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TOXIC EFFECTS OF HERBICIDE TRIBENURON-METHYL ON LIVER TISSUE OF ZEBRAFISH (*DANIO RERIO*)

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ABSTRACT

Tribenuron-methyl (TBM) is a sulfonylurea group herbicide commonly used in weed control and integrated pest control in the world and in our country. As with all pesticides, its incorporation into the aquatic environment is dangerous for non-target organisms. Zebrafish (*Danio rerio*) is a freshwater fish that has been used frequently as a bioindicator species in the field of environmental toxicology in recent years. In this study, zebrafish were fed with pellet feed in tanks filled with chlorine-free water for 10 fish in each aquarium for two weeks. Experimental groups were exposed to 25, 50 and 100 ppm doses of TBM for 96 hours. The experiments were performed in triplicate. At the end of the period the liver organs of the fish were dissected and homogenized immediately. Then, reduced glutathione (GSH), malondialdehyde (MDA), total protein (TP) levels and acetylcholinesterase (AChE) and catalase (CAT) enzyme activities were determined by spectrophotometric methods.

According to the findings, TP level and CAT enzyme activity increased in all doses compared to the control group. MDA levels decreased at all doses. AChE enzyme activity decreased at 25 and 50 ppm doses and increased at 100 ppm dose group. GSH levels increased in the 25 and 100 ppm group, but decreased in the 50 ppm group. In conclusion, TBM showed a detrimental effect on liver tissue enzyme activities and lipid peroxidation levels of zebrafish.

KEYWORDS:
Tribenuron-methyl, Oxidative stress, Zebrafish, Liver

INTRODUCTION

The increase in the use of pesticides in agricultural areas reaches dangerous levels for non-target organisms. Hundreds of newly produced pesticide formulations are used every year in agricultural fields and are being tried to combat unwanted organisms. Herbicides are used to combat unwanted small weeds in agricultural areas [1, 2]. Tribenuron-methyl (TBM) is a sulfonurea group systemic effective herbicide first introduced in 1989. Its chemical formula is $C_{15}H_{17}N_5O_6S$, and its chemical name is Methyl 2 - [[(4-methoxy-6-methyl-1,3,5-tirazin-2-yl) - methylcarbomyl] sulfamoyl] benzoate. The mechanism of action is the inhibition of the enzyme acetolactate synthetase, which catalyzes the first step in the synthesis of amino acids such as valine, leucine and isoleucine. Herbicides inhibit plant growth by disrupting hormonal balance that regulates plant metabolism, such as cell division, cell elongation and protein synthesis [3].

Like all pesticides; herbicides cause some harmful effects on non-target organisms as well as their benefits in agriculture. Zebrafish (*Danio rerio*) is a frequently used model organism in biological, ecotoxicological and genetic fields. Zebrafish have a biological structure and physiological system similar to mammals. It also has the advantage of high fertility and short reproductive cycle. Zebrafish is also a suitable model organism for the evaluation of hepatotoxicity [4].

The transmission of pesticides to the fish through the skin and nutrition leads to the formation of reactive oxygen species (ROS), deterioration of the activities of antioxidant enzymes and the formation of free radicals. Oxidative stress is the deterioration of the balance of hemostasis caused by the increase of free radicals and oxidants. The disruption of this balance between oxidants and antioxidants causes damage to proteins, lipids and DNA, and disruption of physiological functions [1, 5]. The liver is the largest gland found in the body of vertebrates. It plays an important role in the metabolism of carbohydrates, fats, proteins, vitamins, hormones and regulation of detoxification. Therefore, the liver is the target organ for toxic reactions. Liver damage or hepatotoxicity caused by toxic substances is most often caused by drugs and poisons that induce hepatic damage, including oxidative stress and apoptosis. The aim of this study was to determine the effects of different doses of tribenuron-methyl (25, 50 and 100 ppm) on zebrafish liver tissue antioxidant enzymes, lipid peroxidation and total protein levels.

MATERIALS AND METHODS

Experimental design. The zebrafish were purchased from commercial aquarium stores and kept in 10 liters clean glass aquariums at appropriate temperature range (24-28 °C), proper ventilation and light / dark (14:10 hours) conditions. Before exposure, the fish were adapted to the laboratory environment in a dechlorinated water tank for two weeks. During the period, they were fed twice a day with pellet feed and swimming in clean water. The experimental setup was divided into 4 groups with 10 fish in each aquarium. The groups were identified as Group I (25 ppm), Group II (50 ppm), Group III (100 ppm) and control group. The experimental period was planned as 96 hours and the fish were applied in separate aquariums according to their groups.

Fish were sacrificed on ice cold and liver were dissected. The liver tissue of fish were removed with aseptic conditions, cold 0.1 M phosphate buffer (pH 7.4) were added to the liver tissue samples and the samples were homogenized by using glass tissue homogenizers under ice-bath cooling. Then homogenates were centrifuged at 10.000 g for 30 min at 4°C to obtain the supernatant for analysing the enzyme activities. The supernatants were performed under icebath cooling to keep the enzyme activities stable. CAT, MDA and total protein levels were detected using spectrophotometric methods. The experiment was repeated 3 times. At the end of the experiment, dead fishes were destroyed by the medical waste teams of Kadıköy Municipality under appropriate conditions.

Measurement of Catalase (CAT) activity. Catalase enzyme activity was determined according to the method of Aebi. The principle of the assay is based on the determination of the rate constant of hydrogen peroxide decomposition by the CAT. Briefly, the activity was determined by measuring the decrease in absorbance at 240 nm of a reaction mixture consisting of H_2O_2, in phosphate buffer, pH 7.0, and requisite volume of serum sample [6].

Measurement of Acetylcholinesterase (AChE). AChE activity was measured with an assay using the DTNB (dithionitrobenzoic acid) recycling method described by Ellman. The method relies on the spectrophotometric measurement of p-nitrophenol anion quantity formed per thiol molecule as a result of the reaction between DTNB and aliphatic thiol compounds in tissue in slight alkaline medium [7].

Measurement of Total Protein (TP) level. Total protein levels were analysed for liver tissue of zebrafish. Soluble protein concentration was measured with the Comassie Brilliant blue G-250 using bovine serum albumin as a standard [8].

Measurement of Malondialdehyde (MDA) level. The MDA content was measured after incubation at 95°C with thiobarbituric acid in aerobic conditions. The pink colour produced by the reactions was measured with spectrophotometer at 532 nm. Specific activity was defined as the unit of activity per milligram protein [9].

Measurement of reduced Glutathione (GSH) level. GSH concentration was measured with an assay using the dithionitrobenzoic acid (DTNB) recycling method described by Beutler (1974). In this method, the chromophoric product resulting from the reaction of the reagent DTNB and free sulphydryl groups possessed a molar absorption at 412 nm [10].

Statistical analysis. The SPSS 23.0 package program was used for analysis, results of study 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 the differences between the groups. A value of $p<0,05$ was considered statistically significant.

RESULTS AND DISCUSSION

In this study, zebrafish were exposed to 25, 50 and 100 ppm doses of tribenuron-methyl for 96 hours. Glutathione (GSH), malondialdehyde (MDA), total protein (TP) levels and acetylcholinesterase (AChE) and catalase (CAT) enzyme activities were determined by spectrophotometric methods. According to the findings, catalase (CAT) enzyme activity did not show an increase in 25 and 100 ppm group, but increased in 50 ppm group compared to control group. Acetylcholinesterase enzyme (AChE) activity was higher in all dose groups compared to control group. Total protein (TP) levels were high only in 25 and 100 ppm group and close to control in 50 ppm dose group. Malondialdehyde (MDA) levels increased in 25 ppm group, 50 and 100 ppm groups were measured close to the control group. Glutathione (GSH) levels showed a slight increase in 25 and 50 ppm group, while it decreased in 100 ppm group. As a result, it was determined that tribenuron-methyl administered at different doses had a detrimental effect on zebrafish liver tissue enzyme activities and lipidperoxidation levels. The data obtained are shown in Table.1.

All pesticides that interfere with the natural environment leave non-degradable residues on soil, water and living organisms. The catalase enzyme plays an important protective role against the destructive effects of reactive oxygen species (ROS) in animal tissues. Catalase is one of the primary enzymes involved in hydrogen peroxide detoxification and is of particular importance for the removal of

TABLE 1
The mean levels of Catalase (CAT) and Acetylcholinesterase (AChE) enzyme activities, Malondialdehyde (MDA) and Total protein (TP) of liver tissue of zebrafish (*Danio rerio*).

	Liver tissue		
	TBM dose	**Mean**	**STD. Error**
Catalase enzyme (CAT) Activity (U/mg protein)	Group-I - 25 ppm	0.063±0.45	0.01
	Group-II - 50 ppm	2.108±0.10	0.01
	Group-III - 100 ppm	0.653±0.10	0.01
	Control group	**1.739±0.10**	**0.00**
Acetylcholinesterase Enzyme (AChE) Activity (U/mg protein)	Group-I - 25 ppm	0.095±0.21	0.01
	Group-II - 50 ppm	0.027± 0.20	0.01
	Group-III - 100 ppm	0.068±0.21	0.01
	Control group	**0.0105**	**0.01**
Total Protein (TP) Levels (µg/µl)	Group-I - 25 ppm	23.591	0.030
	Group-II - 50 ppm	10.972	0.030
	Group-III - 100 ppm	13.234	0.030
	Control group	**10.651**	**0.042**
Malondialdehyde (MDA) Levels (nmol/g)	Group-I - 25 ppm	0.0910	0.030
	Group-II - 50 ppm	0.0127	0.030
	Group-III - 100 ppm	0.0142	0.030
	Control group	**0.0110**	**0.030**
Glutation (GSH) Levels (nmol/g protein)	Group-I - 25 ppm	0.092	0.01
	Group-II - 50 ppm	0.018	0.01
	Group-III - 100 ppm	0.011	0.01
	Control group	**0.014**	**0.01**

H_2O_2. Also some researchs on various fish species support our study [1, 11, 12, 13].

Plhalova et al. (2017) investigated the subchronic effects of neem-diminutive T/S biopesticide with azadirachtin-A on the growth rates, mortality rates and liver tissue histopathology of juvenile zebrafish. The researchers reported that different concentrations of neem-diminutive T/S adversely affected fish growth rates and had negative effects on oxidative stress indices in the zebrafish fry stage. They also observed histopathological changes in liver tissue [5]. Blahova et al. (2013) reported a significant decrease in CAT activity in all experimental groups of zebra fish exposed to atrazine [2]. Environmental toxicants, such as pesticides, directly increase the cellular concentration of ROS and cause oxidative damage by reducing cellular antioxidant capacity. In our study, there was no increase in catalase activity in liver tissue of zebrafish exposed to tribenuron-methyl for 96 hours in 25 and 100 ppm groups. In the 100 ppm group, CAT activity increased compared to control. This may be a result of cellular oxidative damage due to exposure to different concentrations of tetruren-methyl during the experiment. Therefore, we may think that oxidative balance may play an important role in the prevention of antioxidant enzyme activity due to tribenuron-methyl exposure. Decrease in CAT levels due to high superoxide radicals may lead to inhibition of CAT activity. Zhu et al. (2019) reported that phenobucarb (2-sec-butylphenyl methyl carbamate) causes insufficiency in metabolic organs in fish [14]. Our results also showed that pesticides can induce organ failure in a dose-dependent manner.

There is a great deal of research on the limitation of the activity of enzymes involved in metabolic events as a result of exposure to pesticides in organisms and causing cell damage in tissues. Enzymes are biological catalysts in protein structure that accelerate metabolic events in aquatic organisms. Acetylcholinesterase (AChE) is an enzyme synthesized in the liver. AChE was first isolated from the electric organ of the electric fish (*Torpedo marmoneta*). The task of hepatocyte cells is to produce plasma proteins in the blood, release into the endocrine system, detoxification of environmental pollutants and pharmaceuticals, deamination of amino acids, storage of triglycerides and glucogen [4, 12]. In a study investigating the possible harmful effects of AChE enzyme activity on erythrocytes of Rainbow Trout (*Oncorhynchus mykiss*) after 60 days of chronic exposure of carbosulfan pesticide, physical parameters such as lack of motion, darkening of color, restless behavior and loss of balance were observed. In addition, inhibition of AChE enzyme has been reported to last until the third week after the experiment [15]. In our study, acetylcholinesterase enzyme (AChE) activity was found to be higher in all dose groups compared to control group.

Total protein levels are important biochemical parameters to determine the negative effects of environmental pollutants in aquatic organisms. Proteins are basic macromolecules for the survival of all living species and they are the main components of the

growth and development of tissues and organs in organisms. They are also the precursor of enzymes, hormones, neurotransmitters and cofactors. In one study, *Leporinus obtusidens*, a South American freshwater species, was exposed to different concentrations of clomazoneone for 8 days. Increased protein levels in the liver of fish have been reported at the end of exposure [16]. Moraes et al., also found high total protein levels at different doses of imizapic pesticide [17]. Antioxidant enzyme activity measurements in fish living in nature can be considered as sensitive markers of exposure to various pesticides [18]. In this study, total protein levels were increased in all three dose groups compared to the control group. This may be due to increased doses of tribenuron-methyl in the liver, causing oxidative stress in the liver tissue. Konyalıoğlu and Perçin (2017), in their study, found that Northern Bluefin Tuna samples grown in cages had higher levels of stress-induced antioxidant enzymes in blood tissue compared to free-living individuals in the natural environment. This study is an important research in terms of knowing the biological structures of all kinds of fish living in their natural environment [11]. Vieira et al. (2018) observed that different concentrations of high imidaclobrid (IMI) cause a significant increase in lipid peroxidation levels in liver and kidney tissues in fish. According to the researchers, pesticide exposure has caused oxidative stress on various biological molecules including lipid, proteins and antioxidant enzymes [19]. The results of our study support these findings. MDA is a product of lipid peroxidation and shows oxidative damage in cells and tissues [13]. Changes in MDA levels and activity of antioxidant enzymes are considered markers of oxidative stress [20, 21]. Pesticide residues have serious adverse effects on fish and can biodegrade even in farmed fish. Therefore, there are potential risks to human health in the food chain [1]. Lipid peroxidation (LPO) occurs in the cell membrane. It is one of the most important parameters that determine necrosis in cell degeneration [22]. Environmental pollutants such as heavy metals and pesticides cause oxidative stress in organisms. LPO is frequently used as an indicator of oxidative stress in living cells [23, 24]. Therefore, malondialdehyde is one of the parameters indicating lipid peroxidation damage in all living organisms. In our study, malondialdehyde (MDA) levels increased in 25 ppm group. However, it was measured in 50 and 100 ppm groups close to the control group. The results of our study indicate that the increase in MDA level in 25 ppm group may be an indicator of lipid peroxidation due to free radicals. Maharajan et al. (2018) reported that high concentrations of piriproxifen may have an adverse effect on the early developmental stages of eggs and larvae of zebrafish. It has been shown that these damages that may occur during the developmental stages of organs and tissues such as liver, pancreas and heart will affect the whole life of the fish [12]. In our study, glutathione levels increased slightly in the 25 and 50 ppm group but decreased in the 100 ppm group. The increase in glutathione level can be considered as an adaptation response to tribenuron-methyl. The decrease seen in the 100 ppm group may be due to increased oxidative stress. Oxidative damage occurs to proteins, lipids, and DNA, when the concentration of reactive species is not controlled by counteracting antioxidants such as tocopherols, ascorbic acid, and glutathione or enzymes involved in oxygen radical scavenging such as catalase, peroxidase, and superoxide dismutase, leading to cytotoxicity, genotoxicity, and even carcinogenes [25]. In conclusion, it was determined that tribenuron-methyline applied in different doses had negative effects on zebrafish liver tissue enzyme activities and lipid peroxidation levels.

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Conflict of Interest. The authors declare that they have no conflict of interest.

REFERENCES

[1] Clasen, B., Loro, V.L., Murussi, C.R., Tiecher, T.L., Moraes, B., Zanella, R. (2018) Bioaccumulation and oxidative stress caused by pesticides in *Cyprinus carpio* reared in a rice-fish system. Science of the Total Environment. 626, 737-743.

[2] Blahova, J., Plhalova, L., Hostovsky, M., Divisova, L., Dobsikova, R., Mikulikova, I., Stepanova, S., Svobodova, Z. (2013) Oxidative stress responses in zebrafish *Danio rerio* after subchronic exposure to atrazine. Food and Chemical Toxicology. 61, 82-85.

[3] EPA (U.S. Environmental Protection Agency). (2005) Mancozeb facts, EPA 738-F-05-XX, (http://www3.epa.gov/pesticides/chem_search/reg_actions/reregistration/fs_PC-014504_1-Sep-05.pdf).

[4] Qiu, L., Jia, K., Huang, L., Liano, X., Guo, X., Lu, H. (2019) Hepatotoxicity of tricylazole in zebrafish (*Danio rerio*). Chemosphere, 232: 171-179.

[5] Plhalova, L., Blahova, J., Divisova, L., Enevova, V., Casuscelli di Tocco, F., Faggio, C., Tichy, F., Vecerek., Svobodova, V. Z. (2017) The effects of subchronic exposure to NeemAzal T/S on zebrafish (*Danio rerio*). Chemistry and Ecology. 34(3), 199-210.

[6] Aebi, H. (1974) Catalase in vitro in: Methods of enzymatic analysis. Edited by H.U. Bergmeyer (2nd ed. FL) 121.
[7] Ellman, G. L., Courtney, K. D., Andes, V., Featherstone, R. M. (1961) A new rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88-92.
[8] Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 72, 248-250.
[9] Ledwozyw A., Michalak D., Stepien A., Kadziolka A. (1986) The relationship between plasma triglycerides, cholesterol, total lipids and lipid peroxidation products during human atherosclerosis. Clinica Chimica Acta. 155, 275-280.
[10] Beutler, E. (1975) Red Cell Metabolism. In: A manual of biochemical methods. Beutler, E. (ed), New York. Grunef and Strottan. 67-69.
[11] Konyalıoğlu, S., Perçin, F. (2017) The comparison of lipid peroxidation, glutathione land antioxidant enzyme activities in blood obtained from captive and wild Northern Bluefin Tuna (*Thunnus thynnus* L., 1758). Free Radical Biology and Medicine. 108, S18-S107.
[12] Maharajan, K., Muthulakshmi, S., Nataraj, B., Ramesh, M. (2018) Toxicity assessment of pyriproxyfen in vertebrate model zebrafish embryos (*Danio rerio*): A multi biomarker study. Aquatic Toxicology. 196, 132-145.
[13] Zengin, H. (2018) Oxidative stress and antioxidant defenses after starvation in *Oncorhynchus mykiss* (Walbaum, 1792) larvae reached free swimming stage. Ege Journal of Fisheries and Aquatic Sciences. 35(4), 387-396.
[14] Zhu, X.Y., Xia, B., Wu, Y.Y., Yang, H., Li, C.Q., Li, P. (2019) Fenobucarb induces heart failure and cerebral hemorrhage in zebrafish. Aquatic Toxicology. 209, 34-41.
[15] Çapkın, E. (2011) Effects of carbosulfan on erythrocyte acetylcholinesterase (AChE) activities of rainbow trout (*Oncorhyncus mykiss*)). Journal of Fisheries Sciences. 5 (3), 240-249. (in Turkish).
[16] Miron, D.S., Prettob, A., Crestani, M., Glusczak, L., Schetinger, M.R., Loro, V.L. Morsch, V.M. (2008) Biochemical effects of clomazone herbicide on piava (*L. obtusidens*). Chemosphere. 74, 1–5.
[17] Moraes, B.S., Clasen, B., Loro, V.L., Pretto, A., Toni, C., De Avila, L.A., Marchesan, E., De Oliveira Machado, S.L., Zanella, R., Reimche, G.B. (2011) Toxicological responses of *Cyprinus carpio* after exposure to a commercial herbicide containing imazethapyr and imazapic. Ecotoxicology and Environmental Safety. 74, 328-335.
[18] Karaca, M., Varışlı, L., Korkmaz, K., Özaydın, O., Perçin, F., Orhan, H. (2014) Organochlorine pesticides and antioxidant enzymes are inversely correlated with liver enzyme gene expression in *Cyprinus carpio*. Toxicology Letters. 230, 198-207.
[19] Vieira, C.E.D., Perez, M.R., Acayaba, R.D., Raimundo, C.C.M., Martinez, C.B.R. (2018) DNA damage and oxidative stress induced by imidacloprid exposure in different tissues of the Neotropical fish *Prochilodus lineatus*. Chemosphere. 195, 125-134.
[20] Pascual, P., Pedrajas, J., Toribio, F., López-Barea, J., Peinado, J. (2003) Effect of food deprivation on oxidative stress biomarkers in fish (*Sparus aurata*). Chemico-Biological Interactions. 145(2), 191-199.
[21] Morales, A.E., Pèrez-Jimènez, A., Hidalgo, M.C., Abellán, E., Gabriel C.G. (2004) Oxidative stress and antioxidant defenses after prolonged starvation in *Dentex dentex* liver. Comporative Biochemistry and Physiology C. 139(1-3),153-161.
[22] Tabassum, H., Dawood, A.Q., Sharma, P., Khan, J., Raisuddin, S., Parvez, S. (2016) Multiorgan toxicological impact of fungicide propiconazole on biochemical and histological profile of freshwater fish *Channa punctata* Bloch. Ecological Indicators. 63, 359-365.
[23] San, A.T., Yonar, M.E. (2017) Determination of oxidative stress in scaly carp (*Cyprinus carpio* Linnaeus, 1758) exposed to deltamethrin in different water temperature. Ege Journal of Fisheries and Aquatic Sciences. 34(3), 281-286.
[24] Tsaboula, A., Papadakis, E.N., Vyrzas, Z., Kotopoulou, A., Kintzikoglou, K., Papadopoulou-Mourkidou, E. (2016) Environmental and human risk of pesticides: A prioritization method, based on monitoring, hazard assessment and environmental fate. Environment International. 91, 78-93.
[25] Süleyman, H., Gül, V., Erhan, E. (2018) Oxidative Stress and Tissue Damage. Erzincan Medical Journal. 1(1), 1-4. (in Turkish).

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