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Research Article

Investigation of Genotoxic Effects of Imazamox Herbicide on Zebrafish (*Danio rerio*) Using Micronucleus Assay

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ABSTRACT

Imazamox is an imidazolinone herbicide; it is an acetalactacetase (ALS) / acetylhydroxyacid (AHAC) inhibitor and is widely used for weed control. In this study, the genotoxic effect of the commercial formulation of imazamox was investigated in zebrafish peripheral blood cells *in vivo* using the micronucleus (MN) method. Fishes were treated at three different concentrations of imazamox (3, 6, 12 mg L^{-1}) for 24, 48, 72 and 96 hours. At the highest concentration (12 mg L^{-1}), it was observed that all fish died in all treatment periods. According to 24, 48 and 96 h treatment results; compared to the negative control, it was determined that the MN frequency increased as the concentration increased. These results show us that imazamox herbicide may have genotoxic potential for aquatic organisms. Further studies should be conducted to investigate the mechanisms that are effective in the genotoxicity of imazamox.

Keywords: *Imazamox, Imidazolinon herbicide, Micronucleus formation*

İmazamoks Herbisidinin Zebra Balığı (*Danio rerio*) Üzerine Olan Genotoksik Etkisinin Mikronukleus Yöntemi Kullanılarak Araştırılması

ÖZ

İmazamoks (IMA), imidazolinon grubunda yer alan, asetalaktatsentaz (ALS) / asetilhidroksiasit (AHAS) inhibitörü olan ve yabani ot kontrolü için yaygın olarak kullanılan bir herbisittir**.** Bu çalışmada imazamoks'un ticari formülasyonunun genotoksik etkisi zebra balığı periferal kan hücrelerinde *in vivo* olarak mikronukleus yöntemi kullanılarak araştırıldı. Balıklar imazamoks'un üç farklı konsantrasyonuna (3, 6, 12 mg L^{-1}) 24, 48, 72 ve 96 saatlik boyunca muamele edildiler. En yüksek konsantrasyonda (12 mg L^{-1}) tüm muamele sürelerinde balıkların tamamının öldüğü gözlendi. 24, 48, 72 ve 96 saatlik muamele sonuçları; negatif kontrolle karşılaştırıldığında, konsantrasyon arttıkça MN(mikronükleus) frekansında artış olduğu belirlendi. Bu sonuçlar bize imazamoks herbisitinin sucul canlılar için genoksik potansiyale sahip olabileceğini göstermektedir. İmazamoks'un genotoksisite oluşturmasında etkili olan mekanizmaların araştırılmasına yönelik ileri çalışmalar yapılmalıdır.

Anahtar Kelimeler: *İmazamoks, İmidazolinon herbisiti, Mikronukleus oluşumu*

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I. INTRODUCTION

Excessive human population growth has increased the need for food since the last century. Therefore, the use of pesticides has become necessary in recent modern agricultural activities in order to increase product yield. However, uncontrolled pesticide use causes environmental pollution in addition to potentially reaching excessive levels that lead to toxic effects in both human and animal populations. Additionally, pesticides such as insecticides, herbicides can poison and kill a wide range of non-target organisms by contamination of irrigation and natural waters, and thus are considered a threat for aquatic ecosystems [1].

Herbicides are chemicals that are used to kill or control weeds. Since herbicides prevent the synthesis of leucine, isoleucine and valine amino acids from branched amino acid chains in sensitive plant species, plant growth stops and then death occurs [2]. There are many studies on the genotoxicity of herbicides on different test systems [3-8].

Imazamox (IMA) is an imidazolinone group herbicide widely used for weed control in the cultivation of foods such as soybeans, peas, alfalfa, beans, corn and peanuts. The environmental protection agency (EPA) has identified a family of pesticides, including IMA, as a potential water pollutant. Because IMA dissolves well in water, it is a prime factor for water pollution. In *in vivo* and *in vitro* investigations, USEPA (U.S. Environmental Protection Agency) [9], FAO **(**Food And Agriculture Organisation **)**/WHO(World Health Organisation**)** [10] and EFSA (European Food Safety Authority) [11] stated that IMA was neither mutagenic nor genotoxic. Contrary to these statements', previous studies' conducted on IMA have shown it to have positive results on mutagenic and recombinogenic activity on *Drosophila melanogaster* [12] and hepatic and pancreatic toxic effects in rats [13]. While it has been demonstrated that IMA poses a low risk to aquatic invertebrates [11], there is however no data regarding its genotoxicity effect on zebrafish.

There are many methods used to investigate the genotoxic effects of chemicals. One of them is theMN method, which is widely used to evaluate both aneugenic and clastogenic effects together. Micronuclei occur due to chromosome breakage and gene differentiation during mitosis; they are small nucleolus-like formations in the cytoplasm consisting of whole chromosomes or irregular chromosome pieces and have the same characteristics as the nucleus. The numerical increase in the amount of MN is the most important indicator of genetic damage. Due to this feature, the MN test is a widely used test to detect DNA damage in aquatic organisms.

Although there are many studies on the genotoxic effects of polluted environments, bivalves and fish are frequently used in these studies. The use of fish is appropriate because of their role in the aquatic food chain and bioindicators. Zebrafish, *Danio rerio*, is a freshwater fish species belonging to the family Danionidae. Recently, their use has increased greatly in scientific research due to their suitability to serve as a functional model, with subsequent advantages for aquatic toxicity studies. First of all, they are easy to feed and are sensitive to chemicals. They also have a short reproductive cycle and rapid development. Therefore, they are an ideal ecotoxicological model to observe the effects of chemicals on survival, growth and reproduction [14].

In this study, we aimed to reveal the genotoxic effects of IMA on peripheral blood cells of *Danio rerio* by using the MN assay.

II. MATERIAL AND METHODS

A. TEST CHEMICALS

In the present study, trade formulation of IMA (Simirna®-X) (CAS No: 114311-32-9) was used as the test material. The chemical structure of IMA is given in Figure 1. Giemsa (CAS no. 51811–82–6) and Methanol (CAS no. 67-56-1) were purchased from Merck® and Sigma®, respectively.

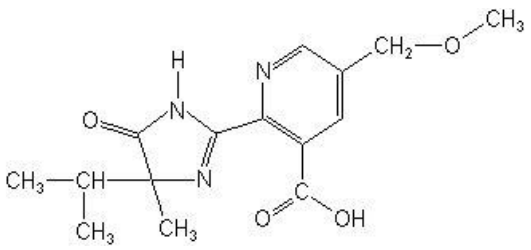

Figure 1. *Imazamox Chemical Structure [15]*

B. ANIMALS AND EXPOSURE PROTOCOL

D. rerio used in the study were obtained from a licensed commercial company. Before the experiment, the fish were kept in the experimental conditions for 2 weeks without any IMA application for them to acclimate to the experimental environment. During the study, the fish were kept in a 50 L glass aquarium with a light period of 12:12 hours day and night, the water and ambient temperature was fixed at 27 ± 1 °C with thermostats, and aquariums were continuously oxygenated with air motors. Fish food was given once a day and filtered tap water (rested for 24 hours prior administration) was used. The experiment was approved by the Ethics Committee of Duzce University in Turkey (No: 2021/03/10).

The LC_{50} value of IMA for fish is >122 mg L^{-1} [16]. Therefore, in our study, 3 different doses of IMA (LC_{50}/10, LC_{50}/20, and LC_{50}/40) were administered to fish for 24, 48, 72 and 96 hours. Dechlorinated tap water was used as the negative control.

C. MICRONUCLEUS ASSAY

At the end of the 24, 48, 72 and 96 treatments, blood samples were taken from the ventral main artery with heparinized syringes under anesthesia. Blood samples from each sample were dripped onto clean numbered slides and spread rapidly to form a thin layer on the surface. Three blood preparations were prepared for each sample and left to dry. Dried blood preparations were fixed in 98% ethanol for 20 minutes, and then the dried slides were stained in 5% Giemsa solution for 15 minutes. The dried preparations were turned into permanent preparations and examined under light microscope. In the evaluation of micronucleus formation, a total of 3000 cells were counted for each sample. The micronucleus percentage was calculated according to the formula % MN = number of cells containing MN/total number of cells.

D. STATISTICAL ANALYSIS

The normal distribution of the data obtained from the micronucleus test was checked with the Kolmogorov-Smirnov test. Evaluation of normally distributed data groups was done with one way ANOVA.

III. RESULTS AND DISCUSSION

When the MN frequencies obtained from the treatment groups were compared with the negative control, it was seen that the MN formation increased depending on the concentration. After 24, 48 and 96 hours of IMA exposure, MN frequencies in the treatment groups were found to increase compared to the negative control group (Table 1). Accordingly, the highest MN frequency was found to be 4.75 for 24 h treatment and at 6 mg L^{-1} concentration of IMA ($p<0.05$).

Table 1. *Frequency of micronucleus in peripheral blood cells of D. rerio exposed to IMA*

	MN-24	**MN-48**	**MN-72**	**MN-96**
Negative control	0.50±0.22	0.33±0.21	0.16±0.16	0.16±0.16
IMA (3 mg L^{-1})	1.00±0.36	1.00±0.70	1.16±0.47	1.16±0.33
IMA (6 mg L^{-1})	4.75±0.70***	3.00±1.51	1.00±0.26	2.50±0.50**
IMA (12 mg L^{-1})	ND	ND	ND	ND

ND: Not determined due to death

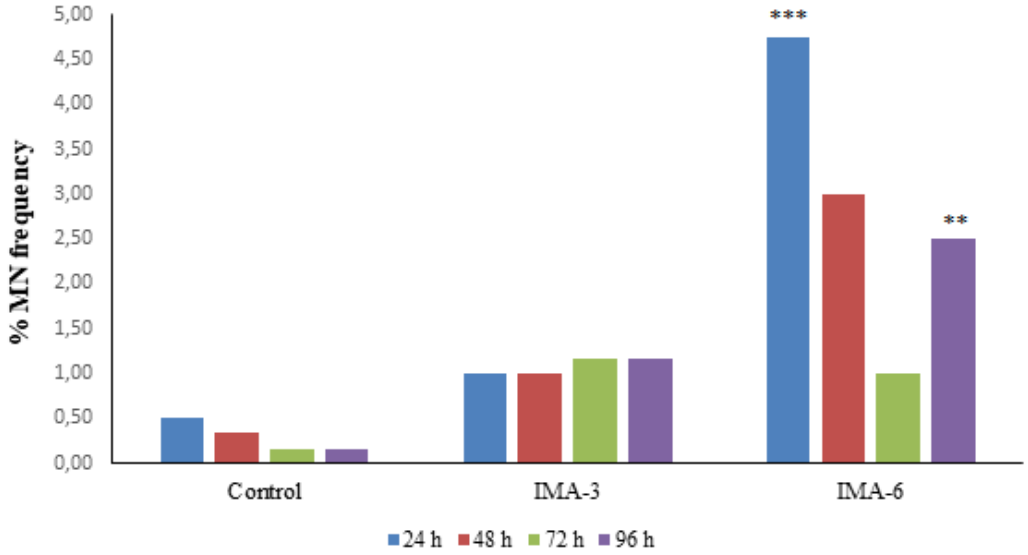

Figure 1. *Percentage of micronucleated erythrocytes after exposure to different concentrations of IMA for 24, 48, 72 and 96 hours in peripheral blood cells of D. rerio.*

The frequency of MN is shown in Figure1 according to the concentrations and exposure periods. In the negative control, a decrease was observed according to the exposure time. At 3 mg L^{-1} concentration of IMA, the frequency of MN increased at 72 and 96 hours, but these increases were not significant. The frequency of MN peaked at 24 hours at the 6 mg L^{-1} concentration of IMA, but it decreased at 48 and 72 hours and then increased again at 96 hours. These reductions at 48 and 72 h were not statistically significant. It was determined that the increase at the 96 h was significant. The increases at 24 and 96 hours differ significantly from the same exposure at 48 and 72 hours. At the highest concentration of IMA (12 mg L^{-1}), it was observed that all fish died in all treatment periods.

Our study is the first report to reveal the genotoxicity of IMA on peripheral blood cells of *Danio rerio* and IMA was genotoxic at 6 mg L^{-1} concentration for 24 and 96h, and at 12 mg L^{-1} for all treatment periods. There are a few studies on the genotoxicity of IMA. Demirci et al. [17] indicated that IMA cleaved plasmid DNA in the presence and absence of Cu(II) ions. Additionally, EFSA [11] reported that IMA has a low risk to soil and aquatic organisms. Fragiorge et al. [12] stated that high doses of IMA (10.0 and 20.0 mM) have a genotoxic potential in *D. melanogaster*. These results are in agreement with results of our present study on the positive genotoxicity of IMA.

Imazamox (IMA) is an imidazolinone group herbicide. Imidazolinone group herbicides are effective as they block the acetolactate synthase (ALS) and acetohydroxy synthase (AHAS) enzymes in chlorophyll. When they are applied to a plant, the synthesis of isoleucine, leucine and valine amino acids in the plant stops, and since this amino acid is not present, many proteins cannot be synthesized and the plant dies.

The results show that high concentrations of commercial formulation of IMA increased micronucleus frequency in erythrocytes of *Danio rerio* and induced genotoxic effects. Excessive use of herbicides can

cause serious ecotoxicological problems by causing undesirable effects on humans and the environment in the short or long term through soil and water resources.

IV. CONCLUSION

In conclusion, the present study used micronucleus assay to evaluate that a commercial formulation of IMA may be genotoxic to zebrafish peripheral blood cells. Therefore, dose selection should be considered when using this herbicide. The genotoxic and cytotoxic activity of imazamox on different test systems needs to be investigated using molecular methods.

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