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EVALUATING THE BIOCHEMICAL EFFECTS OF SUBLETHAL EXPOSURE TO GLUFOSINATE-AMMONIUM ON MODEL ORGANISM ZEBRAFISH (*Danio rerio*) TISSUES

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ABSTRACT

Pesticides are widely used chemicals in agriculture, but these chemicals reach aquatic animals and pollute their habitats. In our study, the effects of Glufosinate-ammonium, a widely used herbicide, on the liver and gill tissues of the zebrafish (*Danio rerio*), which is a model organism, were examined. Primarily, the dose that would kill fifty percent of these fish was determined. In the next step, zebrafish were exposed to sublethal doses (50 mg/L and 100 mg/L) for 120 hours. The effects of the herbicide on oxidative stress parameters were evaluated as enzymatic (Catalase-CAT and Acetylcholinesterase-AChE enzyme activity) and non-enzymatic (Glutathione-GSH, Malondialdehyde-MDA and Total Protein levels). In conclusion, MDA, Total Protein, GSH levels and CAT enzyme activities decreased at low dosing (50 mg/L) in laboratory conditions in zebrafish liver and gill tissues associated with oxidative stress, while it increased at high dosing (100 mg/L). AChE enzyme activity showed a similar picture in liver tissue, but increased in gill tissue at both doses. It can be said that the effect of Glufosinate-ammonium may increase depending on the dose and its presence may pose a serious threat to fish.

KEYWORDS:
Zebrafish, herbicide, oxidative stress, model organism

INTRODUCTION

Pesticides are commonly used in agriculture. The reason for this is that it increases the product quality and production amount [1]. Although they are so important, they carry a huge risk for natural life because of their biological accumulation and toxic effect [2]. The transportation of pesticides used in agricultural areas to the aquatic environment can be by means of surface flows, groundwater sources and uncontrolled elimination of destruction waters [3]. Contamination of pesticides in water also creates potential ecotoxicological risks for non-targeted aquatic organisms at different trophic levels.

Glufosinate-ammonium is a broad spectrum, non-selective and low toxicity organophosphate herbicide [4]. Glufosinate-ammonium with its glutamate-like structure, effects weeds with irreversible inhibition of glutamine synthetase which is necessary for purification of ammonia from its toxic effects and results in high ammonia accumulation [5].

Pesticides cause changes in various parameters of water quality that comply with the living standards of many aquatic organisms. Changing water parameters adversely affect the quality of life of aquatic organisms. With sudden discharges and severe exposures, it can cause chronic diseases and even death. Toxicity experiments applied in aquatic organisms have become a widely used method used to evaluate the bad effects of chemicals that are likely to be toxic on the aquatic life [6]. Fish could play an important marker role for the aquatic life in traditional ecological toxicity tests [7]. Depending on the fish species, they can be affected differently by various pesticides. Pesticides can act in fish in different ways, such as changes in behavior, hematological changes, histopathological disorders, genotoxicity, biochemical changes, antioxidant defense systems change, endocrine system disruption, nutritional profile disorder, alteration of enzyme systems, and changes in nutritional biology [8].

Oxidative damage is caused by an imbalance between oxidants and antioxidant levels, which increase the formation of reactive oxygen species (ROS). ROS reacts with biomolecules such as lipids and proteins that affect cell viability [9]. Oxidative stress induction is one of the main mechanisms of action of many pesticides [10]. Membrane lipids, protein and DNA are damaged by oxidative stress. The effects caused by these damages are measured and oxidative stress assessment can be made [11].

Zebrafish (*Danio rerio*) is a model organism that is widely used in evaluation the effects of various studies on biological organisms because of its advantages such as ease of rearing, brief ripening, high fertility. In addition to all these features, it shows high homology to mammal species [12].

In this study, the impacts of Glufosinate-ammonium herbicide on the model organism zebrafish were evaluated. The effects on the liver and gill tissues of zebrafish exposed to this herbicide at different doses were evaluated in the context of oxidative stress parameters.

MATERIALS AND METHODS

Experimental organism. Zebrafish (*Danio rerio*) is one of about 2000 species of the Cyprinidae family. Zebrafish is a model organism for metabolic, physiological and ecotoxicological studies. It is a strong model organism, not only as a teleost model, but also for the study of human biology and diseases [13, 14]. Animal welfare standards were complied with during the experiments. The experimental animals ethics committee report that allowed the experiment was obtained from the Marmara University Experimental Animals Local Ethics Committee (Protocol code: 89.2018.mar).

Experimental design. In this study, primarily, the dose that killed 50 percent of experimental organism (LD50) value of Glufosinate-ammonium was calculated as 130 mg/L in the 120-hour study on zebrafish. The study was carried out by determining two values below this calculated value. The study was designed as the control group without pesticide exposure, and groups exposed with 50 mg/L and 100 mg/L Glufosinate-ammonium. Before the study, zebrafish were kept in stock tanks. Chlorine-free, rested, ventilated tap water was used. The fish were fed with pellet feed twice a day during the conditioning phase. A 14/10 hour light/dark photoperiod was applied before and during the study. Just before starting the study, random selection was made from the stock tank with n=10 in each group and divided into groups.

After 120 hours of Glufosinate-ammonium application, cold shock was applied to the fish and then dissected and organs to be used in experiments were obtained. Cold tissue homogenate buffer (pH=8) was added to the tissues and homogenization was achieved with the help of a dismembrator. Homogenates were centrifuged for 30 minutes at 4°C with the aid of a cooled centrifuge. The supernatant was then removed and kept in cold conditions until the tissues were studied. Experiments were done in 3 replicates. Spectrophotometric methods were used to measure oxidative stress parameters.

Determination of glutathione activity (GSH). This method is based on the spectrophotometric evaluation of the yellow colored product formed in the reaction of the Elmann's reagent and sulfhydryl groups against the control group [15]. GSH levels in the prepared homogenate are obtained by calculating the dilution factor and extinction coefficient in μg/mg protein from the absorbance measurements at 412 nm.

Determination of catalase (CAT) enzyme activity. This method, which is based on the calculation of the degradation rate constant of hydrogen peroxide with catalase enzyme; it is determined by measuring the decrease in absorbance at 240 nm depending on the dilution of the H_2O_2 reaction mixture in phosphate buffer and the volume of the serum sample required in the appropriate ratio [16].

Determination of malondialdehyde (MDA) level. In the malondialdehyde method, the degree of lipid peroxidation (LPO) is determined by spectrophotometrically measuring the MDA formed as a result of oxidative stress to form a pink-colored complex with thiobarbituric acid (TBA) at an absorbance value of 532 nm [17]. Specific activity is defined in units of activity per milligram protein.

Determination of acetylcholine esterase (AChE) enzyme activity. AChE enzyme is an enzyme that inhibits acetylcholine in tissues. Determination of AChE activity is based on adding Ellman's reagent to tissue homogenates and vortexing and then adding sodium buffer and keeping it at 37°C for 5 minutes following vortexing. After the procedures, acetylcholine iodide is added and kept at 37 ° C for 10 minutes, and the method is completed by reading the absorbance at 412 nm [18].

Total protein analysis. Total protein levels of zebrafish were analyzed separately for all tissues. In this measurement, the Bradford Method is used at an absorbance value of 595 nm [19].

Statistical analysis. SPSS 23.0 package program was used for analysis, study data were given as arithmetic mean and standard error. One-Way ANOVA and post-hoc Tukey HSD (Honestly Significant Difference) test were used to determine the significance of differences between groups. A p value of <0.05 was considered statistically significant.

RESULTS

Glufosinate-ammonium was administered to zebrafish at 50 mg/L and 100 mg/L for 120 hours, and the non-exposure group was considered as the control group. Following this application, CAT and AChE enzyme activities, GSH, MDA and total protein levels were measured by spectrophotometric methods in the liver and gill tissues.

Antioxidant defense systems are biomarkers that are extremely important to use in living systems in the process of monitoring the effects of environmental pollution [20]. The oxidative stress that occurs can create major imbalances in prooxidant and

antioxidant defense mechanisms [21].

Induction of hepatic GSH level has been reported in many studies conducted on fish exposed to environmental toxicity [22]. It was recorded that the GSH levels in the liver tissues of the zebrafish used in our study decreased for both doses compared to the control group. In zebrafish gill tissues, 50 mg/L Glufosinate-ammonium exposure showed a decrease in GSH level compared to the control group, while an increase was observed in 100 mg/L Glufosinate-ammonium exposure (Figure 1, Table 1).

CAT enzyme activity may increase or decrease in contaminated environments depending on the properties of the chemical substance. This situation may cause errors in interpretation. In our study, it was determined that CAT enzyme activities in liver tissues of zebrafish exposed to Glufosinate-ammonium decreased at both doses compared to the control group. In zebrafish gill tissues, 50 mg/L Glufosinate-ammonium exposure showed a decrease in CAT enzyme activity compared to the control group, while an increase was observed at 100 mg/L exposure (Figure 1).

Lipid peroxidation, which is widely used among oxidative stress markers, is an extremely important position in the evaluation of the oxidative response to pollutants. In our study, lipid peroxidation was determined with MDA levels in liver tissues of zebrafish exposed to Glufosinate-ammonium decreased at both doses compared to the control group. In zebrafish gill tissues exposed to Glufosinate-ammonium, a decrease in MDA level was observed at exposure to 50 mg/L dose compared to control, while an increase in dose of 100 mg/L was observed (Figure 1).

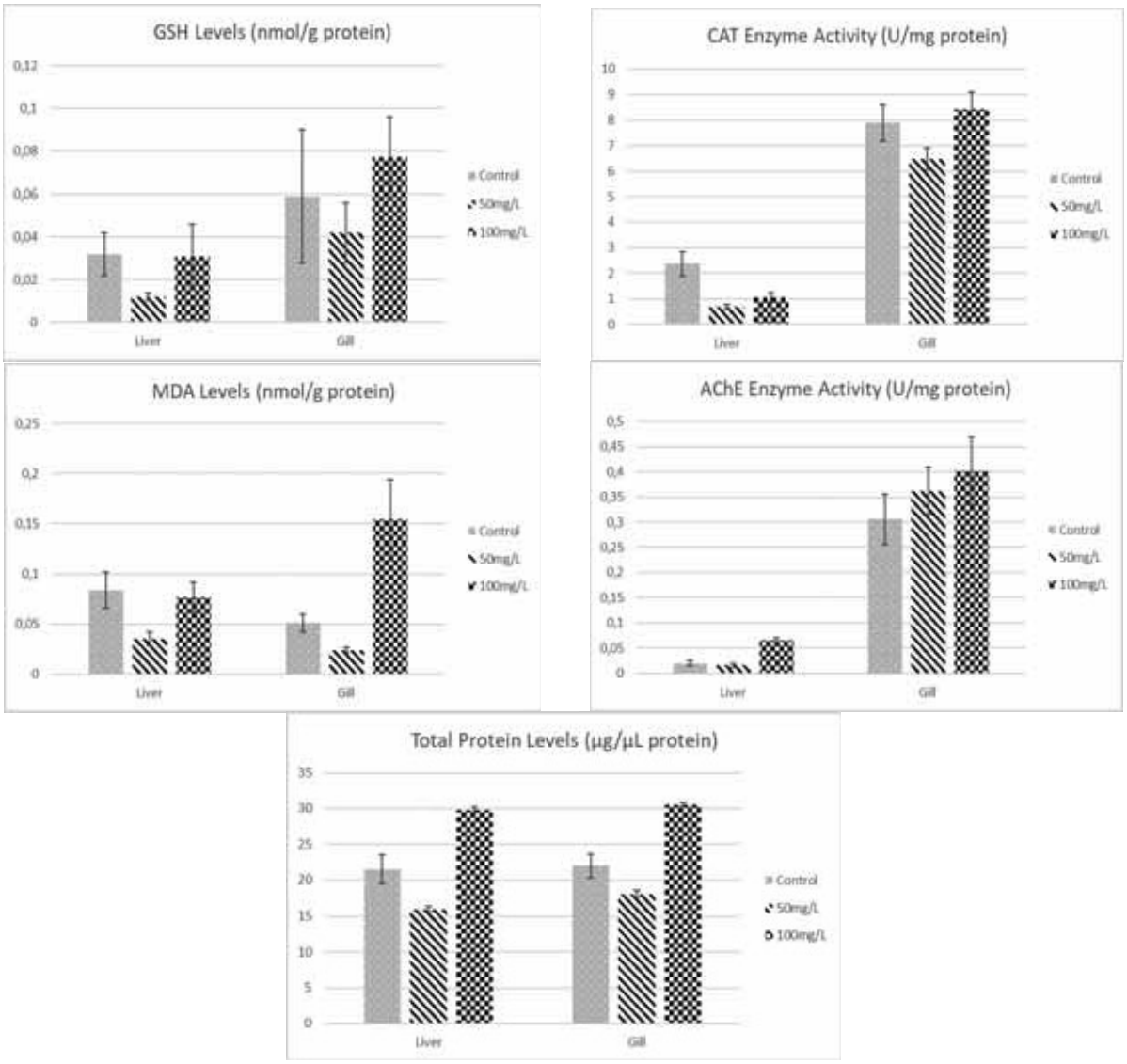

FIGURE 1
Figures show that GSH, MDA, Total Protein levels, CAT and AChE enzyme activities at zebrafish liver and gill tissues. Data are denoted as mean ± standard error.

TABLE 1
The mean levels of GSH, MDA, Total Protein levels, CAT and AChE enzyme activities at zebrafish liver and gill tissues. Data are denoted as mean ± standard error.

Tissues	Groups	GSH (nmol/g protein)	MDA (nmol/g protein)	Total Protein (µg/µL protein)	CAT (U/mg protein)	AChE (U/mg protein)
Liver	Control	0,032±0,01	0,084±0,018	21,512±1,997	2,373±0,474	0,02±0,005
	50mg/L	0,012±0,002	0,035±0,007	15,992±0,355	0,688±0,103	0,016±0,004
	100mg/L	0,031±0,015	0,077±0,015	29,813±0,368	1,055±0,172	0,065±0,006
Gill	Control	0,059±0,031	0,051±0,009	21,96±1,71	7,899±0,7	0,306±0,05
	50mg/L	0,042±0,014	0,024±0,003	18,127±0,429	6,477±0,434	0,362±0,047
	100mg/L	0,077±0,019	0,154±0,04	30,575±0,224	8,43±0,659	0,402±0,068

Aquatic pollutants can cause oxidative damage with the formation of reactive oxygen species at cellular and subcellular (nucleic acid, protein and enzyme structure, etc.) levels in aquatic organisms [8]. It can also affect enzyme systems such as AChE. It may cause oxidative stress in the antioxidants of free oxygen radicals leading to ROS formation and changes in antioxidants. The levels of AChE enzyme activity in liver tissues of zebrafish used in our study decreased at a dose of 50 mg/L compared to the control group, but increased at a dose of 100 mg/L. In zebrafish gill tissues, an increase was observed at both doses compared to the control group (Figure 1).

Protein metabolism may be affected as a result of fish exposure to toxic substances (including pesticides) [23]. Total protein levels in the liver and gill tissues of zebrafish used in our study decreased at a dose of 50 mg/L compared to the control group, whereas it increased at a dose of 100 mg/L (Figure 1).

DISCUSSION

Aquatic pollution studies have gained great importance today. For this purpose, many researchers have reached results by evaluating the changes in aquatic organisms and their oxidative stress parameters. Pesticides are one of the pollutants that affect aquatic organisms the most[24]. Our study was carried out in this direction and zebrafish was chosen as the target organism. The results of our study were evaluated as stated in the previous section. Studies similar to our study and differing from our study will also be included in the discussion section.

One of the potential effects of GSH is that it protects cells from toxic substances and provides defense against ROS. It also plays an important role in apoptosis and gene expression mechanisms. Therefore, reduced GSH levels is an extremely important oxidative stress parameter. In a study by Sharbidre et al. (2011), it was observed that the GSH level decreased in the brain, liver and gill tissues of *Poecilia reticulata* treated with methyl parathion. It has been interpreted that a decrease in GSH in this way may increase the oxidative risk [25]. In a study by Ji et al. (2012), *Carassius auratus* exposed to both Benzo (k)fluoranthene (BkF) alone and in a binary mixture with dichlorodiphenyltrichloroethane (DDT) and a triple mixture with DDT and polychlorinated biphenyls (PCB118). A critical decrease in hepatic GSH levels was found in this study. GSH levels in fish exposed to triple mixtures decreased with prolonged exposure and showed a good concentration-response relationship for 15 days exposure [26]. In a study conducted by Temiz (2019), while the effects of 0,5 ppb and 2,5 ppb concentrations of the fungicide propiconazole (PPC) on the liver tissue of *Oreochromis niloticus* on the 7th and 28th days, the amount of GSH increased on the 7th day; on the 28th day, the amount of GSH decreased and thiobarbituric acid reactive substances (TBARS) levels increased in PPC effect depending on the time and concentration [27].

There are a number of antioxidant systems in the cell that suppress the formation of free radicals or ROS. If these are damaged, intense amount of free radicals may be formed in the cell. One of the most important enzymes in preventive and converting systems is the CAT enzyme. Similar results were obtained in the study conducted by Sayeed et al. (2003)found significant decreases in CAT enzyme activities in liver, kidney and gill tissues of *Channa punctatus*, where they were exposed to deltamethrin [28]. In addition this study, Crestani et al. (2007), observed a decrease in liver catalase activity in *Rhamdia quelen* exposed to herbicide clomazone [29]. In a different study, *Oreochromis niloticus* exposed to chlorpyrifos; CAT enzyme activity was determined to decrease [30]. In contrast to our study, a study with imazephaly and imazepic herbicides observed an increase in CAT enzyme activity on the seventh day in the liver of *Cyprinus carpio* exposed to these herbicides [31].

Another of the oxidative stress markers is the determination of the concentrations of peroxidation by-products, a group that includes MDA. For this reason, it has been evaluated with the method applied. An increase in MDA levels in the brain, liver and gill tissues of *Poecilia reticulata* was observed

when exposed to chlorpyrifos, one of the organophosphate insecticide species. It has been determined that the most affected tissue is the gills [25]. In another study, found increases in TBARS levels for *Rhamdia quelen* exposed to glyphosate. It also observed a decrease in TBARS levels during the recovery period of the exposed fish [32].

AChE is an enzyme that has an important role in neural transmission mediated by acetylcholine. It functions in the central nervous system. Determination of AChE activity provides information in terms of determining whether the enzyme is irreversibly inhibited. Similar to our study, in a study conducted in 2019, *Oreochromis niloticus* was exposed to emamectin benzoate at doses of 110 µg/L and 220 µg/L for 96 hours and 14 days. As a result of this exposure, an increase in AChE enzyme activity in liver tissues was observed in all concentrations of the insecticide depending on the time [33]. Sharbidre et al. observed a dose-dependent inhibition of AChE enzyme activity in the brain tissue of *Poecila reticulata* exposed to chlorpyrifos. [25]. In a study where we studied the toxic effects of the herbicide tribenuron-methyl, increased AChE enzyme activity was observed in the liver tissues of zebrafish treated with 25, 50 and 100 ppm herbicide in response to all doses [24].

Reactive oxygen species have effects on membrane proteins and other proteins; it can increase the aggregation of proteins and prevent the formation of side chains and three-dimensional structures. For this reason, the measurement of proteins is carried out totally. According to a study by Soyingbe (2012), it was found that the protein profile of *Oreochromis niloticus* exposed to diazinon insecticide in muscle, liver, gill and kidney tissues changes depending on concentration. A decrease in protein concentration in the organs tested was observed due to the increase in concentration [34].

CONCLUSION

Pesticides are chemicals that are preferred due to their agricultural benefits, however that can reach non-target organisms. The Glufosinate-ammonium we used in our study is preferred in agricultural use. In our study representing non-target organisms, liver and gill tissues, which are the tissues that are likely to be affected by the zebrafish, were preferred. Ezymatic and non-enzymatic oxidative stress parameters were used as an exposure marker. In conclusion, MDA, Total Protein, GSH levels and CAT enzyme activities decreased at low dosing (50 mg/L) in laboratory conditions in zebrafish liver and gill tissues associated with oxidative stress, while it increased at high dosing (100 mg/L). AChE enzyme activity showed a similar picture in liver tissue, but increased in gill tissue at both doses (Table 1). It can be said that the effect of Glufosinate-ammonium may increase depending on the dose and its presence may pose a serious threat to fish. Identifying these types of pollutants is very important in order to obtain a more livable environment. It is among our responsibilities as human beings towards nature.

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