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Effect of a glyphosate-based herbicide in *Cyprinus carpio:* Assessment of acetylcholinesterase activity, hematological responses and serum biochemical parameters

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ABSTRACT

The objective of this study was to investigate the toxicity effects of acute and sublethal of Roundup® as a glyphosate-based herbicide on acetylcholinesterase (AChE) activity and several hematological and biochemical parameters of *Cyprinus carpio*. The LC_{50}-96 h of Roundup® to *C. carpio* was found to be 22.19 ppm. Common carp was subjected to Roundup® at 0 (control), 3.5, 7 and 14 ppm for 16 days, and the AChE activity is verified in tissues of gill, muscle, brain and liver. After 5 days, a significant decrease was observed in the AChE activity of muscle, brain and liver tissues. Besides, a time- and dose-dependent increase in mean cell hemoglobin (MCH) and mean cell volume (MCV) was observed. In contrast, a significant decrease was found in the quantities of hemoglobin (Hb), hematocrit (HCT) and, red (RBC) and white (WBC) blood cell count. Also, the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) in Roundup® treated groups were significantly higher than the controlled group at experimental periods. However, the level of alkaline phosphatase (ALP) had a significant reduction behavior during the sampling days. It seems that the changes in hematological and biochemical parameters as well as AChE activity could be used as efficient biomarkers in order to determine Roundup® toxicity in aquatic environment.

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1. Introduction

Dramatic increase in growth of world population along with development and optimization of agricultural production, has led to an increase in application of chemical components such as herbicides in agriculture and public health sectors (Ferenczy et al., 1997). Among different types of herbicides, Roundup® (glyphosate 48%) as a glyphosate-based formulation has been utilized as one of the most commonly applied herbicides around the world (Jiraungkoorskul et al., 2002). In fact, it is commonly utilized in agriculture as a non-selective herbicide to eradicate annual and perennial plants, grasses and also broad-leaved woody species (WHO, 1994). The formulation of Roundup® is composed of isopropylamine (IPA) salt of glyphosate 480 g/L, water and polyethoxylated amine surfactant (POEA) (Jiraungkoorskul et al., 2002), which is believed to be much more toxic to aquatic organisms compared to the active ingredient itself (Giesy et al., 2000). This is related to the point that its water solubility and half-life in soil are 15,700 mg/L and 30–90 days, respectively (Cox, 1998). The half-life of glyphosate in aquatic environments is normally in range of 7–14 days. It was also demonstrated that the value of LC_{50}-96 h of Roundup® depending on the different factors including fish species, test circumstances, life stage and herbicide formulation is between 2 and 55 mg/L (Giesy et al., 2000). Measurements of polluted water bodies and aquatic animal health biomarkers (*e.g.*, hematological, biochemical and enzymological parameters) have been broadly used as primary diagnostic approaches (Al-Sabti and Metcalfe, 1995; Vanzella et al., 2007). Fish blood has been studied in toxicological research and environmental monitoring (Piner and Üner, 2012) as a possible biomarker of physiological and pathological alterations in fishery management and disease investigations,

In this regards, diverse hematological factors including white blood cell count (WBC), red blood cell count (RBC), hemoglobin (Hb), and hematological indices like mean cell hemoglobin (MCH), mean cell volume (MCV) and mean cell hemoglobin concentration (MCHC), and also biochemical parameters such as plasma glucose and protein are widely used to assess the toxic stress. Enzyme activities as one of the other categories of sensitive indicators have been also employed to identify tissue damage of fish exposed to diverse group of water pollutants (Saravanan et al., 2011a,b).

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Among different types of enzymes, both alanine aminotransferase (ALT) and aspartate aminotransferase (AST) have been proved to play an essential role in protein and carbohydrate metabolism. Thus, they appear to be useful as reliable biomarkers with which the tissue damage caused by the toxicants can be recognized Nemcsok and Benedeczky (1990). In addition, as a result of the chemical stress and anaerobic capability of tissue, another type of enzyme called the lactate dehydrogenase (LDH) has been identified to be used as an alternative bioindicator. Alkaline phosphatase (ALP) is also an enzyme found in certain body tissues such as liver, which is produced by the cells lining the small bile ducts (Speit and Hartmann, 1999).

It was reported that acetylcholinesterase (AChE) enzyme has a key role in the cholinergic transmission of the nervous systems (Ferenczy et al., 1997). Since the activity of this enzyme changes with different contaminant concentrations, it could be an appealing biomarker for measurement of the neurotoxicity changes (Ferenczy et al., 1997; Sturm et al., 1999; Miron et al., 2005). Many researchers previously proved that the AChE enzyme activity can inhibit by several pesticides such as organophosphate, carbamate and glyphosate (Glusczak et al., 2007; Yogesh et al., 2009; Modesto and Martinez, 2010a,b; Cattaneo et al., 2011). This inhibition was negatively affected on the growth, survival, orientation to food and reproductive behavior of fishes exposed to different pollutants (Dutta and Arends, 2003).

Previous investigations suggested that the regular tissues affected by inhibition activity of AChE are mostly brain and muscle tissues (Cattaneo et al., 2011; Glusczak et al., 2007; Moraes et al., 2011), and only a few numbers of studies have been conducted on gill and liver tissues (Oruç and Usta, 2007; Piner and Üner, 2012; Xing et al., 2012).

Due to having similar biochemical responses to those found in mammals, teleost fish including common carp might be consequently a good indicator of contamination caused by a wide range of pollutants. The common carp (*Cyprinus carpio* L.) is a fish species that is cultivated and consumed widely throughout the world.

Therefore, the objectives of this study were to determine the 96 h-LC_{50} value of the common carp subjected to glyphosate stress and the effects of its sub-lethal concentrations on the AChE activity of different tissues (brain, muscle, gill and liver), hematological and biochemical parameters.

2. Materials and methods

2.1. Experimental fish specimen and chemicals

The common carp (*C. carpio* L.) with almost same sizes (Length 10.12 ± 2.01 cm, Weight 41.03 ± 0.15 g) were collected from a local fish farm (Cilver carp, Rasht, Iran) and used for the experiment. Fish specimens were subjected to a prophylactic treatment by bathing twice in 0.05% potassium permanganate (Merck Chemical Co., Darmstadt, Germany) for 2 min to avoid any dermal infections. Fish was adapted to laboratory conditions under natural photoperiod (12 h:12 h L/D) for at least 25 days before exposure. The fishes were kept in continuously aerated water at low densities in three glass tanks (1000 L). The quality characteristics of used water were as follows: temperature 20 ± 1 °C, pH 7.02 ± 0.07, dissolved oxygen 6.50 ± 0.12 mg/l, water hardness 163 ± 5 mg/L $CaCO_3$ and daily water exchange rate 5%. Fish was fed twice a day with commercial carp pellets at the manufacturer's recommended rate (2% of their body weight twice a day). Also, the fish health was evaluated based on behavioral changes during direct observations.

A commercial formulation of glyphosate (N-(phosphonomethyl) glycine), Roundup®, (Monsanto company, St. Louis, Missouri USA) containing isopropylammonium salt of glyphosate at 480 g/l as the active ingredient (equivalent to 360 g glyphosate per liter) and POEA as surfactant was used in this study. All the other chemicals were purchased from Merck Chemical Co. (Darmstadt, Germany).

2.2. Measurement of sub-lethal concentrations and experiment design

A total of 180 immature *C. carpio* were used in acute toxicity bioassays to determine the LC_{50}-96 h value of Roundup®. This test was performed according to the OECD guideline test 203 under static-renewal test conditions (OECD, 1992). Nominal concentrations of active ingredient tested were 0 (control), 19, 22, 25, 28 and 31 mg/l and each of the concentration was set in triplicate in 300 L fiberglass tanks. 10 fish specimens in each tank were randomly exposed to each of the six Roundup® target concentrations. The water was changed daily to reduce the buildup of metabolic wastes. In the next step, the mortality of fish after Roundup® exposure for 24, 48, 72 and 96 h was recorded. LC_{50}-96 h value was determined by the Probit analysis test according to the applied method by Aydın and Koprucu (2005). Based on the obtained value for LC_{50}-96 h, three test concentrations of Roundup® including 1 (~15% of LC_{50}=3.5 ppm), 2 (~30% of LC_{50}=7 ppm) and 3 (~60% of LC_{50}=14 ppm) were determined in order to design the sublethal tests for the *in vivo* experiment. Common carps were exposed for 16 days to glyphosate based on the half-life of the herbicide (Cattaneo et al., 2011), 50% of the water was renewed and additional herbicide was transferred to the tanks by the 4th day after the beginning of experiment to sustain the expected concentration.

The analysis of glyphosate concentration in water was carried out using an HPLC system as previously explained by Shiogiria et al. (2012). A Knauer (Berlin, Germany) HPLC system has been used which included a K-1001 HPLC pump, a K-1001 solvent organizer, an on-line degasser, a dynamic mixing chamber and a scanning HPLC fluorescence detector (Model RF-10XL) with the excitation and emission wavelengths set at 266 nm and 316 nm, respectively. The separation was performed on an ACE® 5C 18 reverse phase ($4.6\ mm \times 250\ mm \times 5\ \mu m$). The mobile phase was acetonitrile:dihydrogenphosphate buffer (3:97 v/v) at a flow rate of 1.0 mL/min. The chromatographic data was collected and recorded using EuroChrom 2000 software from Knauer, which was controlled under Windows XP platform.

After the onset of Roundup® exposure, the rate of six fishes per treatment at 1, 3, 5, 9 and 16 days were removed, and immediately anesthetized with benzocaine (0.15 ± 0.05 g/L). Blood samples were collected through tail vein puncture from the control and treated groups. Whole blood was used for the estimation of hematological parameters. The residual blood sample were centrifuged at 4500 rpm and at 4 °C for 15 min and the plasma was removed and stored at −70 °C the biochemical analysis. In the next step, they were killed by medullar section and the required tissues (gill, muscle, brain and liver) for the analysis were removed by dissection. Then, these tissues were washed in ice-cold physiological saline solution (0.59% NaCl) and stored at −80 °C for the analysis.

2.3. AChE activity assay

The gill, muscle, brain and liver tissues were thawed and homogenized in ice (5–10 volume) in potassium phosphate buffer (0.1 M, pH 7.5), and then centrifuged at 12,000 rpm and at 4 °C for 15 min. The obtained supernatant was applied for analysis of the AChE activity. This assay was carried out according to the colorimetric technique of Ellman et al. (1961) developed by Alves Costa et al. (2007). The final concentration of the AChE iodide substrate was used, and the Ellman's color reagent (5,5′-dithiobis-(2-nitrobenzoic acid) or DTNB) were 9 and 0.5 mM, respectively. An ELISA microplate reader (Beckman Colter, Fullerton, CA, USA) was used to determine absorbance amount at 415 nm. The AChE activity was expressed as nmol DTNB/min/mg protein. Also, the protein concentration in brain samples was determined by the method of Lowry et al. (1951). Bovine albumin was used as standard.

2.4. Hematological analysis

The counts of RBC and WBC, blood Hb (g/dl) content, hematocrit (HCT) percentage and differential leukocytes were determined using a hemocytometer according to the method applied by Klont et al. (1994). The blood indices such as MCV, MCH and MCHC were calculated according to the method applied by Saravanan et al. (2011a,b).

2.5. Serum enzyme assay

Serum ALT, AST, ALP and LDH concentrations were determined using an automated chemical analyzer (Auto analyzer (Ependrof)) and the commercial diagnostic kits (Pars Azmoon Co., Tehran, Iran).

2.6. Statistical analysis

All analytical experiments were performed in triplicate, and the results were presented as a mean of the three values with the standard deviation. All the data were tested for normality (Kolmogorov-Smirnov test). The results were subjected to two-way analysis of variance (ANOVA) and Tukey multiple-range tests using SPSS 15.0 (SPSS Inc., USA) software. A value of $p < 0.05$ was considered statistically significant.

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