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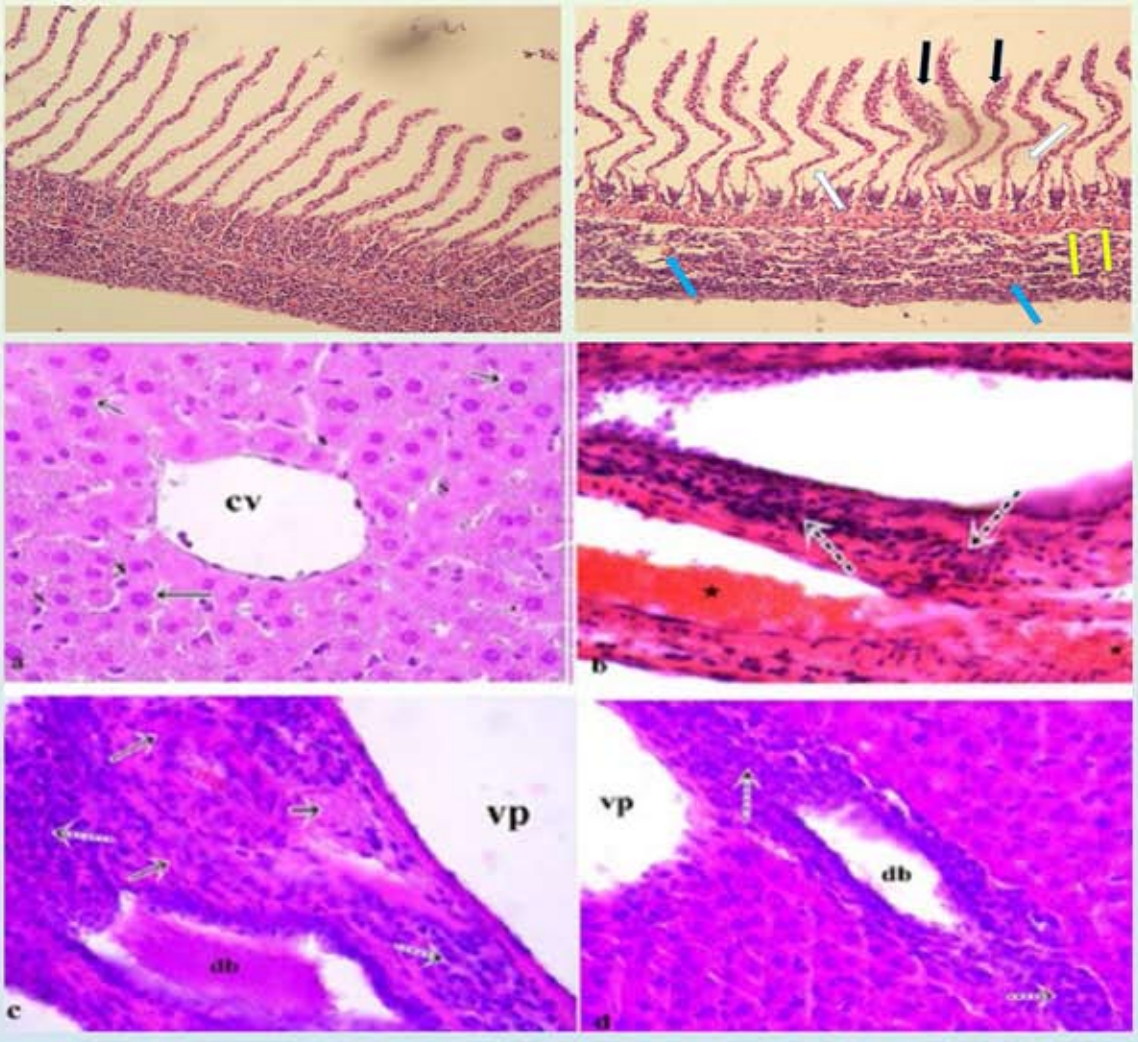

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AIM AND SCOPES
Journal of Cellular Neuroscience and Oxidative Stress is an online journal that publishes original research articles, reviews and short reviews on the molecular basis of biophysical, physiological and pharmacological processes that regulate cellular function, and the control or alteration of these processes by the action of receptors, neurotransmitters, second messengers, cation, anions, drugs or disease.

Areas of particular interest are four topics. They are;

A- Ion Channels (Na^+- K^+ Channels, Cl^- channels, Ca^{2+} channels, ADP-Ribose and metabolism of NAD^+, Patch-Clamp applications)

B- Oxidative Stress (Antioxidant vitamins, antioxidant enzymes, metabolism of nitric oxide, oxidative stress, biophysics, biochemistry and physiology of free oxygen radicals)

C- Interaction Between Oxidative Stress and Ion Channels in Neuroscience
(Effects of the oxidative stress on the activation of the voltage sensitive cation channels, effect of ADP-Ribose and NAD^+ on activation of the cation channels which are sensitive to voltage, effect of the oxidative stress on activation of the TRP channels in neurodegenerative diseases such Parkinson's and Alzheimer's diseases)

D- Gene and Oxidative Stress
(Gene abnormalities. Interaction between gene and free radicals. Gene anomalies and iron. Role of radiation and cancer on gene polymorphism)

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Biophysics	Biochemistry
Biology	Biomedical Engineering
Pharmacology	PhysiologyGenetics
Cardiology	Neurology
Oncology	Psychiatry
Neuroscience	Neuropharmacology

Keywords
Ion channels, cell biochemistry, biophysics, calcium signaling, cellular function, cellular physiology, metabolism, apoptosis, lipid peroxidation, nitric oxide, ageing, antioxidants, neuropathy, traumatic brain injury, pain, spinal cord injury, Alzheimer's Disease, Parkinson's Disease.

RESEARCH ARTICLE

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Histopathological and biochemical responses to the oxidative stress induced by glyphosate-based herbicides in the rainbow trout (*Oncorhynchus mykiss*)

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Abstract

This study aimed to determine the effects of glyphosate, a herbicide commonly used in weed control, on aquatic life. For this purpose, 30 one-year-old rainbow trout with an average weight of 150-165 g were obtained from a local trout production station in Mazmanlı (Hatay, Turkey) and transferred to our laboratory, where they were allowed to adapt to the new environment in polyethylene tanks approximately for 7 days. Three groups each containing 10 fish were formed: a control group with no treatment, the group treated with 1.25 mg/l glyphosate-based herbicide (GBH), and the group treated with 2.5 mg/l glyphosate-based herbicide. At the end of 14 days of treatment, blood samples were taken from the caudal vein of the fish under anaesthesia, and their sera were separated. Total oxidant/antioxidant levels and paraoxonase activity were analysed in the obtained serum samples. Also, for histopathological examination, gill tissues were removed and fixed in 10% buffered formalin. After the fixation and routine tissue processing (graded alcohols, methyl benzoate and benzol processing), the tissues taken were embedded in paraffin and 5 μm serial sections were taken by microtome from the blocks to slides pre-coated with chrome alum gelatine. Histopathological changes were examined at the light microscopic level by staining the sections with haematoxylin-eosin. According to the evaluation of the biochemical parameters obtained from the groups, antioxidant capacity and paraoxonase activity decreased and oxidant level increased in the group treated with 1.25 and 2.5 mg/l glyphosate-based herbicide as compared with the control group. In the histopathological examination of the sections, it was observed that the primary and secondary lamellae had a normal structure in the gill sections obtained from the control group. Irregular secondary lamellae and

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List of Abbreviations;

GBH, Glyphosate-based herbicide; **RDT**, Roundup transorb; **POEA**, polyoxyethylene tallow amine; **EPSPS**, 5-enolpyruvoylshikimate-3-phosphate synthase; **CAG**, Chrome alum gelatine; **MS222**, tricaine methanesulphonate; **TAS**, Total antioxidant status; **TOS**, Total oxidant status; **PON**, Paraoxonase; **HDL**, High density lipoprotein; **CAT**, Catalese; **SOD**, Superoxide dismutase; **GPx**, glutathione peroxidase; **AST**, aspartat aminotransferase; **ALP**, alkaline phosphatase; **LDH**, lactate dehydrogenase; **ABTS**, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid); **$NaHCO_3$**, Sodium bicarbonate; **Fe^{2+}**, Iron; **Fe^{3+}**, Ferric ion; **H_2O_2**, Hydrogen peroxyde

epithelial hyperplasia were observed in the gill sections obtained from the group receiving 1.25 mg/l GBH. And in the gill sections obtained from the group receiving 2.5 mg/ l GBH, swelling in chloride cells, degeneration in secondary lamellae, and areas of necrosis were detected. In conclusion, results of the study suggests that glyphosate, which is widely used in agricultural activities and has a potential to leak into aquatic ecosystems, may cause oxidative stress due to reactive oxygen species formed in the rainbow trout, and may therefore has a toxic effect.

Keywords: *Oncorhynchus mykiss*; Glyphosate-based herbicide; Oxidative stress; Histopathology; Gill.

Introduction

All around the world, herbicides are widely used in agricultural activities, and they are followed by some other pesticides such as insecticides and fungicides (He et al., 2012; USEPA, 2011). Glyphosate, a broad-spectrum, non-selective, organophosphate herbicide, is a weak organic acid containing glycine and phosphomethyl. Roundup®, the first glyphosate herbicide produced by Monsanto in the early 1970s, has since been used extensively around the world, particularly for weed control, with many formulas and by adding various salts into its content (Cox, 2004; Dallegrave et al., 2003). With the use of herbicides, the inhibitory effect of weeds on the production of commercial crops has been greatly reduced. Due to their systemic effect, glyphosate-containing herbicides have great success in controlling perennial plants. For this reason, glyphosate is a leading and increasingly utilised chemical around the world (Baylis, 2000). It is frequently used in agricultural areas, especially in rice, corn and soybean fields, for garden care, forestlands, and to get rid of unwanted plants with large leaves in pastures and green areas (Dallegrave et al., 2003).

Glyphosate is an herbicide that inhibits the synthesis of aromatic amino acids such as tryptophan, tyrosine and phenylalanine in plants (Santos et al., 2007). In plants, the enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) is inhibited by glyphosate. EPSPS is present in the plastids as a primary, and its inhibition causes the accumulation of shikimate-3-phosphate, thereby inhibiting the production of aromatic amino acids, which means inhibition of protein synthesis. Although EPSPS is the only enzyme known to be targeted by glyphosate, it is known that many physiochemical and physical processes are also affected by glyphosate (Baylis, 2000). Since glyphosate is considered to have less toxicity than other herbicides, transgenic plants produced with herbicide resistance have also been reported to be able to tolerate this herbicide easily (Vollenhofer et al., 1999; Williams et al., 2000). The use of glyphosate has been increasing in parallel to the increase of transgenic plantations for boosting agricultural production in the world (Giesy et al., 2000; Pline et al., 2001).

Glyphosate may leak into water bodies after agricultural use or when directly applied to water systems to control macrophyte plants (Soso et al., 2007). Since glyphosate has a high solubility in water, both soils and aquatic systems are continually being contaminated. So, it may lead to developmental, morphological, physiological and biochemical modifications on non-target organisms (Tate et al., 1997). Although there have been numerous studies on the toxicity of herbicides on ecosystems and animals, information on aquatic ecosystems is limited (Medina et al., 1994; Piska and Wagray, 1997). Fish are the most used bioindicators for determining the conditions and changes of an aquatic environment. It is therefore very important to know the physiological, biochemical and pathological responses of fish to such changes in their ecosystem. One of the materials used as a biological indicator in the studies made for this purpose is blood and various tissues taken for pathological studies. These materials that constitute biological indicators also show the effects of environmental and human based stress factors and ecosystem sensitivity (Luskova, 1997; Ozkan et al., 2009, Gül et al., 2008; Aksu et al., 2008).

The aim of this study is to demonstrate, with biochemical and pathological lesions, the effects of commercial glyphosate, which is widely used all over the world and the toxicity of which producers consider to be very low, on fish as non-target organisms.

Materials and Methods

Chemicals

Trademarked chemicals were used for the purposes of the study. Boxer 48 SL (active agent 480 g/l glyphosate, *N*-(phosphonomethyl)gylcine) (Natural chemicals and agrochemicals Inc.) was used as glyphosate. MS-222 (tricaine methanesulphonate) (Sigma-Aldrich, CAS: 886-86-2) was used as an anaesthetic for fish.

Experimental design

The rainbow trout used in the study were acquired from the Trout Production Farm of Mazmanlı (Hassa district of Hatay province). All study work was carried out in the Aquarium unit of Islahiye Vocational High School, Gaziantep University. Nearly 1-year-old rainbow trout weighing approximately 160 g ± 25 g and appearing healthy were taken to the laboratory where the experiment would be performed. Seven days were allowed for the fish to adapt to the new environment. Six tanks were used in the experiments, and 5 fish were placed in each tank. The oxygen level in the tanks was increased with an air-stone by connecting air pumps into the water. The fish were fed to satiety twice daily (morning and evening) with commercial dry pellets and were not fed in the 24h prior to experiments. The rainbow trout were daily fed at 2% of their live weight (Çamlı BioAqua® sinking food for trout, crude protein 46%, crude oil 16%, and crude cellulose 2%). According to the procedure on 'experiments in a renewed environment', water and the glyphosate-based herbicide were repeatedly added into the water every day in order to ensure that wastes of food and faeces were removed from the environment. The physicochemical properties of the water used in the study were measured as temperature (°C): 15 ± 2, dissolved oxygen (as mg/l): 9-10, and pH: 7.0-7.4. Experiments were conducted in 2 replicates. After 14 days of treatment, the fish were anesthetised by taking them into aquariums containing MS222 (buffered with 50 mg/l, 100 mg/l $NaHCO_3$) (Ross and Ross, 2008). While the fish were under MS222 anaesthesia, blood samples were taken from their caudal vein and they were euthanised by cervical dislocation. The sera from the blood samples were separated, and total oxidant/antioxidant levels and paraoxonase activity were analysed in the obtained serum samples. After euthanasia, gills of the fish were removed and subjected to histopathological analyses.

In this study we constituted 3 groups;

1. Control group: This group included *Oncorhynchus mykiss* not treated with any herbicide.

2. Glyphosate group I: This group included *Oncorhynchus mykiss* treated with 1.25 mg/l glyphosate-based herbicide.

3. Glyphosate group II: This group included *Oncorhynchus mykiss* treated with 2.5 mg/l glyphosate-based herbicide.

Biochemical Analyses

Twenty four hours after the last treatment, blood samples were collected from the caudal vein of the fish into heparinised tubes. These samples were centrifuged at 3000 rpm for 15 minutes, and plasmas were obtained. They were kept in deep freeze at a temperature of -20°C. Total antioxidant status (TAS) was determined by the automatic measurement method based on the principle of lightening the characteristic colour created by the free radical 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) by the antioxidants contained in the sample added into the medium (Erel, 2004). The results were given as mmol Trolox equivalent/l. Total oxidant status (TOS) measurement was made by the automatic measurement method (Erel, 2005). The oxidants in question have a function of converting the ferrous ion complex to ferric ion. Ferric ion (Fe^{3+}), which is formed by oxidation of iron (Fe^{2+}) to its more stabilised form (Fe_2O_3), forms colour with xylenol orange in an acidic environment. The intensity of the spectrophotometrically colour is related to the total amount of oxidant molecules present in the sample. The measurement was calibrated with hydrogen peroxide (H_2O_2) and the results were reported as micromolar H_2O_2 equivalent (μmol H_2O_2 equiv./l) per litre. Plasma TAS and TOS analyses were performed according to the methodology developed by Erel (2004; 2005). The measurement of PON1 activity was performed according to the methods of Eckerson et al. (1983), and Gülcü and Gürsu (2003). The PON1 activity was determined by spectrophotometric measurement of the absorbance (at 25°C and 412 nanometres) of the colour

product of the 4-nitrophenol produced at enzymatic hydrolysis of the paraoxon as a substrate. For the paraoxonase activity, the enzymatic activity of the enzyme contained in 1 mL of serum that converts 1 nmol paraoxon to 4-nitrophenol in 1 minute was defined as the unit, and the results were given as U/l.

Histological Analysis

At the end of the experiment period, the gill tissues of the fish euthanised by cervical dislocation under general anaesthesia were fixed in 10% buffered formalin solution. After the fixation and routine tissue processing (graded alcohols, methyl benzoate and benzol processing), the tissues removed were embedded in paraffin and 5 μm serial sections were taken with microtome from the blocks to slides pre-coated with chrome alum gelatine (CAG). Histopathological changes were examined at the light microscopic level by treating the sections with haematoxylin-eosin as a histological staining method (Presnel and Schreibman, 1997).

Statistical analysis

Statistical analysis of the data obtained from the study was carried out in the SPSS package program (IBM SPSS Statistic 22). One-way analysis of variance (ANOVA) was used to determine whether there was a difference between the means of the experimental group and, if there was such a difference between the means of the experimental group, the "Anova-Duncan" test was performed on the group means in order to determine the group or groups from which this difference was derived, and the value $p<0.05$ was considered statistically significant.

Results

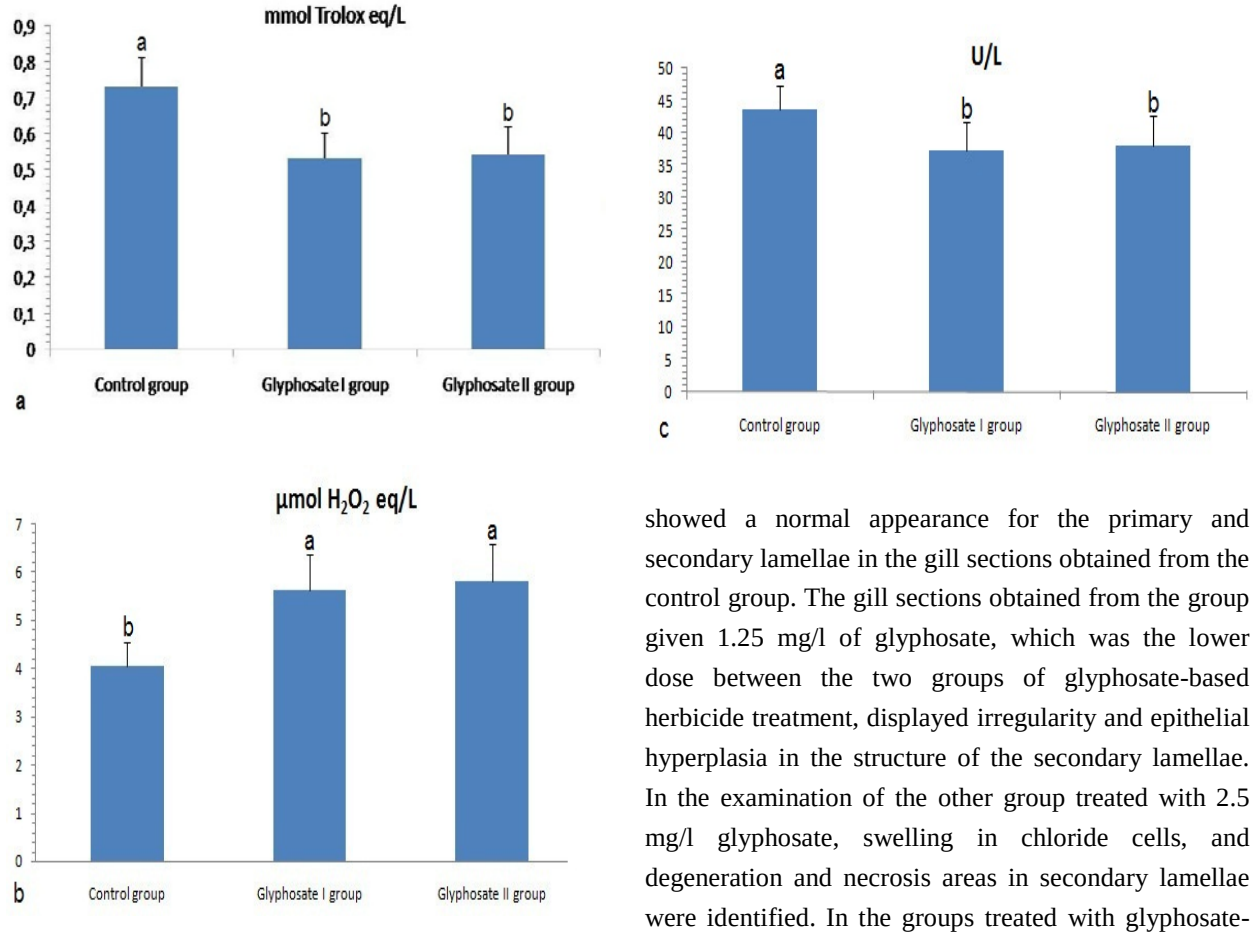
In the end of the treatments, the fish in the tanks were taken to an aquarium containing MS222 (tricaine methanesulphonate) (buffered with 50 mg/l and 100 mg/l NaHCO3) and they were anesthetised (Ross and Ross, 2008). After anaesthesia, heparinised injectors were used to take blood samples from the caudal vein, and then euthanasia was performed by cervical dislocation. As a result of the evaluation of the plasma obtained from the blood extracted for biochemical analyses, a comparison of the groups in terms of the amount of TAS demonstrated that the difference between the glyphosate groups (glyphosate I and glyphosate II) and the control group was significant ($p<0.01$). The internal difference between the glyphosate I and glyphosate II groups was statistically insignificant ($p>0.05$). When the TOS concentrations of the groups were compared, there was a difference between the glyphosate groups (glyphosate I and glyphosate II) and the control group ($p<0.01$). There was no statistical difference between the glyphosate I and glyphosate II groups ($p>0.05$). When the PON activities of the groups were evaluated, the difference between the control group and both glyphosate groups was statistically significant ($p<0.05$). The difference between glyphosate I and II groups is insignificant ($p>0.05$) (Table 1, Figure 1a, b, c).

Table 1. TAS and TOS, PON activity as a result of biochemical data obtained from the groups.

Biochemical parameters	Groups (Mean±SD)			p<
	Control group	Glyphosate I group (1.25 mg L^{-1})	Glyphosate II group (2.5 mg L^{-1})	
TAS (mmol Trolox eq/L)	0.73 ± 0.082[a]	0.53 ± 0.069[b]	0.541 ± 0.079[b]	*
TOS (µmol H_2O_2 eq/L)	4.04 ± 0.51[b]	5.62 ± 0.75[a]	5.80 ± 0.79[a]	*
PON (U/L)	43.72 ± 3.49[a]	37.2 ± 4.62[b]	38.04 ± 4.75[b]	**

*p<0.01 : Statistically significant difference, **p<0.05 : Statistically significant difference, [a, b, c] : Horizontally, values with different letter indicate significant differences, SD: Standart deviation.

Fig. 1. Total antioxidant and total oxidant levels and paraoxonase activity in experimental groups. a. TAS, b. TOS, c. PON, [a, b, c]: Horizontally, values with different letter indicate significant differences.



At the end of the experimental procedure, the histological examination of the gill tissue obtained from groups after fixation and routine tissue processing showed a normal appearance for the primary and secondary lamellae in the gill sections obtained from the control group. The gill sections obtained from the group given 1.25 mg/l of glyphosate, which was the lower dose between the two groups of glyphosate-based herbicide treatment, displayed irregularity and epithelial hyperplasia in the structure of the secondary lamellae. In the examination of the other group treated with 2.5 mg/l glyphosate, swelling in chloride cells, and degeneration and necrosis areas in secondary lamellae were identified. In the groups treated with glyphosate-based herbicide, the histopathological changes observed in both the biochemical values and in the gill may be due to increased reactive oxygen species arising from the oxidative stress caused by glyphosate (Figure 2-4).

Fig. 2. Histological appearance in the fish gill in the control group. Normal structures are observed along with the secondary lamellas (H & E, x100).

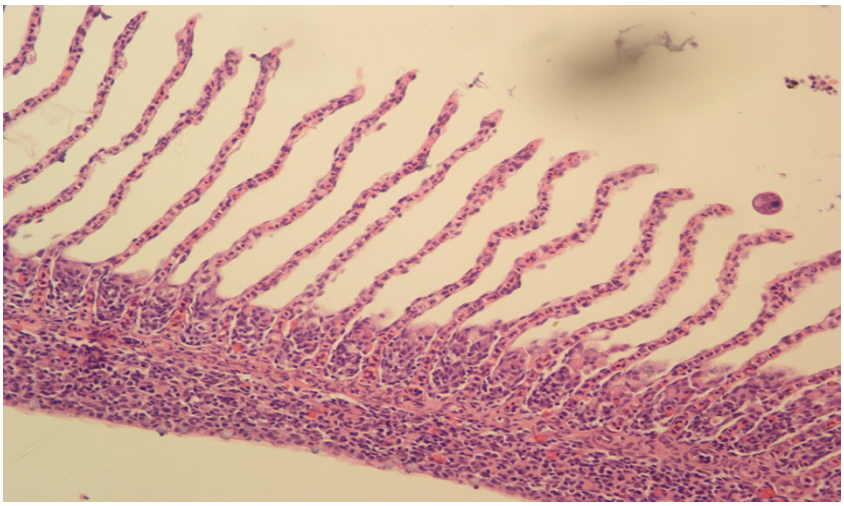


Fig. 3. Histological appearance of the gill tissue belonging to the fish in the group treated with 1.25 mg/l glyphosate-base. Irregular secondary lamellae (white arrows), capillary blood vessels (blue arrows), oedema (osmotic swelling) and epithelial hyperplasia in secondary lamellae (black arrows) and chlorite cells (yellow arrows) are observed (H & E, x100).

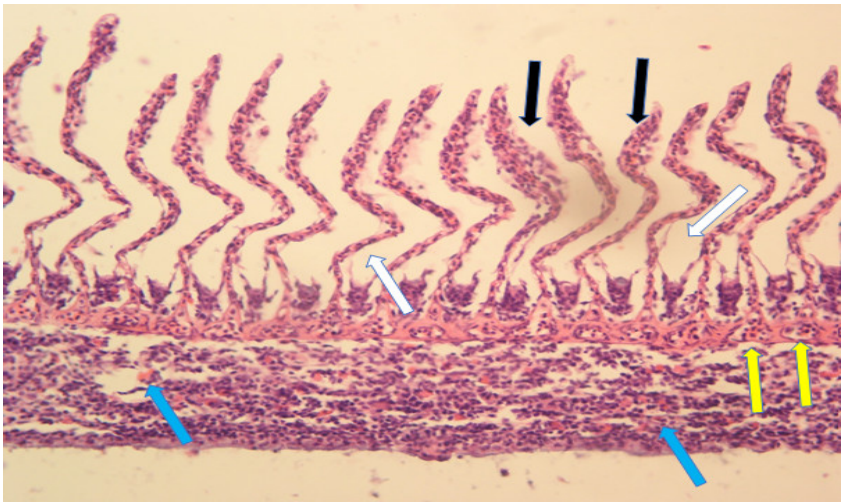
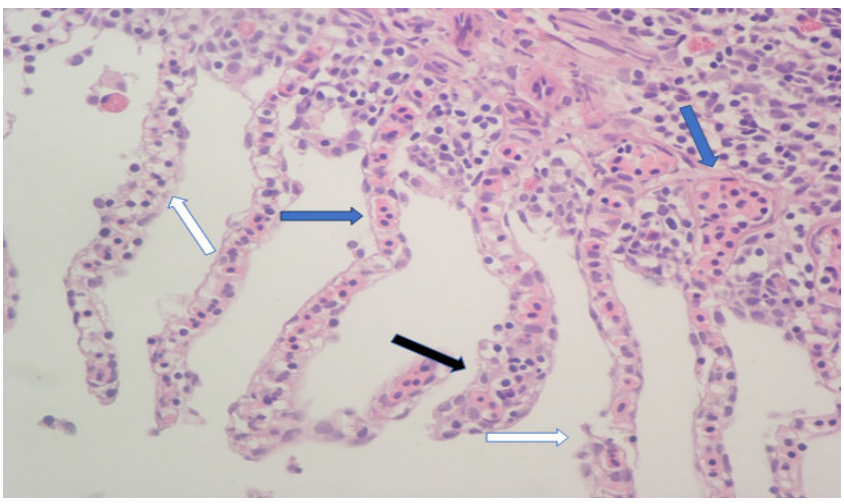


Fig. 4. Histological appearance of the gill tissue belonging to the fish in the group treated with 2.5 mg/l glyphosate. Swelling in chloride cells (blue arrows), degeneration in the secondary lamellae, necrosis (white arrows) and oedema (osmotic swelling) in the secondary lamellae (black arrows) are observed (H & E, x200).



Discussion

Although glyphosate kills the weeds in the fields it is applied to, it also contaminates soil and aquatic ecosystems due to its high solubility in water. This situation causes environmental destruction and threatens the health of living beings. Studies in aquatic organisms demonstrated that the toxicity of glyphosate-containing commercial herbicide preparations is greater than the toxic effect of glyphosate alone (Kolpin et al., 2006). The half-lives are 7 to 70 days for glyphosate and 21 to 28 days for POEA, depending on site conditions (Giesy et al., 2000). Therefore, toxicity studies using relatively high concentrations of glyphosate are environmentally relevant, especially when fish are acutely exposed immediately following Roundup application (Çavas and Könen, 2007). It was reported that the toxic effects on fish vary according to fish species and that Roundup is 30 times more toxic than glyphosate in fish (Mitchell et al., 1987). Glyphosate residues can enter the food chain and its metabolites can leak into rivers. Glyphosate residues were identified in rivers and found to damage river ecosystems (Cox, 1998). Fish, birds, many useful insect species, organisms living in soil, and mammals are in danger because of the damage such resides cause in soil and aquatic ecosystems (Cox, 1995). For this reason, non-target organisms are also affected, and morphological and physiological changes occur in these organisms (Tate et al., 1997). Haematological values are used not only for diseases but also for determining the effects of nutritional and environmental factors. Especially, they are widely used for determining the effects of pollutants in aquatic environments (Atamanalp et al., 2011; Kayhan et al., 2009; Güven et al., 2008). The cause of stress in fish can be variations in endogenous and exogenous factors. Such variations primarily affect blood parameters, and can be observed in a short time. In the case of stress, fish try to restore the metabolism to normal levels through homeostatic mechanisms. However, the metabolic response may be inadequate as a result of a long-term effect of the stress factor (Duran and Erdem, 2013). A study reported that Roundup, which is a glyphosate-derived herbicide, augments oxidative stress in the gold fish even if a little (Lushchak et al., 2009). It was also observed in studies with other fish species such as piava (Leporinus obtusidens) and silver catfish (Rhamdia quelen) that Roundup induces oxidative stress (Glusczak et al., 2006; Glusczak et al., 2007). In a study that used sub-lethal doses of cypermethrin in the rainbow trout (O. mykiss), antioxidant capacity decreased due to an elevation of AST, ALP and LDH enzymes and a decrease of leukocyte count in parallel to histopathological findings (Atamanalp et al., 2002a; Atamanalp et al., 2002b). An increase in hematocrit values and in red and white blood cell counts as a result of acute toxicity induced by the doses of 1 mg/l and 5 mg/l of glyphosate (RDT: roundup transorb) were reported in Prochilodus lineatus juveniles (Modesto and Martinez, 2010). As a result of treatment with different doses of glyphosate in Kars creek transcaucasian barbs (Capoeta capoeta), a significant decrease in PON, HDL, TAS levels and a significant increase in TOS levels were detected (Deveci et al., 2017).

It is very important to examine the physiological, histological and biochemical parameters of living beings in studies carried out to determine toxic effects (Lendhardt, 1992; Kayhan et al., 2009). In those beings living in aquatic ecosystems, the organ that is firstly exposed to any contaminants that are contained in or carried to the water from environment. Therefore, histopathological studies are extensively utilised to determine the extent of damage to fish tissues (Altınok and Çapkın, 2007). A study investigating the acute toxicity of maneb and carbaryl reported that the gills, kidneys and the liver were the most affected organs, and that hyperplasia was determined in gills (Boran et al., 2010). A histopathological examination of the gills of the carb (C. carpio) exposed to deltamethrin revealed desquamation in gill tissues, deterioration, necrosis and oedema in lamellae, and degeneration in epithelia (Cengiz, 2006). A significant increase was observed in the GPx and CAT activity but not in SOD after 12 hours at an acute those of 2.5 mg/l in the rainbow trout exposed to acute and subacute glyphosate. In the case of subacute treatment at a dose of 5 mg/l, no statistical difference was reported with the control group for the amounts of all antioxidant enzymes except for GPx. In the case of the liver tissue, dose-dependent histopathological findings showed degenerated areas and cellular infiltrations, and an increase in hepatocytes (Topal et al., 2015). Liver lesions resulting from acute glyphosate-based herbicide exposure indicate changes in lipid metabolism, lipid peroxidation mediated by oxidative stress, and lipid accumulation (Nwani et al.,

2013; Guilherme et al., 2012). An increase in plasma glucose was noted as a sign of typical stress response in the neotropical fish (Prochilodus lineatus) exposed to 10 mg/l dose of Roundup. In addition, increased amounts of liver catalase and histopathological findings (nuclear degeneration, cellular hypertrophy, cytoplasmic degeneration, hyperaemia, and pyknotic nuclei) were resulted due to antioxidant defence activation (Langiano and Martinez, 2008). A glyphosate-based herbicide at the dose of 1.8 mg/l was reported to cause histopathological findings in the liver of the guppy (Rezende dos Santos et al., 2017). In our study, glyphosate-based herbicide doses were selected below the LC50 dose determined in static studies in the rainbow trout. We examined gills, which are one of the first organs that interact with pesticides in water bodies, after oxidative stress caused by glyphosate-based herbicides, and histopathological changes (irregular secondary lamellae, epithelial hyperplasia, swelling in chloride cells, and degeneration and necrosis areas in secondary lamellae) were encountered. Our findings are in line with the results from studies on pesticides in general and glyphosate-based herbicides in particular. Additionally, compared with the control group, glyphosate-based herbicide treatment significantly increased plasma total oxidant level but decreased total antioxidant capacity and paraoxonase activity. Biochemical results are in agreement with some studies but not with the biochemical findings obtained from those studies in which antioxidant response was sufficient depending on the specific pesticide treatment.

In conclusion, our histopathological findings in gills, the increase in total oxidant level, and the decrease in antioxidant capacity and paraoxonase activity with the glyphosate-based herbicide treatment tell us that the antioxidant response of the organism against the herbicide administered is inadequate. This study suggests that glyphosate, which is widely used in agricultural activities and has a potential to leak into aquatic ecosystems, may cause oxidative stress in the rainbow trout due to reactive oxygen species formed, and may therefore have a toxic effect.

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Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article

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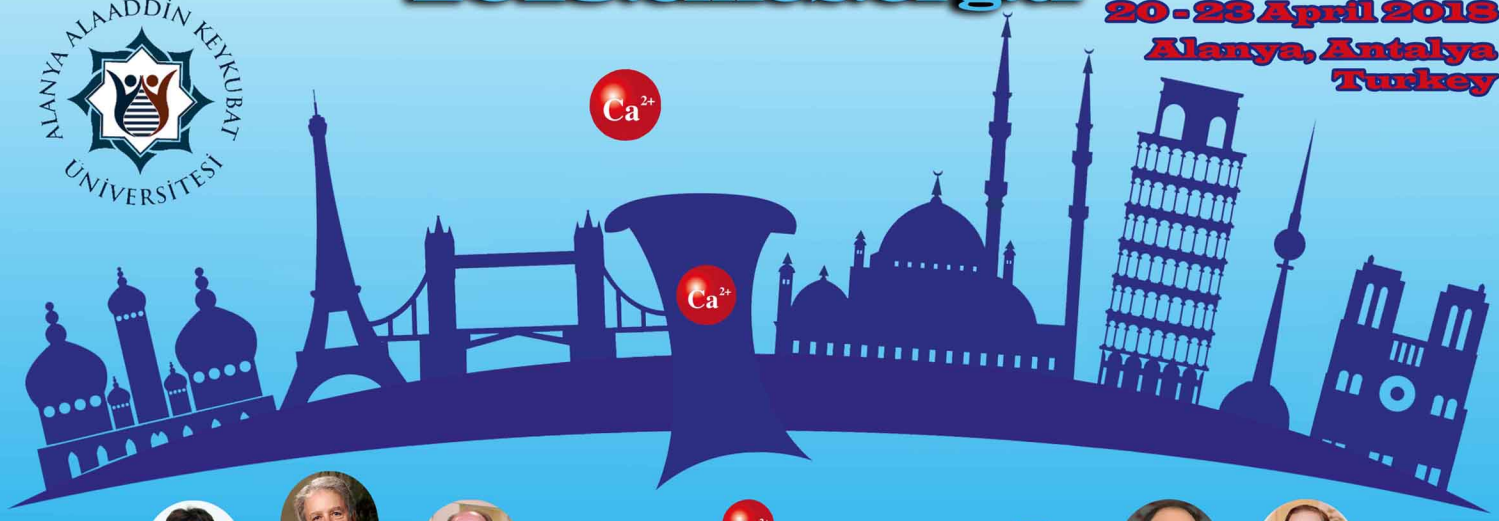


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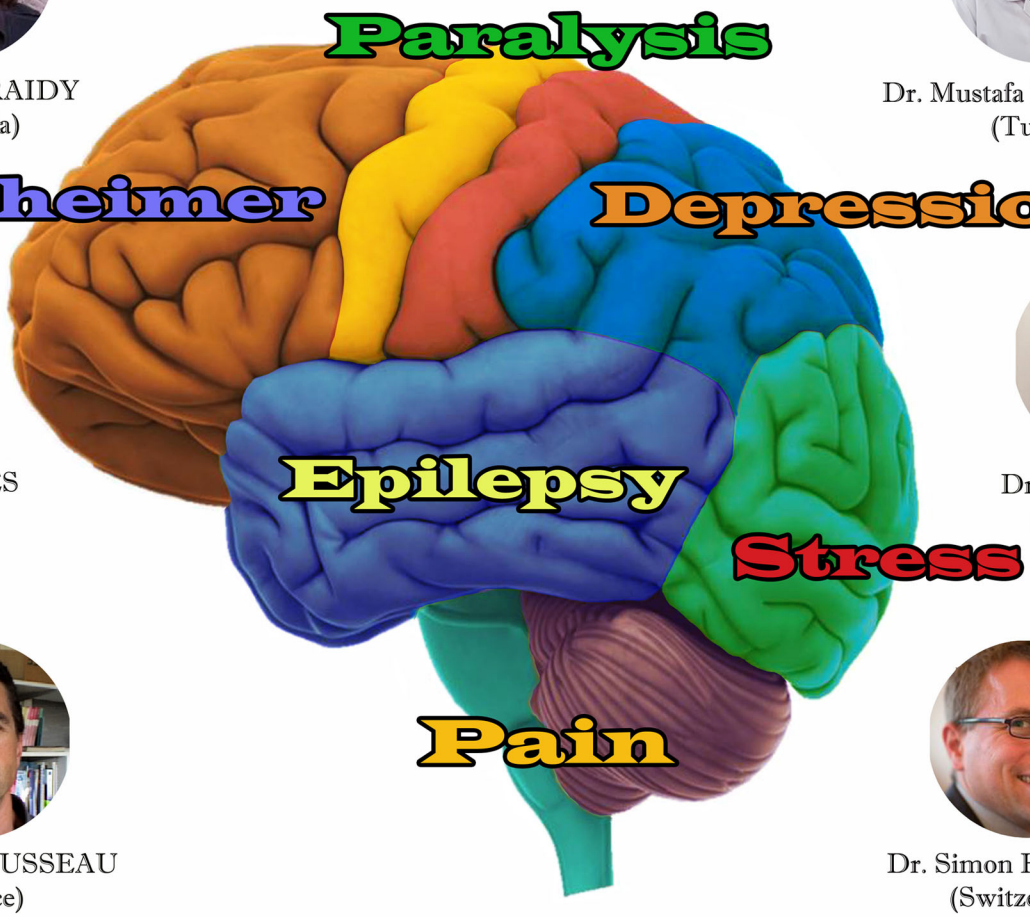

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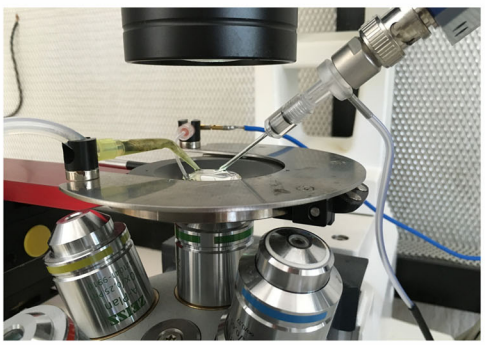

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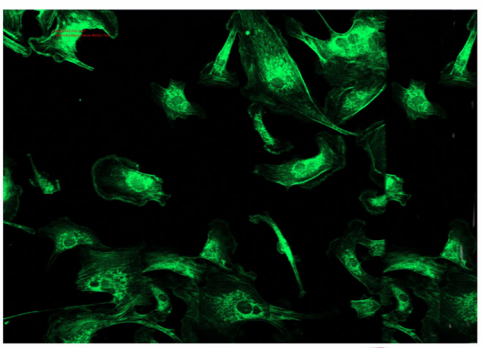

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