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Detection of Biochemical, Cytotoxic, and Genotoxic Damage Caused by Glufosinate-Ammonium on the Zebrafish Cell Line

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ABSTRACT

Pesticides widely used in agriculture can enter aquatic ecosystems and threaten non-target organisms in the environment. In this study, sublethal toxic effects of glufosinate-ammonium on Zebrafish (*Danio rerio*) liver fibroblast cell line (ZFL) were investigated. Cytotoxicity and genotoxicity tests; MTT, neutral red uptake, lactate dehydrogenase, trypan blue tests were observed at 24 hours, 48 hours, 72 hours and 96 hours; apoptosis and necrosis detection at 48 and 96 hours, cell proliferation detection at 72 hours, micronucleus test at 96 hours. A sublethal dose of 2000 mgL^{-1} was determined as the initial concentration and there are dilution differences between the tests. As a result of the tests; Decreases were observed in all applications compared to the negative control in MTT, neutral red uptake, trypan blue, % necrosis and cell proliferation detection tests; in lactate dehydrogenase and % apoptosis tests, an increase were observed in all applications compared to the negative control. In the micronucleus test, it was determined that glufosinate-ammonium application stimulated micronucleus formation compared to the negative control. Biochemical tests were performed in ZFL cell lines with 96-hour application. Selected groups; control group, 250 mgL^{-1}, 500 mgL^{-1} and 1000 mgL^{-1} glufosinate-ammonium doses. Evaluated biochemical parameters; lipid peroxidation determination, reduced glutathione determination, catalase enzyme activity determination, acetylcholinesterase enzyme activity determination and total protein determination. As a result of biochemical experiments; lipid peroxidation level at 1000 mgL^{-1}; catalase enzyme activity 250 mgL^{-1}; total protein levels in all concentrations increased compared to the control. lipid peroxidation level at 250 mgL^{-1} and 500 mgL^{-1}; catalase enzyme activity 500 mgL^{-1} and 1000 mg/L; GSH level and AChE enzyme activity decreased at all glufosinate-ammonium dose applications. It is thought that the obtained results will provide important contributions to the literature and shed light on further research.

Keywords: Pesticide, Zebrafish, Oxidative stress, Cytotoxicity, Genotoxicity.

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Introduction

The rapid increase in the world population and the development of technology have led to the widespread use of pesticides that aim to protect plants and animals from unwanted pests. This practice is considered a part of integrated farming. The extensive use of pesticides should be regarded as a global phenomenon [1]. Despite its numerous advantages, pesticide use has raised many concerns in terms of public health and environmental pollution. [2]. Pesticides used to protect the target organism and control pests are usually found in aquatic ecosystems [3]. According to Mellanby, pesticides can lead to bioaccumulation in both aquatic and terrestrial flora and fauna through the food chain, often resulting in undesirable consequences for the ecosystem [4]. Pesticide residues are generally considered harmful to fish in most cases [5]. Excessive pollutants reaching surface and groundwater from various sources contribute to water pollution, becoming one of the major environmental issues worldwide [5]. There are four main ways pesticides reach water: they can drift outside the intended area during spraying, percolate from the soil to groundwater, be transported towards surface waters, or accidental over-discharge can occur [6].

Many studies have reported changes in lipid metabolism in aquatic organisms due to external stress factors such as pesticides [7,8]. The liver has a vital role in maintaining lipid homeostasis. Changes in lipid functions can lead to metabolic problems and damage to other potential pathways [7]. Additionally, the liver takes responsibility for part of the reproductive system in fish, along with the gonads, pituitary, and brain. In case of damage, the ability to reproduce in these creatures will be affected [9]. Glufosinate-ammonium (D, L-phosphinothricin or 2-amino-4-(hydroxymethylphosphinyl) butanoic acid) is a broad-spectrum herbicide belonging to the organophosphorus chemical family. It is used to control various weeds or for vegetation control in non-cropped areas [10]. Glufosinate disrupts photorespiration, leading to reduction of molecular oxygen, which generates reactive oxygen species. It has an unusual physicochemical profile compared to most herbicides currently used worldwide and is the most important herbicide targeting glutamine

synthetase (GS) [11]. Environmental conditions can significantly affect the performance of glufosinate-ammonium used in the field, and only a few weed species worldwide have developed glufosinate resistance. The European Commission has suggested glufosinate-ammonium as "one of the few alternatives to glyphosate" currently used in the world. Since there are very few studies evaluating the effects of glufosinate-ammonium in the literature, it was thought that it was necessary to examine them.

Zebrafish liver cell line (ZFL) is used to simulate the metabolic processes of toxins entering the liver. The zebrafish genome is preferred for generating preliminary hypotheses about human disease mechanisms since it shares many orthologs of human genes [12]. The use of cell lines is beneficial for obtaining toxicological data by measuring parameters such as cell metabolism, morphology, viability, cell membrane permeability, cell adhesion/detachment, and proliferation or growth kinetics in order to simulate the metabolic processes of toxins entering the liver [13]. In numerous cytotoxicity studies, a positive correlation has been established between *in vitro* and *in vivo* EC50 (Effective Concentration 50) values in fish exposed to different pollutants. These studies emphasize the value of using fish cell lines in the field of toxicology [12,13]. Furthermore, the advantages of using cell lines, such as facilitating mechanistic studies, producing less toxic waste, and reducing animal use, should be increasingly emphasized. Fish cell lines are gaining increasing importance in ecotoxicology and genotoxicology as they represent standardized laboratory systems that are conducted in a controlled environment, providing rapid, cost-effective, and ethically sound results[14]. In this study, the use of cell lines as an alternative method to animal experiments was preferred. The use of cell lines together with the evaluation of oxidative stress parameters, cell survival/damage results, and genotoxic parameters brings the study to a valuable point in the field.

The purpose of this study is to examine the effects of exposure to various doses of glufosinate-ammonium on the ZFL cell line. In order to gain more insight into the potential mechanisms of action of the glufosinate-ammonium compound, unlike many studies, various parameters such as DNA damage, cell proliferation, cell viability and oxidative stress levels were simultaneously selected and evaluated.

Materials and Methods

Chemicals and Reagents

Chemicals were supplied by the following companies: ZFL CRL 2643 cell line from ATCC; MTT test solution from Biofroxx ; LDH Test kit and Neutral Red Uptake Test kit from Abcam; BrdU Cell Proliferation Assay Kit from Biovision; Propidium Iodide from Sigma-Aldrich ; Hoechst 33342, Trypan blue solution, DMSO, Trypsin-EDTA, dPBS and Colchicine from ThermoFisher Scientific; Giemsa solution, Thio Barbituric Acid, Trichloroacetic acid, H_2O_2 , DTNB, Bradford reagent from Merck.

Cell Culture

The zebrafish liver cell line (ZFL), which is considered as a model organism, was preferred to determine the metabolic processes of possible toxicological effects of Glufosinate ammonium, was obtained from the American Type Culture Collection (ZFL-CRL- 2643). The culture medium was prepared according to the manufacturer's instructions (consisting of; 50% L-15, 35% DMEM, 15% Ham's F12, 0.15gL^{-1} sodium bicarbonate, 15mM HEPES, 10µgmL^{-1} bovine insulin, 50ngmL^{-1} mouse EGF, 5% FBS, and 0.5% Trout Serum). The cells were incubated in 75 cm^2 flasks at 28°C 100% air. The doubling time for adherent cells was determined to be 72 hours under laboratory conditions. At the end of 72 hours, when the cells were examined under a microscope and it was understood that they reached 75-80% confluency, the plate was first washed with dPBS to remove cellular waste. After removing dPBS, adherent cells were trypsinized and the cells were removed from the surface. Cells separated from the flasks were stained with 0.4% trypan blue stain (50 trypan blue: 50 cell line %) and total cell count was performed in the Thoma chamber. Cell viability was expressed as the percentage of live cells, and when cell viability was equal to or greater than 80%, further experiments were conducted. A stock solution of glufosinate-ammonium was prepared in the culture medium and diluted in wells. Negative and positive controls were also prepared in the same culture medium for each test. For cytotoxicity tests, including cell proliferation, apoptosis, necrosis, cell viability, and survival, 10^4 cells/well were seeded in a 96-well plate. Measurements were taken at 24, 48, 72, and 96 hours according to the experimental plan. For oxidative stress experiments, 10^6 cells/well were seeded in a 6-well plate. The application was terminated after 96 hours to measure the endpoint. Negative and positive controls were examined in parallel (Figure 1).

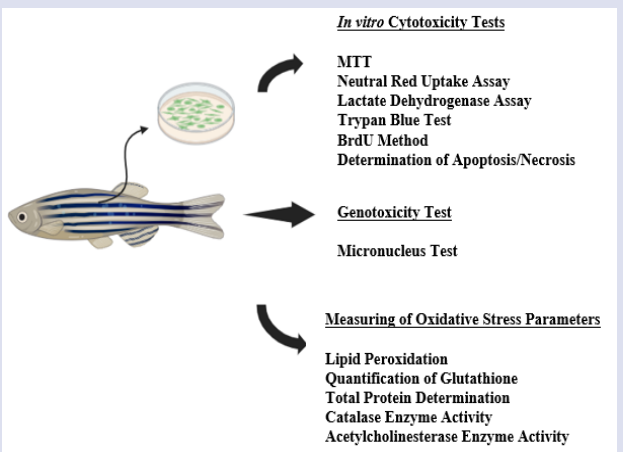

Figure 1. Summary of the tests performed in this study to evaluate cytotoxic, genotoxic, and oxidative stress parameters on the zebrafish liver cell line.

In vitro cytotoxicity tests

MTT

The MTT test is based on the conversion of the yellow tetrazolium salt into the blue-purple colored soluble formazan product by the enzyme mitochondrial succinate dehydrogenase [15]. Cells were seeded in a 96-well, 4-plate format with 10^4 cells per well. Glufosinate-ammonium application doses were added to the wells, and as a positive control 2.5% DMSO solution was added. Plates were incubated at 28°C for screening every 24 hours. At the end of the incubation period, the medium was removed. All wells were washed with dPBS. Fresh medium (100 µL) was added to each well. In a dark environment, 10 µL MTT solution (Biofroxx) was added to each well. Plates wrapped in aluminum foil were shaken at 200 rcf for 2 minutes at room temperature. They were then incubated at 28°C for 4 hours. After 4 hours, the solution was removed. For the dissolution of formazan crystals, 100 µL DMSO was added and shaken at 200 rcf. Formazan crystals were observed under an inverted microscope. Measurements were taken at 570 nm wavelength and 650 nm reference wavelength. The viability rate of the negative control group was considered to be 100%.

Neutral Red Uptake Assay

Lysosomal integrity was evaluated using the Neutral Red Uptake (NRU) method. Damaged cells exhibit differential uptake of Neutral Red, while dead cells cannot retain the dye [16]. In a 96-well, 4-plate format, 10^4 cells were seeded in each well. After adherent cells attached to the surface, the wells were washed with dPBS. Glufosinate-ammonium application doses were added to the wells, and as a positive control 20 mM doxorubicin (Abcam, ab234039) was added. Plates were incubated at 28°C for screening every 24 hours. At the end of the incubation period, the medium was removed. Each well was washed with 200 µL washing solution as per the kit procedure. Subsequently, 150 µL of Neutral Red dye was added, and the plates were incubated at 28°C for 2 hours. After the incubation, the plate was examined under an inverted microscope. The dye was removed, and each well was washed with washing solution. Then, 150 µL of the dissolution liquid was added, and the plate was shaken for 20 minutes at room temperature and 200 rcf in a shaker. At the end of this period, measurements were taken at an absorbance of 540 nm.

Lactate Dehydrogenase Assay

The Lactate Dehydrogenase (LDH) test measures the release of the LDH enzyme from the cytoplasm of cells to the cell medium. When cells are exposed to toxic agents that damage the membrane, LDH reduces membrane integrity to keep LDH in the cytoplasm. The more damage a cell membrane suffers, the greater the LDH leakage [17]. In a 96-well, 4-plate format, 10^4 cells were seeded in each well. After adherent cells attached to the surface, the wells were washed with dPBS. Glufosinate-ammonium application doses were added to the wells, and a 5 µL positive control solution (Abcam, ab197004) included in the kit was added. Plates were incubated at 28°C for screening every 24 hours. Thirty minutes before measurement, 10 µL of the lysate control solution was added, shaken for 1 minute, and incubated. At the end of the incubation period, the plates were initially shaken at 200 rcf in a shaker. 5 µL of the supernatant were transferred to a new white 96-well plate. Then, 95 µL of the reaction mixture was added to each well according to the kit's instructions. All of these processes were performed in a dark environment to prevent potential interference from light. The plate wrapped in aluminum foil was shaken for 10 minutes at 200 rcf. Fluorescence measurements were taken at Ex/Em = 535/587 nm. The blank wells did not contain cells.

Trypan Blue Test

To determine cell viability, one of the most commonly used methods is the Trypan Blue (TB) method. Due to the integrity of the live cell membrane, Trypan Blue dye cannot pass through; it can only pass through dead cell membranes and stain them [18]. In a 24-well, 4-plate format, $5x10^4$ cells were seeded in each well. After adherent cells attached to the surface, the wells were washed with dPBS. Glufosinate-ammonium application doses were added to the wells, and as a positive control 2.5% DMSO was added. Plates were incubated at 28°C for screening every 24 hours. At the end of the incubation period, the medium was removed, dPBS was used for washing, and Trypsin- EDTA solution was added to the wells. The cells were incubated for 5 minutes at 28°C. Growth medium was added to stop the effect of Trypsin-EDTA, and it was aspirated by gentle pipetting. To remove the Trypsin-EDTA solution, the cell suspension was centrifuged at 125 rcf for 5 minutes. The supernatant was discarded, and the cell pellet was resuspended with growth medium. To count cells, 10 µL of the cell suspension and 10 µL of Trypan Blue were mixed. In the use of Trypan Blue, cells that take up the blue color are considered dead, while those that do not are considered live. Cell counting was performed for each well in triplicate using a Thoma chamber.

5-bromo-2-deoxyuridine (BrdU) Method

Cell proliferation experiments are widely used in life sciences. 5-bromo-2-deoxyuridine (BrdU) is a pyrimidine analog that incorporates into the newly synthesized DNA of proliferating cells in place of thymidine. In a 96-well plate, 10^4 cells were seeded in each well. After adherent cells attached to the surface, the wells were washed with dPBS. Glufosinate-ammonium application doses were added to the wells, and as a positive control 2.5% DMSO was added. The plate was incubated at 28°C for 96 hours. At the end of the incubation period, the steps outlined in the kit procedure were followed (BioVision, K306-200). Measurements were taken at 650 nm absorbance between 5-30 minutes. Then, the stop solution was added, and measurements were taken at 450 nm absorbance.

Determination of Apoptosis/Necrosis

Hoechst 33342 (HOE) is a fluorescent dye that can penetrate both intact and fragmented cell membranes and has a strong binding ability to specific areas in DNA (Adenine-Thymine). When the dye binds to DNA, it emits blue light in fluorescent imaging, allowing the counting of all cells (both living and dead) [19]. Propidium iodide (PI) is a fluorescent dye with the ability to penetrate damaged cell membranes and bind to nuclear DNA by circulating among DNA bases, specifically in dead or damaged cells. In a 96-well, 2-plate format, 10^4 cells were seeded in each well. After adherent cells attached to the surface, the wells were washed with dPBS. Glufosinate-ammonium application doses were added to the wells, and as a positive control 2.5% DMSO was added. The plates were incubated at 28°C for 2 different hours (48 hours and 96 hours). The treated cells were washed with dPBS. HOE prepared as a stock solution (ThermoFisher Scientific) was added to each well (100 µL per well) and incubated at 28°C for 20 minutes. Just before taking measurements, 10 µL of PI solution (Sigma-Aldrich) was added to the wells. Fluorescent imaging was obtained with Texas Red, DAPI, and Brightfield filters using a multi-plate reader. During the area scan, photographs were taken, and a percentage evaluation was made based on 200 counted cells.

In vitro genotoxicity test

Micronucleus test

Micronuclei are formed from chromosomes or fragments of chromosomes. An increase in their numbers indicates genomic instability and damage occurring during the cell cycle [20]. In a 96-well plate, $5x10^3$ cells were seeded in each well. After adherent cells attached to the surface, the wells were washed with dPBS. Glufosinate-ammonium application doses were added to the wells, and as a positive control 2.5% DMSO was added. The plates were incubated for 72 hours, which is the doubling time. At the 50th hour after dose application, colchicine was added to each well at a concentration of 0.20 µg mL^{-1}. Incubation continued until 72 hours had elapsed. After incubation, the colchicine-containing medium was removed. Each well received 100 µL of hypotonic solution and was incubated for 10 minutes. After centrifugation at 1200 rpm for 15 minutes, the supernatant was removed. The cells were treated with a fixation solution and allowed to dry. Upon drying, 30 µL of Giemsa staining solution (Merck) was added to the cells. After waiting for 15 minutes, the cells were rinsed three times with distilled water and allowed to dry. At the end of the process, cells with two or more nuclei and cells with micronuclei were counted and recorded using an inverted microscope.

Measuring the Oxidative Stress Parameters

The imbalance between oxidants and antioxidants causes oxidative stress by resulting in the formation of reactive oxygen species (ROS). Fish are equipped with a defense system to against the effects of ROS, which result from the breakdown of xenobiotics [21].

Lipid Peroxidation

According to the Ledwożyw method, the spectrophotometric evaluation of the pink color produced as a result of the reaction between Malondialdehyde (MDA) and thiobarbituric acid (TBA) at 532 nm will allow the assessment of lipid peroxidation [22].

Quantification of Glutathione

The Ellman reagent is based on the ability of DTNB (5,5'-dithiobis 2-nitrobenzoic acid) to react with compounds containing sulfhydryl groups to form a mixed disulfide (GS-TNB) and 2-nitro-5-thiobenzoic acid (TNB) [23]. This method evaluates reduced glutathion (GSH) levels spectrophotometrically at 412 nm.

Total Protein Determination

Protein determination in tissues based on the Bradford method relies on the measurement of the absorbance at 595 nm of the color solutions formed as a result of Coomassie Brilliant Blue dye binding to proteins [24].

Catalase Enzyme Activity

A quantitative spectrophotometric method defined by Aebi monitors the breakdown of hydrogen peroxide catalyzed by catalase (CAT) by observing a reduction in ultraviolet absorbance of a hydrogen peroxide solution. This decomposition is determined by the decrease in absorbance at 240 nm after the tissue in the experimental solution is allowed to stand for 1 minute [25].

Acetylcholinesterase Enzyme Activity

In the aftermath of oxidative damage, acetylcholinesterase (AChE) enzyme activity is often affected, resulting in an excessive accumulation of acetylcholine. The degree of AChE enzyme activity impairment is measured through spectrophotometric measurements at 412 nm, aiming to determine the damage resulting from oxidative stress [26].

Statistical Analysis

Experiments were carried out with a minimum of three replicates. The SPSS 23 software package was utilized for statistical analyses. The experiments were evaluated using one-way analysis of variance (ANOVA), Tukey's post hoc comparison method, and factorial ANOVA. Differences within the confidence intervals of $p<0.05$ were considered statistically significant. Data is presented as mean ± standard error.

Results

While pesticides tend to become diluted when they enter aquatic ecosystems, they often remain toxic through various mechanisms. For aquatic vertebrates like fish, acute toxicity occurs at low mg/L concentrations. However, the effects of environmentally relevant chronic exposure have not been fully assessed, which is taken into

consideration in our study. Glufosinate-ammonium, classified among non-selective herbicides and widely used in agriculture, has been chosen for this study.

MTT Test

To assess the potential toxicity and genotoxicity at the cellular level induced by glufosinate-ammonium, in vitro cytotoxicity tests were employed. These tests aimed to provide detailed information on cell viability, causes of cell death (apoptosis, necrosis), protein/DNA synthesis processes, and cell division.

The MTT assay revealed a consistent decrease in viability at all time points compared to the group of negative control (Figure 2a). After a 24-hour exposure, statistically significant decreases in viability were observed compared to the group of negative control ($p<0.05$). At 48 hours, a statistically non-significant decrease was identified only at the 500 mg L^{-1} dose. However, viability at all other doses exhibited a statistically significant reduction compared to the group of negative control ($p<0.05$). Significant differences were found between the 24-hour application and the subsequent time points (48, 72, and 96 hours) according to factorial ANOVA ($p<0.001$). Additionally, statistically significant differences were identified within the 48-hour and 96-hour applications ($p<0.001$).

NRU Test

The NRU assay results indicated a decrease in neutral red uptake at all time points and doses compared to the group of negative control (Figure 2b). After a 24-hour application, statistically non-significant decreases were observed at doses of 2000 mg L^{-1} to 31.25 mg L^{-1} compared to the group of negative control ($p>0.05$). At 48 hours, only the 2000 mg L^{-1} dose showed a statistically significant decrease compared to the group of negative control. All other doses exhibited a decrease in comparison to the group of negative control. After 72 hours, a decrease was observed at all doses compared to the group of negative control. A statistically significant difference was found at doses of 250 mg L^{-1} and 31.25 mg L^{-1} after 96 hours ($p<0.05$). Significant differences were identified between the 24-hour and 48-hour applications ($p<0.01$) and between the 48-hour and 72-hour applications ($p<0.05$) according to factorial ANOVA.

LDH Test

The LDH assay results, evaluated at 24, 48, 72, and 96 hours after glufosinate-ammonium application, indicated an increase in lactate dehydrogenase (LDH) activity at all time points and doses compared to the group of negative control (Figure 2c). After a 24-hour application, statistically non-significant increases were observed at doses of 2000 mg L^{-1} to 31.25 mg L^{-1} compared to the group of negative control ($p>0.05$). At 48 hours, only the 1000 mg L^{-1} dose showed a statistically significant increase compared to the group of negative control. All other doses exhibited a non-significant increase compared to the group of negative control. After 72 hours, an increase was observed at all doses compared to the group of negative control, with statistically significant increases at doses of 500 mg L^{-1} and 250 mg L^{-1}. After 96 hours, a statistically significant difference was identified only at the 250 mg L^{-1} dose compared to the group of negative control ($p<0.05$). Significant differences were identified between the 24-hour application and subsequent time points (48, 72, and 96 hours) according to factorial ANOVA ($p<0.001$).

Trypan Blue Test

The trypan blue test results, evaluated at 24, 48, 72, and 96 hours after glufosinate-ammonium application, indicated a decrease in cell viability at all time points compared to the group of negative control (Figure 2d). After a 24-hour application, statistically significant decreases in viability were observed at doses of 2000 mg L^{-1} to 250 mg L^{-1} compared to the group of negative control ($p<0.05$). At 48 hours, statistically significant decreases were identified only at doses of 2000 mg L^{-1} and 1000 mg L^{-1} compared to the group of negative control. All other doses showed a non-significant decrease in cell viability compared to the group of negative control ($p>0.05$). After 72 hours, statistically significant decreases were observed at doses of 2000 mg L^{-1} to 500 mg L^{-1} compared to the group of negative control ($p<0.05$). All other doses continued to exhibit a decrease in cell viability compared to the group of negative control. After 96 hours, statistically significant decreases were identified at doses of 2000 mg L^{-1} and 1000 mg L^{-1} compared to the group of negative control ($p<0.05$). According to factorial ANOVA, there was a statistically significant difference only between the 48-hour and 96-hour applications ($p<0.05$).

BrdU Cell Proliferation Test

The results of the bromodeoxyuridine (BrdU) cell proliferation test were evaluated after a 96-hour application of glufosinate-ammonium. A decrease in all doses was observed compared to the group of negative control (Figure 2e). After the 96-hour application, a statistically non-significant decrease was identified compared to the group of negative control ($p>0.05$). This suggests that cell proliferation was not significantly affected.

Hoechst 33342 and Propidium Iodide Dual Staining Test

The results of the apoptosis/necrosis test were evaluated after 48 and 96 hours of glufosinate-ammonium application. The % Apoptosis test showed an increase at all time points compared to the group of negative control (Figure 2f). After a 48-hour application, a statistically significant increase in the percentage of apoptosis was observed at the 2000 mg L^{-1} dose compared to the group of negative control ($p<0.05$). In all other doses, the percentage of apoptosis increased non-significantly compared to the group of negative control ($p>0.05$). After the 96-hour glufosinate-ammonium application, a statistically significant increase was identified at doses of 2000 mg L^{-1} to 250 mg L^{-1} compared to the group of negative control ($p<0.05$). Statistically non-significant increases were observed at doses of 125 mg L^{-1} and 62.5 mg L^{-1}. According to factorial ANOVA for the % Apoptosis

test, a statistically significant difference was found between the 48-hour and 96-hour applications ($p<0.05$).

The % Necrosis test showed a change at all time points compared to the group of negative control (Figure 2g). After a 48-hour application, a statistically significant decrease in the percentage of apoptosis was identified at the 2000 mg L^{-1} dose compared to the group of negative control ($p<0.05$). While doses of 1000 mg L^{-1}, 500 mg L^{-1}, and 62.5 mg L^{-1} showed statistically non-significant decreases compared to the group of negative control, doses of 250 mg L^{-1} and 125 mg L^{-1} exhibited statistically non-significant increases ($p>0.05$). After the 96-hour glufosinate-ammonium application, a statistically significant decrease was identified at doses of 2000 mg L^{-1} to 250 mg L^{-1} compared to the group of negative control ($p<0.05$). Statistically non-significant decreases were observed at doses of 125 mg L^{-1} and 62.5 mg L^{-1}. According to factorial ANOVA for the % Necrosis test, a statistically significant difference was found between the 48-hour and 96-hour applications ($p<0.01$).

Micronucleus Test

The results of the micronucleus test were evaluated after a 72-hour application of glufosinate-ammonium, corresponding to the doubling time of ZFL cells. A change in micronucleus rates was observed in doses compared to the group of negative control (Figure 2h). After the 2000 mg L^{-1} and 1000 mg L^{-1} applications, a statistically significant increase in % micronucleus rates was identified compared to the group of negative control ($p<0.05$). Increases continued at doses of 500 mg L^{-1}, 250 mg L^{-1}, and 125 mg L^{-1}compared to the control, but these increases were not statistically significant. The lowest dose application, 62.5 mg L^{-1}, showed a statistically non-significant decrease compared to the control.

Figure 2. Analysis Findings of ZFL Cells Over Time; A) MTT Analysis Results B) NRU Analysis Results C) LDH Analysis Results D) Trypan Blue Analysis Results E) BrdU Method for Cell Proliferation Analysis Results F) % Apoptosis Analysis Results G) % Necrosis Analysis Results H) % Micronucleus Analysis Results (* $p<0.05$)

Oxidative Stress Tests

In order to assess potential biochemical changes in the ZFL cell line, measurements of total protein, MDA, and GSH levels, as well as CAT and AChE enzyme activities, were conducted. Sublethal exposure doses of 250 mg L^{-1}, 500 mg L^{-1}, and 1000 mg L^{-1} were selected. The evaluation of biochemical test parameters is presented as mean values (± standard error). Statistically significant results ($p<0.05$) were observed in the low and medium doses for total protein and GSH (Table 1).

Table 1. Biochemical Analysis Findings of ZFL Cells (* $p<0.05$)

Parameters	Control	250 mg L^{-1}	500 mg L^{-1}	1000 mg L^{-1}
MDA	0,018±0,002	0,017±0,004	0,015±0,003	0,019±0,005
GSH	0,037±0,007	0,008±0,006*	0,016±0,001*	0,025±0,005
CAT	9,759±0,421	10,097±0,478	8,786±0,243	8,519±0,492
AChE	0,103±0,009	0,088±0,002	0,099±0,010	0,091±0,006
Total Protein	13,571±0,304	15,899±0,309*	15,443±0,274*	14,769±452

Discussion

In our study, the ZFL cell line was chosen as a suitable model because it has the genotypic and phenotypic features of liver cells that have the ability to metabolize many xenobiotics to a large extent. The exposure durations applied in our study were kept broad to provide a reliable estimate of glufosinate-ammonium toxicity. Similar to our research, some studies also identified time-dependent effects. A study conducted in 2007 shows a five-fold increase in differential toxicity between the active ingredient glyphosate and a formulation of Roundup after 72 hours of exposure [27]. Additionally, short-term exposures were hypothesized to result in different effects on tested compounds' direct toxicity compared to long-term exposures [28].

In our study, a reduction in cell viability compared to the group of the control has been observed in all hours and doses where glufosinate-ammonium application was administered. In a study related to determining the cytotoxicity of apigenin at different concentrations on CHO-K1 cells, our results similarly indicate a dose-dependent decrease in cell viability [29].

In another study similar to ours, Bonomo et al. (2019) exposed the ZFL cell line to various doses (1, 10, 100, and 1000 ng mL^{-1}) of the flavonoid metal-insecticide magnesium-hesperidin complex (MgHP) for 24 hours, and the mitochondrial activity (MTT test) of ZFL cells showed a dose-dependent decrease. When the exposure time was extended to 96 hours, a decrease in mitochondrial activity was reported at the concentration of 10 ng mL^{-1} and higher concentrations of MgHP. However, no significant difference was found in the trypan blue and LDH tests assessing cell membrane integrity compared to the control group [30]. Goulart et al. conducted a study on ZFL cells, exposing the cell line to various doses of the herbicide glyphosate, the insecticide carbofuran, and a combination of these pesticides. In the ZFL cell line treated with glyphosate, a significant decrease in plasma membrane integrity (i.e., viability) was observed even at the lowest tested concentration (67.7 µg L^{-1}, 94% ± 0.7) ($p<0.05$). At higher tested concentrations a decrease in cell viability was determined in correlation with the integrity of the plasma membrane. Additionally, a significant decrease in medium viability was observed at medium and high concentrations due to mitochondrial activity. Regarding the integrity of the plasma membrane in ZFL cells exposed to the insecticide carbofuran, when compared to the control, cytotoxicity was found to cause a significant reduction in cell viability for three tested concentrations ($p<0.05$). In the case of mixed application, the effects on membrane integrity exhibited a statistically significant reduction in cell viability compared to the group of the control, although no statistically significant difference was observed compared to separate application of the mixture [31].

Our study aligns with a study conducted by Kanat and Selmanoğlu in 2020, which produced results similar to our NRU test findings. In their research, the effects of the organochlorine insecticide fipronil on the human neuroblastoma cell line SH-SY5Y were evaluated at different durations (24 and 48 hours) and concentrations (125, 250, and 500 µM). Similar to our NRU test results, this study also demonstrated a dose-dependent decrease in cell viability. The most significant decrease in cell viability was observed in the group with the highest application dose of 500 µM. The lowest cell viability was calculated as 52.22% for 24 hours and 30.77% for 48 hours. In the same study, the LDH test conducted after 24-hour and 48-hour applications revealed a significant increase in each concentration group compared to the group of the negative control. The highest cytotoxicity was observed at the highest dose (500 µM) [32].

Our study indicates an increased LDH leakage compared to the group of negative control. It has been noted that there may be a significant difference between time-dependent applications. In a study, Karacaoğlu conducted MTT and LDH leakage tests to evaluate the cytotoxic effects of the triazole fungicide flusilazole at concentrations of 25, 100, and 200 µM on the SerW3 cell line. Similar to our study results, this research also showed a dose-dependent reduction in SerW3 cell viability based on the results of the MTT test during a 24-hour incubation period of flusilazole application. Cell viability statistically decreased in all flusilazole application groups compared to the group of the control. Moreover, the cell viability in the group treated with the highest flusilazole dose (200 µM) was statistically different from the group treated with 25 µM flusilazole. In the same study, an LDH test was conducted. The results revealed that increasing

concentrations of flusilazole caused cell membrane damage. While the percentage of LDH leakage increased during 25 and 100 µM flusilazole applications, it did not show a statistically significant difference from the control group. However, the percentage of LDH leakage in the 200 µM flusilazole application group was found to be statistically significantly different from the control group [33].

In a study conducted in 2019, it was determined that ZFL cells exposed to various doses of MgHP (1, 10, 100, and 1000 ng mL-1) for 10, 24, and 96 hours did not show a significant impact on apoptotic or necrotic processes. Furthermore, when comparing exposure durations of 24 hours and 96 hours, it was observed that the effects of MgHP after 96 hours were not as severe as those observed after 24 hours. The researchers associated this phenomenon with the adaptive capacity developed by the cells against stressful conditions or the xenobiotic metabolism capability of liver cells [30]. In a study, Karacaoğlu utilized the Acridine Orange/Propidium Iodide (AO/PI) staining method to determine the type of cell death induced by the fungicide flusilazole in SerW3 cells. The evaluation of this staining method revealed that the 24-hour application of flusilazole induced concentration-dependent apoptotic cell death. The increase in the percentage of apoptotic cell death in all treatment groups showed statistical significance when compared to the control group. It was also determined that necrotic cell death occurred at high concentrations of flusilazole [33].

The potential of substances to induce toxicity is known to cause harm to the cellular DNA structure. In this regard, our study aims to determine cell proliferation using the Bromodeoxyuridine (BrdU) method. Following a 96-hour application, a decrease in cell proliferation compared to the group of negative control was observed. In a thesis conducted by Orta Yılmaz in 2017, various concentrations of fluoride (0.01, 0.05, 0.1, 1, 5, 10, 50, 100, and 200 ppm) were applied to TM3 Leydig cells for 24 hours, and BrdU test results were evaluated. According to this study's findings, a decrease was observed in all concentrations compared to the control group. With the exception of the lowest dose, all administered doses demonstrated statistical significance [34].

In another study conducted in 2020, it was observed that as the concentration of the flavonoid metal-insecticide magnesium-hesperidin complex increased, genotoxicity also increased, leading to nuclear abnormalities after 24 hours of exposure. When the exposure period was extended to 96 hours, improvements in nuclear abnormalities were detected, along with the stimulation of DNA repair mechanisms. In the same study, at high concentrations, it influenced cell stability by increasing ROS levels in both exposure durations (24 and 96 hours). After 24 hours of exposure, CAT enzyme activity decreased, while GSH levels increased. Consistent with our findings, it was determined in this study that there was no deviation from the group of negative control when examining lipid peroxidation levels [35].

In a study conducted on *Cnesterodon decemmaculatus* in 2019, chlorpyrifos and glyphosate were administered to the fish separately and in combination for a duration of 42 days. It was observed that the formation of micronuclei in fish was not statistically significantly affected, while nuclear abnormalities showed a significant increase [3].

ROS formation is associated with oxidative damage and can occur when the antioxidant and detoxification systems are out of balance. It becomes particularly significant when active intermediates produced by xenobiotics and their metabolites need to be neutralized. Lipid peroxidation is a valuable indicator of cellular components resulting from oxidative stress [36]. Furthermore, variations in antioxidant responses can be explained to some extent by differences in the intensity of oxidative stress. ROS may induce the biosynthesis of antioxidant enzymes and GSH to enhance antioxidant capacity. Additionally, significantly higher ROS levels may also lead to inactivation of antioxidant enzymes. Following increased ROS production, a moderate oxidative stress may occur, characterized by a further increase in oxidatively modified components but a decrease in ROS-sensitive parameters due to enzyme inactivation caused by the ROS source. GSH, non-protein thiol, serves as a primary reducer in cells. It is the most abundant antioxidant and is commonly reported as a focal point in assessing oxidative stress in zebrafish. Typically, the concentration of GSH decreases upon induction of oxidative stress.

Consistent with our findings, Gasnier et al. study in 2010 on glyphosate-based herbicides reported that the treatment of a human hepatic cell line with glyphosate and four formulations led to a decrease in glutathione transferase levels [37].

Lipid peroxidation is likely the most reliable indicator of the systemic damage caused by ROS and, therefore, is implicated in the toxicity of pesticides through this mechanism. In our study, no statistically significant differentiation in MDA levels was observed. Similarly, Larsen et al. (2012) in their research noted that, akin to our study, rats exposed to glyphosate through drinking water did not show significant changes in lipid peroxidation levels, although TBARS production tended to be lower. They suggest that one reason for this could be the absence of cell damage or cell death, and another reason might be the creation of specific oxidative radical groups by glyphosate that do not attack lipids [38].

CAT, located in peroxisomes, is an enzyme that facilitates the removal of hydrogen peroxide by metabolizing it into molecular oxygen and water. The SOD-CAT system provides the first line of defense against oxygen toxicity and is often used as a biological indicator of ROS production [39]. A study conducted by Karacaoğlu in 2022 has reported similar results to our research. After applying the fungicide flusilazole to SerW3 cells at doses of 25, 100, and 200 µM, a spectrophotometric method was employed to determine CAT enzyme activity. Compared to the control group, statistically insignificant decreases in CAT activities were observed at low and

medium flusilazole concentrations, while a significant decrease occurred at high concentrations [33].

Conclusion

With the contribution of the increasing world population and developing technology, pesticide use has become more widespread. Despite the many contributions of pesticides, the view that they are also an important public health problem has begun to spread. In addition to affecting target organisms throughout the ecosystem, they also cause non-target organisms to be affected. In this study, the cytotoxic, genotoxic, and oxidative stress effects of glufosinate-ammonium on the zebrafish liver cell line were evaluated. The zebrafish liver cell line was used to simulate the metabolic processes of toxins entering the liver. It is of critical importance due to its role as a biological model organism. The findings show that agents used to support production, such as glufosinate-ammonium, can trigger oxidative stress in non-target organisms and potentially strain the antioxidant defense system of this organism. As a result of the resulting stress, cell survival may decrease, DNA breaks may occur, and micronucleus formation may be stimulated.

These results, which can be seen in non-target organisms, can tell us that we need to be more careful about pesticide use and that the smallest change that can occur in nature can affect the survival of all organisms with the butterfly effect. The application of a holistic approach will make it easier for all living things to live in prosperity. In order to investigate the toxic effects of glufosinate-ammonium on aquatic organisms more comprehensively, its chronic effects should be evaluated by including different methods.

Conflicts of interest

There are no conflicts of interest in this work.

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