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The 2,4-D Herbicidal Effect of Defense Enzyme Activities and AChE Levels in Liver and Gill Tissues of *Xiphophorus hellerii* (Swordtail fish)

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ABSTRACT

In the present study, herbicide 2,4-D was investigated for its potency to induce oxidative stress and its effects on antioxidant systems in gill and liver tissues of (*Xiphophorus hellerii*). Animals were exposed to sublethal doses of 2,4-D for 96 hours except for the control group. Protein, malondialdehyde, catalase and acetylcholine esterase enzyme activity (AChE) were determined using spectrophotometric methods. The results show that protein levels were reduced in all experiments when compared to control group. Levels of malondialdehyde were increased in each group. Catalase enzyme activity was significantly decreased in all groups. In addition, an increased AChE activity was observed.

Introduction

Fish are vulnerable to alteration of environmental factors caused by the introduction of industrial wastes, heavy metals, pesticides, oils, pills and other agents that directly affect the aquatic ecology. Toxic effects of pesticides on the biochemical and physiological systems of a fish can be determined through the study of malondialdehyde levels (MDA) and acetylcholine esterase enzyme activity (AChE), among other methods [1]. Biochemical biomarkers can provide information about the process of pesticide detoxification.

When in contact with an organism, the toxic agent can be biotransformed by enzymes, which act to make the xenobiotic substance a less toxic compound and facilitate its excretion. Lipid peroxidation may be the first step of cellular membrane damage and can be induced by environmental pollutants as herbicides. Organisms have both enzymatic and non-enzymatic antioxidant defenses against reactive oxygen species (ROS) [2].

One of the first enzymes that act in defense against ROS is superoxide dismutase (SOD). It catalyses the conversion of reactive superoxide anions ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2), which is subsequently detoxified by catalase (CAT) and glutathione dependent peroxidase (GPx). GPx catalyses the metabolism of H_2O_2 to water involving a concomitant oxidation of reduced glutathione (GSH), one of the most important non-enzymatic antioxidants in the cell [3]. The activity of AChE is another biochemical biomarker normally used to monitor aquatic environments mainly contaminated by pesticides. This enzyme can be inhibited by different types of agrochemicals [4].

Pesticide pollution can be observed in increasing amounts in water and soil as a result of its uncontrolled and unaware usage in many countries. These pesticides can be removed by rain and irrigation water. The number of scientific studies show that the agricultural usage of pesticides have negative ecological effects and toxic effects on living things in the medium, environment in addition to the effects on immune systems and various tissues, which respectively increases day by day [5-8].

Two groups of herbicides can be divided in terms of creation of oxidative stress: The first is known to be directly responsible for the enhancement of free radical generation, entering redox cycles and constantly generating ROS. The second group of herbicides consists of several classes of compounds known to be mainly inhibitors of antioxidant enzymes, such as SOD and CAT [2].

2,4-Dichlorophenoxyacetic acid (2,4-D) has been the most commonly used acidic phenoxy herbicide in agriculture and forestry since 1940. 2,4-D is one of the most common and most toxic herbicides. 2,4-D has a notorious past. It was one of the two chemicals in the defoliants Agent Orange and Agent Purple. It was also one of the two chemicals in Agent White. The toxicity of 2,4-D depends on its chemical forms, including salts, esters, and an acid form. The ester forms of 2,4-D can be highly toxic to fish and other aquatic life. 2,4-D generally has moderate toxicity to birds and mammals, is sorely toxic to fish and aquatic invertebrates, Herbicidal activity of 2,4-D may also be due to an increase in the production of ROS. The objective of this study is to understand the effects of the herbicide 2,4-D on swordtail fish tissues by using a set of biochemical markers. To this aim, CAT and AChE enzyme activities and MDA levels were measured using spectrophotometric methods in liver and gill tissues of Swordtail fish (*Xiphophorus hellerii* Heckel, 1848).

Material and Method

The applied doses of 2,4-D were prepared using commercial 2,4-Dichlorophenoxyacetic acid salt emulsions. *X. hellerii* was obtained from local commercial aquarists. The animals were acclimatized for two weeks in stock tanks under laboratory conditions. Millipore water was used in this experiment. During the acclimatization period, fish were fed ad libitum with pellet twice a day. The fish were randomly selected and divided into four experimental groups, arranged

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aquarium tanks to hold 10 fish in each. The groups are: 0,05 ppm, 0,1 ppm, 0,2 ppm and the last one was used as control group.For each concentration, three replicates were maintained.After 96 hours,gill and liver tissues were removed. Tissues were homogenized in a buffer containing. 15 % KCl, to obtain 1:10 (w/v) whole homogenate. The homogenates were centrifuged at 18.000 g for 30 minutes at 4°C, and then used for determination of MDA concentrations, CAT and AChE enzyme activities. Total protein concentrations were measured spectrophotometrically at 595 nm according to Bradford Method (1976) [9].

Concentrations of MDA were determined according to the method described by Ledwozyw (1986), the lipid peroxidation in the tissue samples were measured using the thiobarbituric acid reaction [10].

The quantification of the thiobarbituric acid reactive substances was determined by comparing the absorption with the standard curve of malondialdehyde equivalents, generated by the acid-catalyzed hydrolysis of 1,1,3,3-tetramethoxypropane.

AChE was measured with an assay using the dithionitrobenzoic acid (DTNB) recycling method described by Ellman (1961) [11].

This method relies on the spectrophotometric measurement of p-nitrophenol anion quantity formed per thiol molecule as a result of the reaction between DNTB and aliphatic thiol compounds in tissue in slight alkaline medium.

CAT activity was determined according to the method of Aebi (1974) [12].

The principle of the assay was based on the determination of the rate constant of hydrogen peroxide decomposition by the CAT enzyme.

The SPSS 16.0 package program was used in statistical analyses.

Study data were given as arithmetic means and standard deviations. The one-way analysis of variance and student t-test were used for the determination of the significance of differences between the groups.

A value of $p<0.05$ was considered statistically significant.

Results

Statistically significant differences were observed in the MDA levels of the treatment groups compared to the control.

The MDA levels increased in all groups exposed to 2,4-D swordtail fish gill and liver tissues (Fig. 1) ($p<0,05$). CAT activities showed a statistically meaningful decrease in all treatment groups compared to control group tissues (Fig. 2) ($p<0,01$). This result showed that 2,4-D inhibited to CAT enzyme activities and in this way it caused oxidative stress.

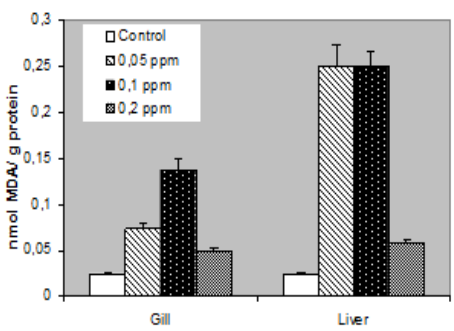

Figure 1. Lipid peroxidation measured throughout MDA levels (nmol MDA/ g protein) in the gill and liver of swordtail fish exposed to herbicide 2,4-D ($p<0,05$) (n=10).

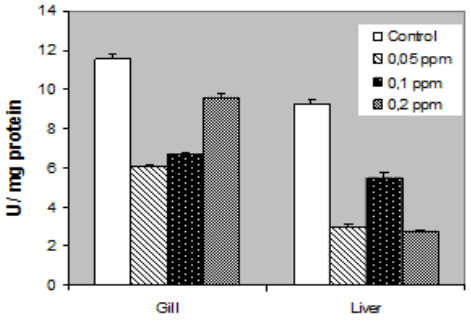

Figure 2. CAT enzyme activity (U/ mg protein) in the gill and liver of swordtail fish exposed to herbicide 2,4-D ($p<0,01$)(n=10).

Compared to the control group, a statistically significant decrease in total protein levels were seen in the groups that were administered with all doses of 2,4-D (Table 1) ($p<0,01$). AChE enzyme activity has shown an important increase, while a significant decrease was found in 0,2 ppm dose group of liver tissue (Table 1) ($p<0,05$).

Table 1. AChE activity (U/ml) ($p<0,05$) and total protein level (µg/ µl) ($p<0,01$) in the gill and liver of swordtail fish exposed to herbicide 2,4-D.

*** Results were expressed as mean±SE for each 10 fish.**

Tissues	Gill		Liver	
	AChE (U/ml)	Total protein (µg/µl)	AChE (U/ml)	Total protein (µg/µl)
Control	0,068±0,007	0,606±0,019	0,253±0,034	0,808±0.008
0,05 ppm	0,525±0,077	0,048±0,012	0,947±0,089	0,186±0,023
0,1 ppm	0,835±0,177	0,117±0,011	1,441±0104	0,188±0,02
0,2 ppm	0,117±0,009	0,56±0,019	0,130±0,016	0,713±0,022

Discussion

Gill tissues are always in contact with the environment and thus they are first to be exposed to aquatic pollutants [13]. The functional damages on gills caused by pollutants may seriously harm the fish health. Therefore fish are thought to be the most suitable indicators for aquatic pollution levels [14]. The biotransformation, detoxification and storing processes occur in the liver.

Liver is essential for the pollution exposed fish to survive. It's simple for the contaminants to enter the fish, accumulate in sensitive tissues like liver and cause disturbance in tissue metabolisms. In the current study, a general toxic trend on the antioxidant systems was observed in gill and liver tissues of *X. hellerii*.

MDA is one of the products formed through lipid peroxidation and it's a commonly used parameter to show oxidative damage. High MDA levels point out to lipid peroxidation.

In this study, all experimental groups have shown an increase of MDA levels compared to the control (Fig. 1). The nonoccurrence or low occurrence of lipid peroxidation indicates the defensive activity of oxidative enzymes. Menezes et al. stated that under oxidative stress, Clomazone herbicide increases the MDA levels in tissues of liver, brain and muscle of the fish *Rhamdia qualen* [15].

In some researches that studied the effects of pesticies on antioxidant systems of aquatic organisms, an increase of lipid peroxidation in tissue and organs is proclaimed [16-18]. According to Xing et al. who have studied the acute effects of atrazine and chlorpyrifos pesticides on liver and gill tissues of carp, it is discussed that reactive oxygen products cause damage on cell membrane lipids and an increase of MDA

levels [19]. Atamaniuk et al. exposed to goldfish high doses of 2,4-D (1, 10 and 100 mg/L) and ascertained that lipid peroxidation increased in gill tissue of fish [20].

Antioxidant enzymes are vitally important for cell stability and their induction are a result of a reaction against pollutants such as pesticides [21]. Overproduction of oxygen radicals can inhibit CAT activity [22].

In this study, it is determined that the activity of catalase decreases in all groups in proportion with the control (Fig. 2).

CAT activity is reported to decrease in various studies about the toxic effects of pesticides [23-26]. A reduction can occur in catalase activity because of the excess production of lipid peroxidation and free radicals. In a study, the CAT activity decreases, while lipid peroxidation levels increase in gill tissues of carps (*Cyprinus carpio*) exposed to fipronil for 90 days in rice fields.

Instability between antioxidant system occurring as a result of pesticide toxicity and pro-oxidant situation is one of the possible reasons for the changes in catalase activity [27].

Generally in the adaptation process observed in aquatic organisms, their physiological responses to pollutants are monitored using biochemical pathways.

The decrease of total protein content in gill and liver tissues detected during the experiment could be due to a generalized stress response.

It is known that different toxicants build up a reduction in protein content of the tissues in the exposed fish. This reduction activated by toxicants demonstrates the physiological adaptability of fish to take care of the stressful position. Fonseca et al. observed a reduction of protein levels in liver tissues of *Leporinus obtusidens* that are exposed to different doses of 2,4-D [28].

Fernandez-Vega et al. informed that the sublethal doses of the herbicide thiobencarb caused a reduction of protein in all tissues of *Anguilla anguilla* [29].

AChE activity has been widely used as a bioindicator of environmental pollution.

AChE enzyme activity is frequently decreased after exposure to pesticides.

However, some pesticide types have shown an enhancement of AChE activity [30-31]. Moraes et al. have treated *Cyprinus carpio*'s habitat with imazethapyr and imazapic for 7, 30 ve 90 days.

It is shown that AChE activites in brain, muscle and liver tissues have changed over time [32].

Atamaniuk et al. ascertained that AChE activity decreased in gill tissue of goldfish exposed to 2,4-D [20]. Cattaneo et al., determinated that AChE activity increased in tissues of *Rhamdia quelen* exposed to 2,4-D [33].

In conclusion, antioxidant alterations and variations in enzyme activity can be used as good biomarkers of pollution in the water bodies affected with herbicide. In this study, oxidative stress resulting from 2,4-D in gill and liver tissues of *X. hellerii* is shown by the increase in lipid peroxidation levels.

It is determined that AChE enzyme activity increases to resist oxidative stress. Oxygen radicals formed as a result of oxidative stress have also been observed to inhibit catalase enzyme activity. According to the results of this study, it can

be analyzed that 2,4-D in sublethal doses can cause damage in gills and liver.

2,4-D is being banned by municipalities, provinces and countries worldwide. Unrestrained applications of herbicides have been observed to cause serious problems in aquatic organisms along with the targeted organisms. Toxic substances affect all organism groups in the food chain. Therefore, pollution studies observing aquatic organisms in every step of the food chain should become widespread. It is believed that the results of this study will be beneficial for environmental pollution studies that investigate multiple variants all together.

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