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Original Article

Sub-lethal toxicity of chlorpyrifos on Common carp, *Cyprinus carpio* (Linnaeus, 1758): biochemical response

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Abstract: Chlorpyrifos, an organophosphate pesticide, is widely used to control pests in agriculture farms and orchards of fruit trees. In this study, the fish were exposed to sub-lethal concentrations of chlorpyrifos which were determined based on numerical value of 96 h LC_{50}. Blood was sampled after 10, 20 and 30 days and biochemical parameters including glucose, total protein, albumin, globulin, triglyceride and cholesterol levels, and aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatine kinase (CK), alkaline phosphatase (ALP) and acetylcholinsetrase (AChE) activities were measured. Behavioral changes in the fish were also recorded during the experiment. Unbalanced swimming, swimming in the surface water and hyperglycemia, increased blood triglyceride, and increased levels of AST, LDH and CK activities as well as decreased levels of AChE activity were important changes that were observed in the specimens exposed to chlorpyrifos during experimental periods. The most important alterations in the blood biochemical parameters were measured in the specimens exposed to 40 µg/L chlorpyrifos on the 20th and 30th day of the trial. In conclusion, results of the present study indicated that exposure to sub-lethal concentrations of chlorpyrifos as low as 40 µg/L may cause biochemical and behavioral changes in *Cyprinus carpio*.

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Introduction

Chlorpyrifos [0, 0-diethyl 0-(3, 5, 6-trichloro-2-pyridinyl)-phosphorothioate] is a broad-spectrum organophosphate pesticide which is widely used in agriculture and in domestic use against harmful insects (Wu and Laird, 2003; Lee et al., 2004). There are many pathways by which chlorpyrifos distribute throughout the aquatic ecosystems. The major route of chlorpyrifos to aquatic ecosystems is through rainfall runoff and air-drift (Xing et al., 2012). Different concentrations of chlorpyrifos were detected in the world's groundwater and surface waters (Turner, 2003). Moreover, chlorpyrifos is moderately persistent in aquatic environments, especially in freshwater and estuaries (Turner, 2003). So, during and after treatment with chlorpyrifos to control pest, non-target animals are probably exposed to the pesticide.

The fishes living in aquatic ecosystems close to agricultural fields are the most important non-target organisms that can be affected by pesticides. In surface waters chlorpyrifos may be absorbed through the gill, skin and digestive system of fish and distributed in different tissues via the blood. Due to the lipophilic property of this pesticide, it accumulates mainly in fatty tissues. Bioaccumulation and detoxification of chlorpyrifos in the liver may effect on the physiological function of cells (Wheelock et al., 2005; Oruç, 2010; Xing et al., 2012; Ural, 2013). Since dysfunction in the

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physiology of a cell exposed to toxic substances can change the levels of biochemical parameters in cytoplasm and extracellular fluids such as blood (Banaee et al., 2011; Saravanan et al., 2011). Monitoring the alterations of the blood biochemical parameters could be a useful tool to diagnose toxicity effects in target organs to determine the physiological status in fish exposed to pesticides (Banaee et al., 2008; Banaee et al., 2011; Banaee, 2012; Banaee, 2013).

Common carp (*Cyprinus carpio*) is one of the most important cultured fish in Iran. Common carp and other species belonging to the family Cyprinidae are found in most rivers in Iran, making it a proper model species to study eco-toxicity of pesticides. Therefore, the aim of this study was to identify the changes in the blood biochemical parameters of carp exposed to sub-lethal concentrations of commercial formulation of chlorpyrifos.

Materials and methods

Experimental animals: Healthy common carp (*C. carpio*) (body weight: 95 ± 15 g (mean ± SD)), were purchased from a local fish farm and acclimated to the laboratory conditions for 2 weeks before the experiments. Fishes were randomly introduced into 12 closed 200-L recirculating tanks supplied with oxygenated water maintaining constant dissolved oxygen at 6.5 ± 0.5 mg/L, temperature at 24 ± 2 °C, pH at 7.4 ± 0.2, water hardness 150 ± 5 mg/L CaCO3 and natural photoperiod. During acclimation and all experimental tests, specimens were fed with commercial carp pellets (Beyza Fedd Mill Co. Iran) at the manufacturer's recommended rate (2% of their body weight twice a day). Fishes were starved for 24 h before sacrifice.

Acute toxicity experiments: 180 immature common carp were used in acute toxicity tests. The acute toxicity test was conducted following the OECD Guideline No. 23 under static-renewal test conditions (OECD, 2000). Test solutions of chlorpyrifos were prepared from a commercial chlorpyrifos, Emulsifiable concentrate (EC) 40.8%, with the active molecule chlorpyrifos [0, 0-diethyl 0-(3, 5, 6-trichloro-2-pyridinyl)-phosphorothioate], were purchased from National Agrochemical Co. Iran. Nominal concentrations of chlorpyrifos were 0.0 (control), 50, 150, 250, 500 and 1000 µg/L. Thus, the acute toxicity was conducted with six different concentrations of pesticides and three replicates for each concentration in 18 fiberglass tanks (200 L). During the 96 h acute toxicity experiment, water was aerated and had the same conditions as the acclimation period. The static-renewal tests exposed the specimens for 96 h to test solution. After 24 h test solution was replaced and all stock solutions were made immediately prior to use. Water was changed daily to reduce the build-up of metabolic wastes and to keep concentrations of chlorpyrifos close to the nominal level. Fish mortality was recorded 0, 24, 48, 72, and 96 h after exposure to chlorpyrifos. LC_{50} values were calculated using the Probit Analysis (Hahn and Soyer (date unknown)).

Sub-lethal toxicity experiments: Ninety common carp were randomly distributed in nine 200-L fiberglass tanks (3 treatments with three replicates) the experiment was run for 30 day period sub-lethal toxicity tests. Every tank had 10 specimens were exposed to test solutions with the following concentrations of chlorpyrifos: 0.0 (control), 20 (10% 96 h LC_{50}) and 40 µg/L (20% 96 h LC_{50}). Sub-lethal concentrations were selected according the previous acute toxicity test. The water was changed daily to reduce the build-up of metabolic wastes and to keep concentrations of chlorpyrifos near the nominal level. Nine fishes per each exposure concentration were captured and anesthetized with extract of clove powder (200 mg/L) after 10, 20 and 30 days of exposure. Blood samples of specimens from each experimental group were collected from the dorsal aorta and stored in sterilized glass vials at 4 °C containing the anticoagulant 1% EDTA. The blood was centrifuged for 15 min at 4000 rpm and the plasma was recovered. Samples were maintained at -21°C until biochemical analyses.

Biochemical Analyses: Glucose, total protein, albumin, globulin, triglyceride and cholesterol of

Table 1. Lethal concentrations of chlorpyrifos at different times.

LC	24h	48h	72h	96h
LC99	2149.55±309.61	1205.68±126.67	774.58±88.90	596.29±66.88
LC90	1570.78±210.25	867.27±84.11	551.41±57.52	419.56±42.75
LC80	1327.08±169.61	724.78±67.42	457.43±45.26	345.14±33.51
LC70	1151.36±141.27	622.03±56.38	389.67±37.24	291.48±27.66
LC60	1001.21±118.20	534.24±48.13	331.77±31.41	245.63±23.66
LC50	860.87±98.27	452.18±42.01	277.65±27.38	202.78±21.23
LC40	720.53±81.15	370.12±38.17	223.53±25.39	159.92±20.55
LC30	570.38±68.35	282.33±37.41	165.63±26.06	114.07±21.93
LC20	394.66±65.07	179.58±41.09	97.87±30.21	60.41±25.81
LC10	150.96±82.75	37.09±52.21	3.90±39.74	0.00±33.65

plasma were determined by standard procedures used in clinical biochemistry laboratories based on manual biochemical kits (Pars Azemon Co, Iran) (Thomas, 1998; Johnson et al., 1999). In measurement of creatine kinase (CK) activity, the enzyme reacts with creatine phosphate and ADP to form ATP, which is coupled to the hexokinase/GDP reaction generating NADPH. Lactate dehydrogenase (LDH) activity was measured based on the conversion of pyruvate to L-lactate by monitoring the oxidation of NADH. Aspartate aminotransferase (AST) was assayed in a coupled reaction with malate dehydrogenase in the presence of NADH. In alanine aminotransferase (ALT) assay, the enzyme reacts with alanine and α-ketoglutarate to form glutamate and pyruvate. Pyruvate is converted by lactate dehydrogenase to make lactate and NAD^+. All these activities were monitored by measuring the change in absorbance at 340 nm. Alkaline phosphatase (ALP) assay is based on the enzyme-mediated conversion of p-nitrophenol phosphate to nitrophenol in an alkaline buffer at 405 nm (Moss and Henderson, 1999). Plasma acetylcholinsetrase (AChE) activity was determined by adding and adequate volume of sample into a cuvette containing 0.1 M phosphate pH 8.0, and acetylcholine iodide (0.015 M) and dithiobis nitrobenzoic acid (0.01 M) as substrates. AChE activity was recorded during 180 s at 405 nm (Knedel and Boetteger, 1967). All biochemical parameters were measured by UV/VIS spectrophotometer (model UNICCO 2100). ***Statistical analysis:*** A significant difference in biochemical characteristics of specimens Statistical exposed to the different concentrations of chlorpyrifos was examined using one-way ANOVA. All the data were examined for normality (Kolmogorov-Smirnov test). The significant means were compared by Duncan's test and a $P < 0.05$ was considered statistically significant. Statistical analyses were performed using SPSS (IBM, Release 19) software. Data are presented as mean ± SD.

Results

LC_{50} of chlorpyrifos at 24, 48, 72 and 96 hours are presented in Table 1. Fish mortality elevated with increasing the concentration of chlorpyrifos. Sub-lethal concentrations of chlorpyrifos were determined according to 96 h LC_{50}.

No mortality was observed during the experiment. Increased mucus secretion, color changes, behavioral changes such as tremors, lethargy, unbalanced swimming, swimming in the surface water and extreme irritability were important changes found in the individuals exposed to chloryprifos.

Alterations in enzymatic activities in plasma are presented in Table 2. Levels of AST activity in fish exposed to 40 µg/L chlorpyrifos were significantly higher than control group from day 10 onwards ($P<0.05$). At the end of experiment, ALT activity in

Table 2. Changes in the level of enzyme activities in blood of the fish exposed to different concentrations of chlorpyrifos.

Parameter (U/L)	Concentration (µg/L)	Duration of exposure (days)		
		10	20	30
AST	0.0	277±44[a]	218±59[a]	265±66[a]
	20	293±42[ab]	324±51[b]	326±62[a]
	40	330±36[b]	343±49[b]	415±76[b]
ALT	0.0	28±8[a]	27±8[a]	25±3[a]
	20	27±6[a]	28±7[a]	30±5[ab]
	40	28±6[a]	30±7[a]	31±7[b]
LDH	0.0	529±116[a]	477±46[a]	496±20[a]
	20	554±79[a]	598±83[b]	623±100[b]
	40	570±87[a]	605±81[b]	643±109[b]
ALP	0.0	81±14[a]	102±23[a]	107±24[a]
	20	81±12[a]	88±15[a]	94±16[a]
	40	81±11[a]	88±13[a]	107±11[a]
AChE	0.0	844±84[b]	839±32[c]	860±52[b]
	20	775±55[b]	556±104[b]	343±89[a]
	40	560±104[a]	393±28[a]	288±51[a]
CPK	0.0	792±79[ab]	816±41[a]	841±54[a]
	20	838±40[b]	869±41[a]	937±25[b]
	40	740±92[a]	865±118[a]	1049±87[c]

plasma of the individuals exposed to 40 µg/L chlorpyrifos increased significantly compared with the control group (P<0.05). LDH activity increased significantly on the 20th and 30th day, in both concentrations. No significant changes were detected in ALP activity. CK activity was significantly higher than the control group on the 30th day. The specimens exposed to 40 µg/L chlorpyrifos indicated a reduced AChE activity on the 10th day and 20th day of the experiment.

Changes in plasma biochemical parameters of fish exposed to different concentrations of chlorpyrifos are presented in Table 3. Although no significant changes were found in plasma total protein and globulin on the 10th day (P>0.05), both decreased significantly on the 20th and 30th day of trial in the two concentrations. However, a significant reduction was found in plasma albumin of the specimens exposed to 20 µg/L chlorpyrifos on the 30th day (P<0.05). There were no significant changes in albumin levels in plasma of fish exposed to chlorpyrifos on the 10th and 20th days of experiment (P>0.05). Glucose and triglyceride increased significantly at 40 µg/L. Cholesterol increased at 40 µg/L on the 20th day.

Discussion

The present results show that chlorpyrifos is highly toxic to common carp. The observed 96 h LC_{50} was 203 ± 21 µg/L. Halappa and David (2009) found a 96 h LC_{50} values for chlorpyrifos in common carp of 160 µg/L, which is highly comparable to our results. The 96 h LC_{50} values of chlorpyrifos in *Oreochromis mossambicus*, *O. niloticus* and *Gambusia affinis* were 154, 25.9 µg/L and 297 µg/L, respectively (Oruç, 2010; Rao et al., 2003; Rao et al., 2005).

The main breakdown product of chlorpyrifos in the environment and in organisms is the chloropyrifos-oxon, which disrupts AChE activity (Chawanrat et al., 2007; Oruç, 2010). Decreases in AChE activity in the plasma of piava (*Leporinus obtusidens*) exposed to glyphosate have been reported (Glusczak et al., 2006). Halappa and David (2009) believed that

Table 3. Changes in blood biochemical parameters of the specimens exposed to different concentrations of chlorpyrifos.

Parameters	Concentration (µg/L)	Duration of exposure (days)		
		10	20	30
Protein (g/dL)	0.0	3.51±0.28[a]	3.85±0.31[b]	3.93±0.18[c]
	20	3.46±0.27[a]	3.26±0.19[a]	3.30±0.19[b]
	40	3.26±0.41[a]	3.15±0.40[a]	3.04±0.20[a]
Albumin (g/dL)	0.0	2.07±0.13[a]	2.04±0.17[a]	2.15±0.10[b]
	20	2.13±0.10[a]	2.04±0.09[a]	2.03±0.12[a]
	40	2.08±0.12[a]	2.15±0.30[a]	2.19±0.11[b]
Globulin (g/dL)	0.0	1.43±0.18[a]	1.81±0.30[b]	1.77±0.21[c]
	20	1.32±0.20[a]	1.21±0.18[a]	1.28±0.25[b]
	40	1.18±0.36[a]	1.00±0.20[a]	0.85±0.26[a]
Glucose (mg/dL)	0.0	78.61±14.16[a]	81.33±15.53[a]	65.29±10.92[a]
	20	93.60±16.30[ab]	95.67±23.23[ab]	91.92±12.30[b]
	40	98.11±16.06[b]	103.01±18.05[b]	116.41±20.39[c]
Triglyceride (mg/dL)	0.0	180.81±18.13[a]	186.39±20.62[a]	185.53±29.84[a]
	20	207.44±20.77[b]	198.11±23.73[a]	210.94±21.66[a]
	40	208.56±12.35[b]	224.68±23.38[b]	245.45±26.89[b]
Cholesterol (mg/dL)	0.0	173.75±34.31[a]	165.35±26.85[a]	189.72±39.46[a]
	20	200.43±37.70[a]	182.50±35.02[ab]	212.24±35.01[a]
	40	196.38±30.87[a]	200.86±38.53[b]	207.99±44.07[a]

chlorpyrifos is not only inhibiting AChE activity, but also has negative effects on functioning of the respiratory and digestive systems of fish.

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), Lactate dehydrogenase (LDH), Alkaline phosphatase (ALP) and Creatine kinase (CK) are found in almost all cells of the body tissues, such as heart, kidneys, liver, skeletal muscle, brain, erythrocyte, intestine and gills. The release of intercellular enzymes into the blood and their increased activity in plasma are the most important clinical signs in the diagnosis of damage to cell membranes (Banaee et al., 2011). So, increase in plasma AST, ALT, LDH and CK activities of fish exposed to the chlorpyrifos may indicate damage to cell membranes, particularly liver cells. Increased activities of AST and ALT were observed in plasma of *Channa punctatus* (Agrahari et al., 2007), *Cyprinus carpio* (Banaee et al., 2008), and *Oncorhynchus mykiss* (Banaee et al., 2011) exposed to organophosphorus pesticides. The observed increase of LDH activity can be attributed to the conversion of accumulated pyruvate into lactate which is transported through muscle to hepatopancreas and regenerated glucose and glycogen to supply energy for fish exposed to insecticides. Similar results have been reported by Goel et al., 2006; Lavanya et al., 2011; Li et al., 2011; Saravanan et al., 2011. The increased activity of CK on the 30 day of trial may be indicative of a disorder in muscle fibers. These results agree with a previous study carried out on carp that had been exposed to bifenthrin (Velisek et al., 2008).

Increase in blood glucose of fish exposed to the chlorpyrifos may reflect an increased need for energy to counteract the effects of stress caused by chlorpyrifos toxicity. Hyperglycemia or elevated blood glucose levels indicate impaired glucose and lipid metabolism and degradation of glycogen in liver (Acker and Nogueira, 2012). Increases in blood glucose levels have been reported in *Heteropneustes fossilis* (Saha and Kaviraj, 2009), *C. carpio* (Banaee et al., 2008), and *O. mykiss* (Banaee et al., 2011) after exposure to cypermethrin and diazinon,

respectively. In stressful situations, glucose is converted to pyruvate in the glycolytic pathway, and pyruvate is metabolized to acetyl-CoA in aerobic tissues, which can be used as a precursor in the synthesis of cholesterol and fatty acids in the citric acid cycle (Murray et al., 2003).

The increase of blood triglyceride and cholesterol may be due to liver dysfunction. Destruction of cell membranes can also lead to increased levels of cholesterol in plasma. Disorder in triglyceride uptake by adipose tissue may also increase triglycerides. Increase of stress hormones such as cortisol in blood of fish exposed to various insecticides, which stimulates lipid breakdown in adipose tissue, were also found in Banaee (2013). Cholesterol and glucose levels in blood of freshwater fish, *Mystus vittatus*, increased after exposure to metasystox and sevin (John, 2007). Similar changes have also been reported by Ibrahim and El-Gamal (2003), Lasram et al. (2009) and Acker and Nogueira (2012).

A reaction between chlorpyrifos and plasma proteins may decrease levels of plasma proteins. Malnutrition, reduced efficiency of the liver in protein synthesis, and reduction of nutrient absorption, especially protein, in the digestive system may be important factors in decreasing plasma total protein. Decrease in plasma albumin and globulin levels of fish exposed to the chlorpyrifos may be due to decreases in total protein levels in plasma. Decreased globulin levels may reduce the resistance of fish to pathogens. Banaee et al. (2011) showed that the levels of total protein, albumin and globulin decreased in fish exposed to diazinon. Similar results have been reported by Banaee et al. (2008).

In conclusion, measuring blood biochemical parameters in the present study was proved to be useful for monitoring the long-term effects of chlorpyrifos in cultured fish. Exposure to sub-lethal concentrations of chlorpyrifos as low as 40 µg/L may cause biochemical changes. These changes may affect the survival of carp and should be taken into consideration when this pesticide is used near freshwater ecosystems.

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