelaar-Bonga, 1997; Ahmadivand *et al*., 2014; Blahova *et al*., 2014).

In this study, oxadiargyl induced mildly to severe histological lesions in gills, kidney, muscle, and spleen of common carp depending on herbicide concentration, which is in agreement with previous studies reporting different histopathological changes in common carp following exposure to herbicides (Poleksić and Karan, 1999; Blahova *et al*., 2014**;** Stoyanova *et al*., 2015). However, these lesions seem to be a result of increased cell activities and reversible in proper conditions.

The observed changes in size and number of melanomacrophage centers (MMCs) in kidney and spleen tissues, depending on the herbicide concentration, confirmed that MMCs can be considered as a biomarker of environmental stress such as pesticides (Ribeiro *et al*., 2011).

In kidney tissue necrosis of tubular epithelium, hyperemia, and protein casts in the tubules (proteinuria) were also observed. Therefore, as kidney showed endocrine, immune and hematopoietic functions, oxadiargyl can produce toxic effects on many important physiological processes in fish. Exposure to oxadiargyl also induced histological changes in muscles tissues of the fish which may result in obstruction of circulation and digestive systems, and lower fillet quality (Wendelaar-Bonga, 1997).

Sublethal exposure to different concentrations of oxadiargyl, induced hyperplasia of lamellar epithelium, hyperemia, inflammatory cells infiltration and aneurysm of secondary gill lamellae in gill tissues. Severity of lesions increased with enhancing oxadiargyl concentration The induced histological lesions in gill tissues can also lead to respiratory distress via reduction in oxygen up-taking by secondary lamellae, subsequently decreasing fish activity and growth performance (Caldwell, 1997). Also, toxic compounds naturally reduce respiratory function of freshwater fish, whereas oxygen consumption rate is applied to maintain cell viability during stress increase.

Significant increase of DNA damage revealed by comet assay in this study indicated genotoxic potential of oxadiargyl in common carp (*C. carpio*). Similarly, it is reported that oxadiazon, belonging to the same chemical class, could cause DNA damages in erythrocytes of *C. carpio* (Zanjani *et al*., 2017).

Our finding had also similarity with results of Klobučar *et al*. (2010) and Mitkovska *et al*. (2017), assessing genotoxic effects of pesticides by comet assay in common carp, confirming that species and method can be considered for assessment pesticides in aquatic environments. Also, results of genotoxic studies on pesticides are intermittently dependent on purity of active ingredient.

In this study, DNA damage in erythrocytes cells was recorded after 30 days of exposure, while Cavalcante *et al*. (2008) reported a non-persistent DNA damage in gill cells of streaked prochilod (*Prochilodus lineatus*) exposed to roundup herbicide which may be due to intrinsic differences in cell turnover and/or the repair enzyme system of erythrocytes (Moreno *et al*., 2014).

In conclusion, this study investigated toxic effects of a widely used herbicide, oxadiargyl, in common carp fingerlings. The results showed that oxadiargyl is highly toxic to common carp and had genotoxic and hematotoxic effects, as well as adverse effects on tissue of vital organs. Moreover, our findings further confirmed that common carp can be considered as a suitable species for assessment of pesticides in aquatic environments. Further studies are needed to find out toxic effects of oxadiargyl on other species that may also naturally be exposed due to their close habitat to the area of the herbicide use.

Acknowledgments

The authors would like to thank M. Ahmadpoor for assisting with histological sections. This study was supported by Research Council of Islamic Azad University, Science and Research Branch, Tehran, Iran.

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