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GLYPHOSATE INDUCES MORPHOLOGICAL AND ENZYMATIC CHANGES IN COMMON CARP (*CYPRINUS CARPIO* L.) LIVER

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Abstract

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The main aim of the present study was to investigate the effects of glyphosate based herbicide on histological and biochemical parameters of common carp (*Cyprinus carpio* L.) liver. We used 20 $mg.l^{-1}$, 40 $mg.l^{-1}$ and 72 $mg.l^{-1}$ of the test chemical under laboratory conditions. Histological lesions which we observed in the liver parenchyma were degenerative and necrotic. Degenerative alterations were as follows: granular, balloon and fatty degeneration. Necrotic alterations in the fish liver were associated with presence of karyopyknosis and karyolysis, respectively. Histological alterations in the liver blood vessels were associated with lymphocyte proliferation and hyperemia. In addition, the degree of histopathological alterations in the liver was increased proportionally to the increasing glyphosate concentrations. We also measured the activity of the hepatic enzymes lactate dehydrogenase (LDH), aspartate aminotransefrase (ASAT) and alanine aminotransefrase (ALAT). We determined that the enzymatic activity of LDH in the exposed fish liver was significantly increased compared to the control group ($P < 0.05$). Moreover, the enzymatic activity of ASAT and ALAT in the exposed fish liver was decreased compared to the control.

Key words: glyphosate, liver, histology, enzymatic activity, common carp

Introduction

Pesticides are one of the most potentially harmful chemicals introduced into the environment. Their adverse effects on non-target organisms are significant (John, 2007; Velcheva et al., 2012). When a herbicide is used to control weeds, sometimes a majority of the compound ends up in the environment. Due to the widespread use of these chemicals over the years, there has been an accumulation of their residues in the environment, which causes alarming contamination in the ecosystems and leads to negative impacts on the biota (Marin-Morales et al., 2013). Glyphosate is one of the most common herbicides used in agriculture, but also in forestry and horticulture (including home use) (EPA, 2011). According to the information from the respective chemical safety data sheets, the glyphosate-based herbicides are classified as hazardous to the aquatic environment (toxic to aquatic life with long lasting effects) (Sihtmäe et al., 2013).

Fish can be exposed to pesticide contamination during their life cycle (Velisek et al., 2009). and they are among the group of non-target aquatic organisms (Velcheva and Baltova, 2002) Fish liver carries out essential body functions, including regulation of metabolism and detoxification of toxic compounds. In general, the liver is a target organ due to its

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large blood supply, which causes noticeable toxicant exposure (Mohamed, 2009). According to Stentiford et al. (2003) numerous categories of liver pathology are present as reliable biomarkers of toxic damage. Histological and ultrastructural changes in the cells can be used as good biomarkers of pollutant stress. Moreover, histopathology makes it possible to detect both acute and chronic changes in the tissue of individual organisms. Thus, the biochemical parameters in fish liver are sensitive for detecting potential adverse effects and relatively early events of pollutant damage (Stentiford et al., 2003) Furthermore, changes in the activity of liver enzymes such as lactate dehydrogenase (LDH), aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) serve as an indicator for normal liver function, and they also can be used as biomarkers for tissue damage (Almeida et al., 2002). Thus, it can be concluded that these enzymes are sensitive biomarkers for the determining stress in the fish subjected to various pollutants present in the waters (Adhikari et al., 2004). Hence, assessment of the biochemical and histological changes in the fish liver has become an important tool for evaluation the environmental pollution under experimental conditions.

As toxicological data on "Nasa 360 SL" herbicide toxicity on fish is relatively scarce, the main objective of the present study is to observe the effects of the glyphosate based herbicide on the morphological structure of common carp liver, as well as, hepatic activity of the enzymes lactate dehydrogenase (LDH), aspartat (ASAT) and alanine (ALAT) aminotransferases.

Materials and Methods

Experimental set up

Glyphosate (IUPAC name N-(phosphonomethyl)-glycine) is an active substance of the test herbicide "Nasa 360 SL".

Forthy healthy common carps were obtained from the Institute of Fisheries and Aquaculture in the city of Plovdiv, Bulgaria. They were of the same size-group (mean length 15.5 cm ± 2.33; mean body mass 43.5 g ± 2.3) with no external pathological abnormalities. After transportation the fish were moved in glass aquaria with chlorine-free tap water (by evaporation) to acclimatize for a week. After acclimatization the fish were divided into four groups (n = 10) in chlorine-free tap water. Fish were not fed 48 hours prior to the experiment.

Three groups of fish were exposed to the herbicide at concentration of 20 $mg.l^{-1}$, 40 $mg.l^{-1}$ and 72 $mg.l^{-1}$. The first concentration of 20 $mg.l^{-1}$ represented LC_{50} for common carp, given in the instructions of the manufacturer. The second concentration of 40 $mg.l^{-1}$ was twice as high as LC_{50} and 72 $mg.l^{-1}$ is four times higher than the LC_{50}, respectively. The fourth fish group served as a control with no added chemical.

All aquaria had a permanent aeration with air pumps and the water was kept oxygen saturated. During the entire duration of the experiment the fish were maintained under a natural light/dark cycle (12:12). Physico-chemical characteristics of the aquarium water such as: pH, temperature, dissolved oxygen; oxygen saturation and conductivity were measured once per day according to standard procedure (APHA, 2005). They were as follows: pH – 7.8; temperature – 23.22^0C ± 0.7; dissolved oxygen – 7.4 $mg.l^{-1}$ ± 0.25; oxygen saturation – 90.78% ± 0.75 and conductivity – 380 ± 10.5 $\mu S.cm^{-1}$.

All experiments were conducted in accordance with national and international guidelines of the European Parliament and the Council on the protection of animals used for scientific purposes (Directive 2010/63/EU).

Histological analysis

Fish dissection was performed according to the international standard procedures given in the EMERGE Protocol (Rosseland et al., 2003). Fish liver was divided into two pieces, for both histological and biochemical analyses. Samples were placed in vials with 10% neutrally buffered formaldehyde solution (pH = 7) for 12 hours. They were rinsed in tap water, dehydrated in a graded series of ethanol concentrations, cleared in xylene, embedded in paraffin wax with melting point of 54–56°C, sectioned to a thickness of 5–7 μm using a semi-automated rotary microtome (Leica RM 2245, Germany) and mounted on sterilized glass slides. Sections were then deparaffinised, stained with hematoxylin and eosin (H&E) for histological examinations and prepared for light microscopy analysis (Romeis, 1989). Histological changes in the liver were observed and photographed by using a light microscope (Nikon, Japan) mounted with a digital camera.

Liver histology of all specimens, including the control fish livers were appraised individually and semi-quantitatively by using the grading system of Mishra and Mohanty (2008). Each grade represented specific histological characteristics and was categorized as follows: (–) – no histological alterations; (+/–) – mild histological alterations; (+) – moderate histological alterations; (++) – severe histological alterations; (+++) – and very severe histological alterations in the hepatic architecture.

Enzymatic analysis

Livers were rapidly thawed on ice and manually homogenized, using a Potter Elvehjem homogenizer fitted with a Teflon pestle in chilled phosphate buffer (50 mM, 300 mM NaCl, pH = 7.4). Homogenates was subjected to centrifugation at 9000 rpm for 15 min in a cooling centrifuge (MPW 351 R, Poland) at 4°C. Supernatant fractions were aliquoted, transferred in new

eppendorf tubes and stored at –80°C for further enzyme assays. All biochemical assays were measured spectrophotometrically (Beckman Coulter Spectrophotometer DU 800, USA) at 25°C. Chemicals used in this study were purchased from Sigma Chemical Co. and were of analytical grade.

Lactate dehydrogenase (LDH, E.C. 1.1.1.27) activity was assayed according to Vassault (1983). Aspartate aminotransferase (ASAT, E.C. 2.6.1.1) and alanine aminotransferase (ALAT, E.C. 2.6.1.2) acitivities were determined by the method of Reitman and Frankel (1957) as described by IFCC (1986) using commercially available kits.

Protein levels were measured by the Bradford (1976) method with Coomassie Brilliant Blue G-250 using bovine serum albumin as standard. Absorbance of samples was detected at 595 nm and expressed as milligram protein per millilitre homogenate.

One unit of LDH, ASAT and ALAT is defined as the amount of the enzyme that consumes 1 mol.l^{-1} of substrate or generates 1 mol.l^{-1} of product per min. Activity was expressed in international units per milligram of protein.

Statistical analysis

Activity of the hepatic enzymes was expressed as mean±SD. Descriptive statistics was performed and software packet STATISTICA version 7.0 for Windows (StatSoft Inc., USA, 2004) was used. Differences between the individual variables were tested for significance using the Student's t-test ($P < 0.05$) and one-way analysis of variance (ANOVA).

Results and Discussion

Histological changes

The present study demonstrated that the control fish liver generally exhibited a normal architecture (Figure 1*a*), which is in agreement with Takashima and Hibiya (1995). On the other hand, histological analysis showed degenerative and necrotic changes in the fish exposed to all glyphosate concentrations (Table 1).

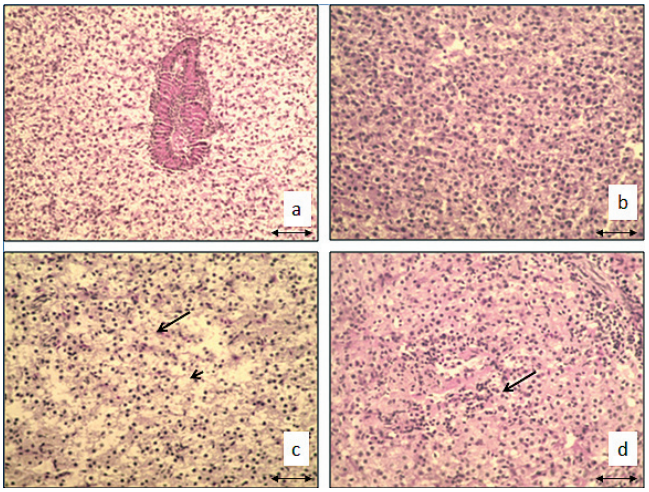

Fig. 1. Histological alterations in common carp liver after glyphosate exposure, × 400, H&E
***a*) control group**
***b*) granular degeneration, 20 mg.l^{-1}**
***c*) fatty degeneration (short arrow); balloon degeneration (long arrow), 40 mg.l^{-1}**
***d*) lymphocyte proliferation, 72 mg.l^{-1}**

We found granular, balloon and fatty degeneration. Furthermore, we determined necrotic alterations in the fish liver which were presented as karyopyknosis and karyolysis. We also found necrotic areas of liver parenchyma which were presented in cell mass where the boundaries of the separate hepatocytes could not be seen. Histological alterations in the hepatic blood circulatory system were lymphocyte prolif-

Table 1

Histological alterations in common carp liver after glyphosate exposure

Histological alterations	Glyphosate concentrations			
	Control	20 mg.l^{-1}	40 mg.l^{-1}	72 mg.l^{-1}
Granular degeneration	–	++	++	+++
Balloon degeneration	–	+	+	++
Fatty degeneration	–	+/–	+/–	+/–
Necrotic alterations:				
Karyopyknosis	–	+/–	+/–	+/–
Karyolysis	–	+/–	+/–	+/–
Necrosis	–	+/–	+/–	+
Lymphocyte proliferation	–	+/–	+	+
Hyperemia	–	+/–	+/–	+/–

(–) – no histological alterations; (+/–) – mild histological alterations; (+) – moderate histological alterations; (++) – severe histological alterations; (+++) – and very severe histological alterations in the hepatic architecture.

eration and hyperemia. Degree of expression of histological alterations increased proportionally with the increasing concentrations of glyphosate. At the lowest concentration of glyphosate (20 mg.l^{-1}) granular degeneration was presented in a severe degree of expression (Figure 1*b*). We determined balloon degeneration in a moderate degree of expression, but fatty degeneration was presented in a mild degree. In addition, we found necrotic alterations and necrosis in a mild degree of expression, respectively. Disturbance in the hepatic blood circulation in the fish group exposed to 20 mg l^{-1} glyphosate was also in a mild degree of expression. Similarly to the previous concentration of 20 mg l^{-1} glyphosate, we found granular degeneration in a severe degree of expression in the fish liver exposed to 40 mg.l^{-1} of the test chemical, as well as balloon and fatty degeneration in a moderate and mild degree of expression. Necrotic alterations, necrotic areas of liver parenchyma and hyperemia were presented in a mild degree of expression. In addition, lymphocyte proliferation was found in a moderate degree. Histological alterations in the common carp liver were most pronounced at the highest concentration of 72 mg.l^{-1} glyphosate. We determined granular degeneration in a very severe degree of expression. Balloon degeneration was in a severe degree. We also found fatty degeneration, necrotic alterations and hyperemia in a mild degree of expression. Fatty degeneration was presented in lipid deposits in single hepatocytes of the liver parenchyma. In addition, necrosis and lymphocyte proliferation were in a moderate degree of expression (Figure 1*c*, *d*).

According to Greenfield et al. (2008) histopathological biomarkers can be good indicators for the link between the action of the toxicant and the histological structure of the test organ. Ayoola (2008) found that the fish liver exposed to glyphosate had infiltration of leukocytes, increased hepatocyte size with pyknotic nuclei and presence of vacuoles. Ahmad et al. (2002) stated that disturbances in the osmotic regulation of cellular membranes resulted in increasing the volume of the nuclei and nucleoli, and thislead to necrosis of liver cells. In addition, vacuolation of hepatocytes in *Cirrhinus mrigala* has been found after exposure to the pesticide lambda-cyhalothrin (Velmurugan et al., 2007), as well as, in *Corydoras paleatus* after exposure to the pesticide methyl parathion (Fanta et al., 2003). McHugh et al. (2011) found that the liver alterations in fish exposed to pesticides were mostly associated with circulatory disturbances, related to pathological conditions of blood and tissue fluid flow, and regressive changes. They included dilation of blood sinusoids, as well as, cytoplasmic granular degeneration and vacuolation of the hepatocytes. Similarly to Ayoola (2008) we consider that necrosis of some areas in the liver tissue were probably resulted from the excessive work required by the fish to get rid of the toxicant from its body during the process of detoxification by the liver.

Biochemical changes in enzymatic activity

Enzymatic activity of LDH, ASAT and ALAT was significantly different at all glyphosate concentrations compared to the control group (Table 2).

Exposure of the fish liver to glyphosate for 96 hours increased LDH activity in all test fish groupswhen compared to the control (Table 2). In addition, LDH activity in the fish groups decreased with increasing the herbicide concentrations in the following order 20 mg l^{-1} < 40 mg l^{-1} < 72 mg l^{-1} glyphosate, respectively ($P < 0.05$). Some significant changes in ASAT and ALAT activities were observed dependent upon the glyphosate concentrations compared to the control group ($P < 0.05$). Similarly to LDH activity, ASAT and ALAT activities decreased with increasing the herbicide concentrations compared to the control group

Lactate dehydrogenase (LDH) is an enzyme which takes part in anaerobic pathway of carbohydrate metabolism (Banaee, 2013). Aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) are liver specific enzymes, participating in the amino acids metabolism. They are sensitive indicators for hepatotoxicity and histophathalogic changes and can be assessed within a shorter time (Balint, 1997). Increase of LDH activity is a diagnostic index widely used to recognize increases of anaerobic metabolism resulting from depletion of energy under anaerobic and

Table 2

Hepatic activities of LDH, ASAT and ALAT in common carp (average±SD) after glyphosate exposure, including enzymatic responses in control fish

Concentration of glyphosate	Enzymatic responses U/mg protein in common carp liver (average±SD)		
	LDH	ASAT	ALAT
Control	0.3±0.05	0.53±0.07	0.56±0.05
20 mg.l^{-1}	1.24*±0.05	0.32±0.05	0.41±0.04
40 mg.l^{-1}	1.03*±0.06	0.25*±0.05	0.29*±0.04
72mg.l^{-1}	0.89*±0.06	0.14*±0.05	0.09*±0.006

*enzymatic activity significantly different than the control (P<0.05)

environmental stress conditions (Banaee, 2013). We agree with Yousafzai and Shakoori (2011) who stated that the the increased enzymatic activities in the liver might be due to increased enzyme synthesis to counter the damage caused by toxicants. In contrast, the decreased activities of some enzymes may be attributed to decreased enzyme synthesis, or it may also be due to changes in permeability of hepatic cells. According to Yousafzai and Shakoori (2011), this could be a result of the induction or inhibition of different enzymes due to the effect of the toxicant, which shows the adverse effect of aquatic pollution on the fish health. Thus, LDH enzyme can be applied as a bio-indicator for demonstrating tissue damage in fish (Saravanan et al., 2011). Das and Mukherjee (2003) reported increased LDH activity in the liver of Indian major carp, *Labeo rohita* exposed to cypermethrin. Similarly to us, Oruç and Üner (1998) also measured increased LDH activity in the fish liver, exposed to different pesticides.

We consider that the increased LDH activity is due to the effect of all test concentrations of glyphosate in our study, which may correspond to induced oxidative stress in the fish organism, resulting to a deficiency of ATP and necessity of regeneration of NADH. They ASAT and ALAT play a crucial role in protein and carbohydrate metabolism and act as an indicator for tissue damage and cell rupture (Svobodova et al., 1997). Therefore, any change in the transaminase activity can be correlated with the protein and carbohydrate metabolism and thereby help in analyzing the metabolic shifts (Beyer et al., 1996). Li et al. (2009) obtained similar to our results when they exposed rainbow trout liver to carbamazepine for 42 hrs. They observed that the chemical inhibited ASAT and ALAT activities in the liver, gills and kidney, and also reported that hepatic enzymes, followed by kidney and the gills seemed to be mostly affected by carbamazepine poisoning. Hence, we think that changes in the activity of ASAT and ALAT transaminases can affect the energy metabolism in fish.

Conclusion

Overall, we can conclude that the observed cellular and tissue alterations in the present study occurred in the common carp liver metabolism under the influence of all tested concentrations of glyphosate. These changes are probably due to oxidative stress. We also consider that the alternated hepatic morphology could be a result of changes in the liver metabolism under the influence of glyphosate. Our study shows that there is a relation between the glyphosate concentrations and severity of expression of all histological alterations, as well as, biochemical changes in the fish liver.

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