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Determining Genotoxic Effect of Thiamethoxam in Rainbow Trout (Oncorhynchus Mykiss) by Micronucleus Test

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Abstract— **The micronucleus (MN) test, which is an indicator of genetic damage accumulated over the lifetime of cells, is one of the most suitable techniques to identify the integrated response to the complex mixture of pollutants. The aim of the study is to evaluate the genotoxic effects of thiamethoxam (TMX), a neonicotinoid pesticide widely used in agricultural activities, on rainbow trout, which is a biomarker in toxicological studies in aquatic ecosystems, by micronucleus technique. In the study, 4 groups including 10 fish in each were formed with two replicates. No substance was applied in the control group. Thiamethoxam doses of 25 and 75 mg/L were applied. As a positive control, 10 mg/L benzene was applied. In the application unit, 5000 erythrocytes were counted for each fish from the blood samples taken from the caudal vein after anesthesia and the micronucleus frequency in 1000 cells on average was determined. Liver tissue was taken after cervical dislocation and evaluated for histopathological analysis. As a result of micronucleus frequency analysis, the average micronucleated erythrocyte rate was 0.3% in the control group, 0.78% in the 25 mg/L thiamethoxam group, 1.72% in the 75 mg/L thiamethoxam group and 5.88% in the positive control group treated with 10 mg/L benzene at the 36th hour. At the end of the 6th day, MN frequency was 0.32% in the control group, 0.92% in the 25 mg/L TMX group, 2.6% in the 75 mg/L TMX group, and 7.06% in the benzene group. In histological examinations of the tissues, liver tissue in the control group was observed to have a normal histologic structure, while slight degeneration was observed in the 25 mg/L thiamethoxam group. In 75 mg/L thiamethoxam group, degeneration, necrosis areas and steatosis were observed in hepatocytes in liver sections. In the positive control group, cell infiltration and bile duct degeneration were observed in addition to hepatocyte degeneration, necrosis, and steatosis. As a result of the data obtained from the study, it was determined that the dose increase in thiamethoxam administration led to an increase in the frequency of erythrocytes with micronuclei compared to the control group. In addition, it was understood that there was an increase in the frequency and severity of lesions seen in the liver tissue with thiamethoxam dose increase. Thiamethoxam was observed to be genotoxic and cytotoxic in aquatic organisms, as well.**

Keywords— **Oncorhynchus mykiss, Thiamethoxam, Genotoxicity, Micronucleus, Liver**

I. INTRODUCTION

In the face of a rapidly increasing world population, the failure to provide sufficient foodstuffs is a serious problem. Thus, it is necessary to increase the production and yield of foodstuffs and to take measures to prevent food losses. In order to increase agricultural productivity, it has become necessary to combat diseases, pests and weeds that cause significant losses in agricultural products. In this struggle, chemical compounds known as pesticides are used [1-3]. Pesticides are chemicals used to kill pests that live on or near people, animals, and plants, and they can also reduce the nutritional value of foods. These pests are parasites carrying various diseases, agricultural and crop insect pests, weeds and fungi, as well as flying and walking creatures such as flies, lice, fleas, ticks, scabies, and cockroaches in humans, animals, environment, and shelters [2, 4, 5]. Since different environmental pollutants/chemicals from different sources are easily and directly mixed in water bodies, organisms in aquatic ecosystems are known to face numerous problems compared to terrestrial animals [6, 7]. The hazard use of chemicals, particularly pesticides in agriculture, pollutes the environment and endangers all aquatic species, including fish. Fishing activities and agricultural activities negatively affect water quality. Therefore, the habitat is destroyed and threatens the future of inland water ecosystems [6, 7].

Micronuclei (MN) are formations that occur when chromosomes or fragments of acentric chromosomes/ chromatids that remain during anaphase of dividing cells and cannot be incorporated into the main nucleus in telophase remain outside the nucleus. They are viewed as small nucleus-like structures surrounded by the nuclear membrane and located in the cytoplasm. The Micronucleus Test is an important criterion to show the toxicity of chemical and physical agents depending on the presence and

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frequency of micronuclei on the DNA chain in the human body, which are the result of genotoxic damage and are mostly used to diagnose diseases such as cancer [8-10]. An increase in the number of MNs is considered as a marker of numerical and structural chromosomal abnormalities in cells caused by various agents, including ionizing radiation, drugs and other chemicals with genotoxic and carcinogenic potential [8, 11, 12].

The aim of the present study is to measure the genotoxicity of thiamethoxam, a neonicotinoid insecticide group, which is one of the pesticides that adversely affects environmental health and aquatic ecosystem, in rainbow trout in aquatic ecosystem, outside target organisms by micronucleus test.

II. MATERIAL AND METHOD

A. Chemical

For the purposes of the study, trademarked chemicals were used. Cayenne Hektaş (active substances 350 g/L thiamethoxam) (Natural chemicals and agricultural chemicals Inc.) was used as thiamethoxam (TMX). In fish, MS-222 (Tricaine methanesulfonate) (Sigma-Aldrich, CAS: 886-86-2) was used as an anesthetic.

B. Experimental Design

The present study was approved by the Local Ethics Committee of Iskenderun Technical University Aquaculture Animal Experiments (approval no: ISTE-SUHADYEK/2024-135336). Rainbow trout, *O. mykiss* (145.10±23.56 g and 20.10±1.23 cm), used as research material, was obtained from Mazmanlı (Hassa/Hatay) trout production farm. The fish were taken from the rearing ponds and transferred to the aquarium conditions in the laboratory for adaptation to the new environment for about one week. Air pumps were connected to the water in the aquariums and the oxygen level in the tanks was increased with an air stone. Fish were fed twice a day (morning and evening) with commercial dry pellets (12 mg/kg fish) and were not fed in the 24 hours before the experiments. Rainbow trout were fed daily at 2% of their live weight (Çamlı BioAqua® sinking feed for trout, 46% crude protein, 16% crude fat and 2% crude cellulose). According to the 'Experiments in a refreshed environment' procedure, water and thiamethoxam were repeatedly applied to the water every day to remove food and fecal wastes from the environment. During the adaptation period, the temperature (°C) was applied as 13-20±1, pH 7.3-7.4, dissolved oxygen ≥7 mg/L and total hardness ($CaCO_3$) (mg/L) 150-170 and a photoperiod of 12:12 hours to figure out the physicochemical properties of water. Experiments were carried out in 2 replicates. At the end of the adaptation period, sublethal concentrations of 25 and 75 mg/L were selected according to the doses specified in the literature [13-19] and toxicity studies were conducted using these doses. The fish were divided into 4 groups; the control group was not subjected to any treatment and the other groups were exposed to 2 sublethal doses of the chemical (25 and 75 mg/L) and benzene (10 mg/l) as a positive control group for 6 days (n=10, number of groups using F tests ANOVA: repeated measures, within-between interactions A priori: compute required sample size-given α, power, and effect size in GPower 3.1 program: 4 (4 concentrations x 2 replicates) and number of fist to be used was determined through power analysis conducted at α=0.05, power=0.88 and f=0.40 [20]. The concentrations selected according to the LC_{50} value of Thiamethoxam given in the literature were prepared by dissolving the stock solutions in the test water and diluted to obtain the concentrations determined in the aquariums. In order to prevent changes in pesticide concentration due to reasons such as evaporation and absorption, fish were transferred to recently pesticide-treated aquariums every day [21]. The behavior of the fish was monitored regularly during the experiments. After 6 days of treatment, fish from each group were transferred to aquariums containing MS222 (50 mg/L, buffered with 100 mg/L $NaHCO_3$) and anesthetized [22]. After anesthesia, the length and weight of the fish were determined, blood samples were taken from the caudal vein and euthanized by cervical dislocation. Tissue samples were taken for histopathological analysis (on 6th day). In the study, weight measurements of the fish were made with a digital scale with precision of 0.1 g, and their total length was measured with a measuring ruler with divisions of 1 mm.

In the present study, 4 groups were formed;

1. Control group: This group includes *Oncorhynchus mykiss* that were not treated with any substance, but only had water periodically exchanged like the other groups in terms of exposure to the same equivalent stress conditions as the other groups.

2. 25 mg/L Thiamethoxam group: Fish in this group were treated with 25 mg/L thiamethoxam-based pesticide.

3. 75 mg/L Thiamethoxam group: Fish in this group were treated with 75 mg/L thiamethoxam-based pesticide.

4. Positive control (benzene, 10 mg/L) group: Fish in this group were treated with 10 mg/L benzene.

C. Micronucleus Test

Before starting the experiments for micronucleus test, the aquariums were washed thoroughly to ensure that nothing was left in them. In the aquariums, 25 mg/L thiamethoxam, 75 mg/L thiamethoxam and 10 mg/L benzene were placed, respectively and 10 fish were placed in each aquarium. The average weight of the fish was 145.10±23.56 g and their length was 20.10±1.23 cm. Control group was formed by placing 5 fish in one aquarium without any substance. In addition, 10 mg/L benzene was used as positive control. Ten fish were placed in the aquarium where the positive control was applied and blood samples were taken from the fish at the end of 36th hour and 6th day, 1000 erythrocyte cells were counted in each preparation and the number of micronucleated cells was determined and the average of the preparations was taken. For the determination of micronucleus frequency, 80 fish (40 fish x 2 replicates) were used. The lighting in the experimental environment was adjusted to 12 hours of light and 12 hours of darkness in accordance with the natural period. At different time intervals (36th hour and 6th day), blood samples were taken from caudal vein of 5 fish from control and experimental groups and 5 preparations were prepared from each blood sample. The preparations were air dried and fixed in methanol for 20 minutes. The preparations were air dried again and stained in 10% giemsa-phosphate buffer solution for 15 minutes. At the end of this period, the preparations were removed from the solution, washed with phosphate buffer solution and allowed to air dry. The

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preparations were examined under a light microscope at x1000 magnification and the number of micronucleated cells was determined by counting 1000 erythrocyte cells in each preparation [10, 11, 23]. In erythrocytes with uniform nuclei in the preparation, round particles separate from the main nucleus were evaluated as micronuclei. In order not to confuse MN with dye particles, the nucleus and MN were checked with microvida and the disappearance and reappearance of the nucleus and MN together ensured this distinction.

D. Histopathological Analysis

At the end of the study, the fish were dissected on the 6th day of thiamethoxam exposure and their tissues were taken for histopathological analysis. Tissue samples taken from the subjects were placed in 10% buffered formalin solution and fixed for 48 hours, then kept overnight in running water to eliminate the fixation solution. They were then passed through alcohol and xylol series and kept in soft paraffin (46-48 °C) and hard paraffin (56-58 °C) in a drying oven for at least two hours and then embedded in paraffin in cassettes. Sections of 4-5 µm thickness were taken from these paraffin blocks, stained with hematoxylin-eosin (HE) method and evaluated under a light microscope (Zeiss Axio Imager 2) to determine possible pathological changes and the toxic effect of the substance on aquatic organisms [24]. Thus, the histopathological findings detected in the tissues were compared with the control group and graded as (-) none, (+) mild, (++) moderate and (++++) severe.

E. Data Analysis

The findings of the present study were evaluated using SPSS (Statistical Package for Social Sciences) 22.0 packaged software. The quantitative values obtained were analyzed by one-way ANOVA in the statistical data program. Statistical significance between groups was evaluated by Tukey HSD multiple comparison test. The value of $p<0.05$ was used as significance threshold. Results are given as mean ± standard error.

III. RESULTS

A. Results of Micronucleus Analysis

In the current study, thiamethoxam, a neonicotinoid pesticide, which is thought to have toxicity effect on rainbow trout (Oncorhynchus mykiss), one of the indicator organisms of aquatic ecosystem, was used. Thus, the fish were exposed to 25 and 75 mg/L thiamethoxam concentrations, which were determined based on LC50 doses for experimental sub-acute modeling, for 36 hours and 6 days at the end of a one-week period for adaptation after being brought to laboratory conditions, and at the end of this period, preparations were prepared with blood samples taken from the fish and evaluated by micronucleus counts under light microscope. Micronucleus (MN) frequencies were determined by examining the stained preparations under a microscope, 5 preparations were prepared with the blood obtained from each fish and 1000 erythrocytes were counted from each preparation and the average of the erythrocytes carrying MN in these erythrocytes (in 1000 cells) was determined.

According to the study procedure, blood samples were taken from rainbow trout at specified times and the fish exposed to thiamethoxam and the fish in the control groups were compared in terms of the frequency of micronucleus formation. In this way, the genotoxic potential of thiamethoxam was tried to be revealed by micronucleus test. Since benzene was only used as positive control for the genotoxicity test, blood was also taken from the fish in this group in order to compare the results of the experiment, and then micronucleus test was performed and MN frequency was evaluated in the preparations prepared from these samples (Figure 1, Table 1).

Table 1: Micronucleus frequency in erythrocytes from control and thiamethoxam treated groups.

Groups	36th hour (Mean±SE)	Lower and upper limits at confidence interval of 95%	6th day (Mean±SE)	Lower and upper limits at confidence interval of 95%
Control	3.00±0.44^{d}	1.75±4.24	3.20±0.37^{c}	2.16±4.23
25 mg/L TMX	7.80±0.73^{c}	5.75±9.84	9.20±0.73^{c}	7.15±11.24
75 mg/L TMX	17.20±0.66^{b}	15.35±19.04	26.0±1.41^{b}	22.07±29.92
Benzene (10 mg/L)	58.8±1.85^{a}	53.65±63.94	70.6±3.04^{a}	62.15±79.04

*: $p<0.05$=Statistically significant difference, a,b,c: Difference between group means with different letters in the same column is significant. n: number of subjects in the group, SE: Standard error.

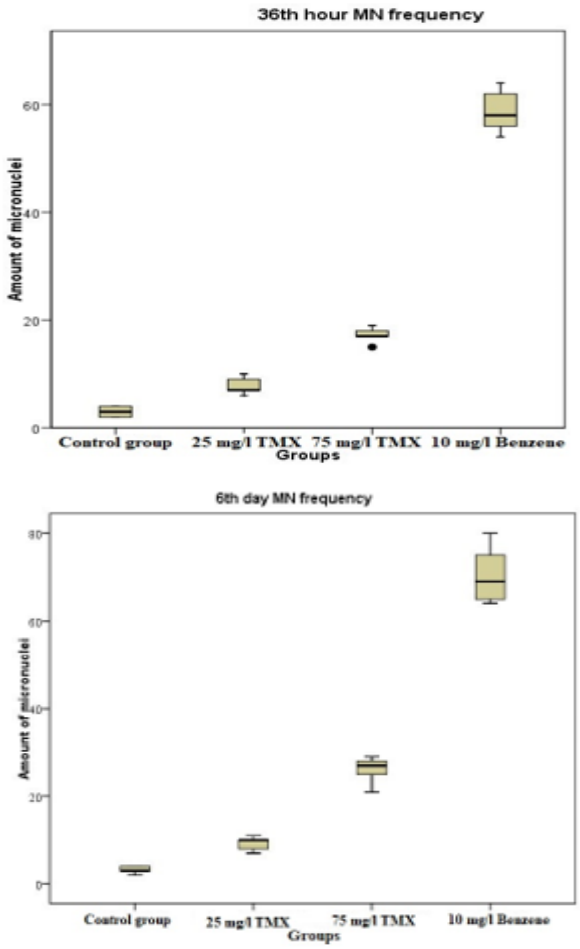

Figure 1: Box plot view of micronucleus levels in erythrocytes of rainbow trout treated with subacute thiamethoxam.

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At the end of 36th hour, the average micronucleated erythrocyte rate was 0.3% according to the counting results of each 1000 erythrocyte cells obtained from the fish materials in the control group (Figure 2). In the preparations obtained from fish samples treated with 25 mg/L thiamethoxam dose, the rate of micronucleated erythrocytes per 1000 erythrocyte cells was found to be 0.78%. In the group treated with 75 mg/L thiamethoxam, the incidence in 1000 erythrocytes was found to be 1.72%. The rate in 1000 erythrocytes obtained from the group treated with 10 mg/L benzene as positive control was 5.88%. At the end of the 6th day, the MN frequency was 0.32% in the control group, 0.92% in the 25 mg/L TMX group, 2.6% in the 75 mg/L TMX group and 7.06% in the benzene group. At 36th hour, the MN rates in erythrocytes obtained from caudal vein blood samples were statistically different from each other in all groups ($p<0.05$). When compared with the control group, it was observed that the rate of erythrocytes with micronuclei increased with the increase in thiamethoxam dose. On 6th day, the statistical difference between the control group and 25 mg/L thiamethoxam group was not significant ($p>0.05$) in the comparison of MN rates in erythrocytes obtained from caudal vein blood samples. Positive control group was significantly different from all other groups ($p<0.05$). Again, the data obtained from the 75 mg/L thiamethoxam group were significantly different from the data obtained from the control and 25 mg/L thiamethoxam groups, respectively ($p<0.05$). When the importance of the time factor in the application was examined, the MN frequency on the 6th day increased by 6.06% compared to 36th hour in the control group, while this increase was 17.9% in the 25 mg/L thiamethoxam group, 15.11% in the 75 mg/L thiamethoxam group, and 12% in the positive control group. These data showed that there was an increase in the frequency of erythrocytes with micronuclei compared to the control group depending on the dose increase and time (Figure 3).

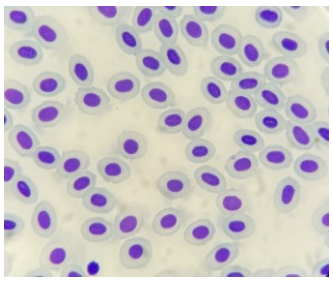
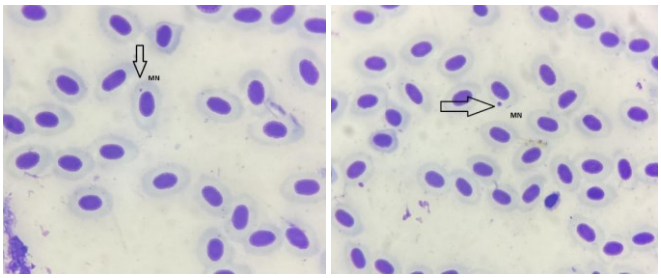


Figure 2: Nucleus image in fish erythrocytes of the control group.



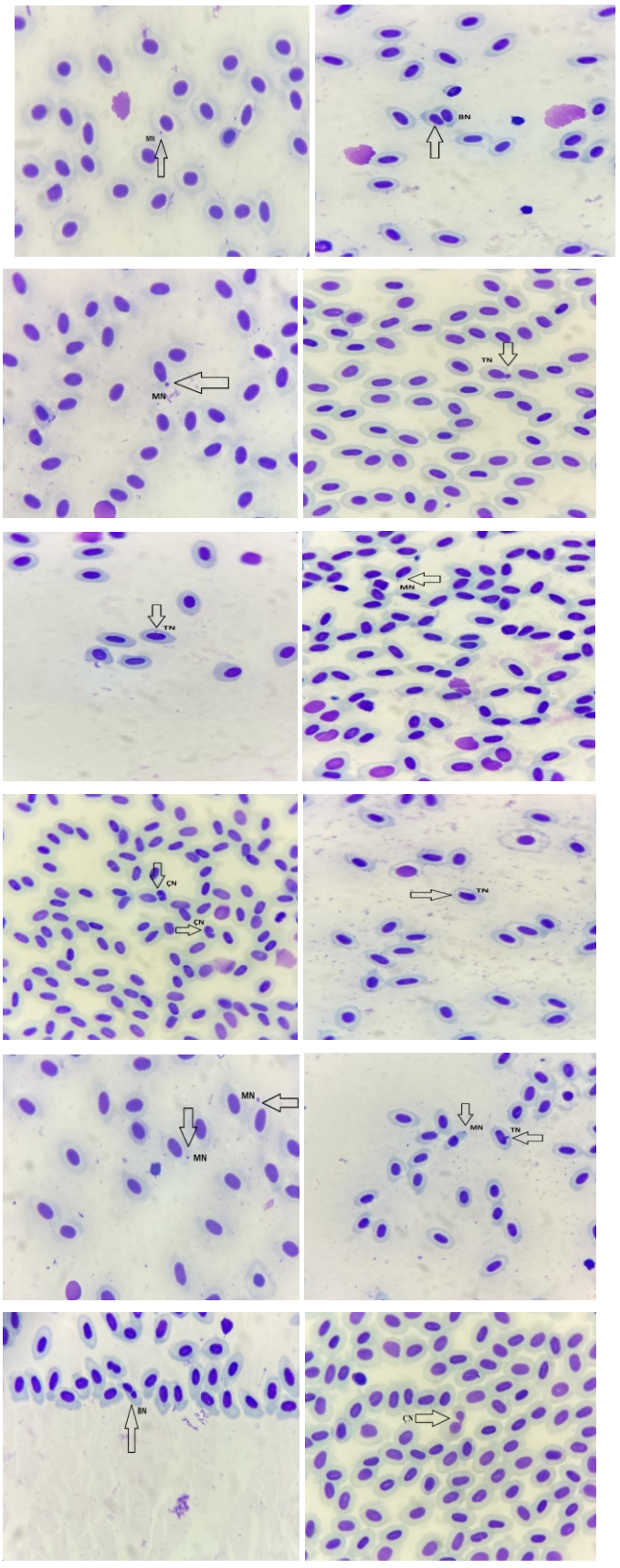


Figure 3: Nucleus morphological irregularities and micronucleus appearance in erythrocytes obtained from thiamethoxam-treated groups (25 mg/L and 75 mg/L) and 10 mg/L benzene group as positive control. MN: micronucleus, TN: blebbed nucleus, BN: binucleated nucleus, CN: notched nucleus.

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B. Histopathological findings

At the end of the experimental procedure, the liver tissues taken on the 6th day were embedded in paraffin after the fixation and tissue follow-up stages and 5 µm serial sections taken from the blocks obtained with the help of a microtome were stained with hematoxylin-eosin and examined at the light microscopic level. As a result of microscopic examination of the liver sections, it was observed that the sinusoids between the cords formed by hepatocytes starting from the vena centralis to the portal region and the central vein structure from which they opened were normal in the control group. Hepatocyte arrangement was regular in the control group (Figure 4a). The appearance of the 25 mg/L thiamethoxam-treated liver sections in terms of lesion was similar to the control group. As a result of the examination of the preparations, mild degeneration of hepatocytes was observed in some areas (Figure 4b). In the sections obtained from 75 mg/L thiamethoxam treated group, degeneration, necrosis areas and steatosis were observed in hepatocytes. Degeneration was also observed in the bile duct (Figure 4c). In the preparations obtained from the group treated with 10 mg/L benzene as positive control group, degeneration, necrosis areas and steatosis were observed in hepatocytes. In addition to cell infiltration, bile duct degeneration was observed at some points (Figure 4d). As the dose of TMX used in the study increased, lesion severity and frequency also increased (Table 2).

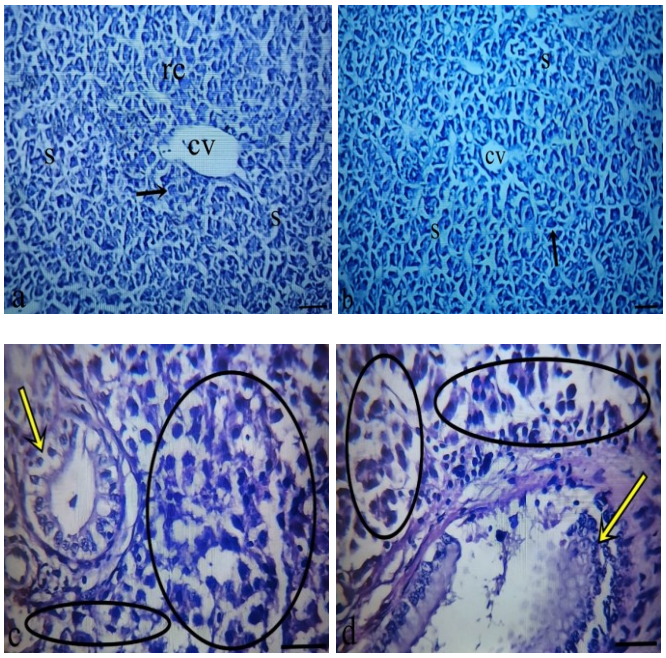

Figure 4: Liver tissue sections of Oncorhynchus mykiss exposed to different doses of thiamethoxam and 10 mg/l benzene as positive control. a. Control group. b. 25 mg/L thiamethoxam group. c. 75 mg/L thiamethoxam group. d. 10 mg/L benzene treated group as positive control. Central vein (cv), hepatocyte (black arrow), sinusoid (s), remark cords (rc), hepatocyte degeneration, necrosis areas and steatosis (black circle), bile duct degeneration (yellow arrow), Magnification: x100 (a, b), x400 (c, d), Bar: 50 µm. H&E.

Table 2: Tissue change grading of histopathological lesions in liver tissue of Oncorhynchus mykiss.

Liver lesions	Control group	Thiamethoxam dose groups		Positive control group (10 mg/L benzene)
		25 mg/L	75 mg/L	
Hepatocellular degeneration	-	+	++	+++
Bile duct degeneration	-	-	++	+++
Necrosis and steatosis	-	-	++	+++

-: no abnormality, +: low frequency of abnormality, ++: moderate frequency of abnormality, +++: high frequency of abnormality.

IV. DISCUSSION AND CONCLUSION

In the modern world, human health and the environment have gained great importance, and controlling the negative effects of environmental pollutants, including pesticides, has become mandatory. In today's world, where our export of a significant amount of agricultural products to many developed countries continues, it is necessary to use pesticides in a very conscious and controlled manner at the standards of developed countries in order to protect health, the environment and our economy. Like many other environmental pollutants, water is one of the main pathways for pesticides to reach different parts of the environment from their application areas. Pesticides show their greatest environmental impact when they contaminate aquatic ecosystems. Today, especially the irreversible use of water reserves has led to an intensification of studies on aquatic ecosystems. Several studies have shown that fish can be effectively used to test genotoxic effects of pesticides in aquatic environments in the laboratory setting [25-27]. Çavaş and Ergene-Gözükara reported that lambda cyhalorthin, an insecticide, significantly increased erythrocyte micronucleus frequencies in Garra rufa (Cyprinidae) [28]. Gül et al., investigated the genotoxic effects of NaOCl on Orthrias angorae in their study. As a result of the study, they reported that NaOCl dose-dependently increased the number of micronuclei and may be genotoxic [23]. In another study, they examined endosulfan, which is widely used in agricultural areas, on Sparus aurata (Perciformes) under laboratory conditions. They observed significant increases in erythrocyte micronucleus and erythrocyte morphological nucleus irregularity frequencies depending on the application dose and duration [29]. Talapatra et al., exposed Heteropneustes fossilis individuals to a solution containing 5, 10, and 30 mg/L Zn and examined MN formation at the end of 24, 48, 72, and 96 hours. They reported that there was a significant increase in MN frequency at all concentrations compared to the control groups, with the highest significant increase at the 30 mg/L concentration at the end of 72 and 96 hours [30]. Rodrigez et al., applied imidacloprid at doses of 250, 125 and 62.5 µg/L to O. niloticus and determined genotoxic damage by comet assay. They found that DNA damage and consequently MN and nuclear anomaly formation increased with increasing concentration [31]. In parallel with the studies of other researchers, the results of the current study also showed an increase in micronucleus frequency due to the increase in

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pesticide dose. Micronucleus formation, which is a marker for the detection of genotoxic potential, can also be examined in other organisms other than fish. In a study, it was reported that 3-Methylcholanthrene caused genotoxicity in mouse bone marrow cells and cysteamine reduced this genotoxic effect caused by 3-Methylcholanthrene [32]. In another study, it was reported that acetamiprid increased micronucleus and chromosomal aberration in mouse bone marrow cells and had a genotoxic cytotoxic effect [33]. In their study, Gül et al., reported that U. dioica essential oil increased the rate of chromosomal aberrations, micronucleus formation, and cytotoxicity in human lymphocyte cells [34]. A study reported that nickel sulfate increased micronucleus frequency in rat bone marrow cells and bromelain, which was used as a protective agent against this increase, did not decrease micronucleus frequency [35]. In addition to their genotoxic character, pesticides also have a cytotoxic effect on living tissues due to the formation of oxidative stress. The liver is an important organ for assessing the effects of pollutants as it stores chemicals. The liver is also considered to be the main site for biotransformation of chemicals, which reduces their toxicity and facilitates their excretion [36]. Nur et al., reported irregular secondary lamellae, edema, epithelial hyperplasia in chloride cells and secondary lamellae, swelling in chloride cells, necrosis and degeneration in secondary lamellae in gills in rainbow trout due to glyphosate-based exposure [37]. Pyknosis, hepatocellular degeneration, hypertrophy of hepatocytes, hemorrhage, sinusoidal enlargement and leukocyte infiltration were reported in the liver due to oxytetracycline exposure in rainbow trout [38]. In carp treated with azadirachtin pesticide, vacuolar degeneration, hydropic degeneration and infiltration were observed in the sinusoidal area of the liver [39]. Pathological changes were reported in liver tissue in C. carpio exposed to atrazine and chlorpyrifos [40] and L. rohita exposed to profenofos [41]. Oxidative stress is a pathological mechanism that causes both structural and functional abnormalities in the liver [42]. In a study by Singh (2013), vascular occlusion, necrosis, diffuse hyperemia and vacuolization were observed in the liver of Cyprinus carpio due to dimethoate treatment [43]. The results of the MN test, which we preferred due to its fastness, reliability and low cost in genotoxicity analyses, and the cytotoxic findings on liver tissue showed that thiamethoxam had genotoxic and cytotoxic characters for organisms other than the target organisms and especially for organisms living in the aquatic ecosystem.

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Conflict of Interests/Competing Interests: None.

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