

Effect of Fungicide Toxicity on Apoptosis, DNA Damage, and Antioxidant Enzymes in Van Fish

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ABSTRACT

The extensive use of tebuconazole, an azole group fungicide, causes undesirable toxicity in non-targeted organisms, including fish in aquatic environments. We investigated metabolic responses by observing apoptosis (caspase-3), DNA damage (8-hydroxy-2-deoxyguanosine (8-OHdG)), malondialdehyde (MDA), and antioxidants (SOD, CAT, GSH-Px) activity in Van fish kidney and muscle tissue after 24, 48, 72, and 96 h of tebuconazole exposure at a concentration of 2.5 mg/L. The obtained results indicated that caspase-3, 8-OHdG, and MDA levels significantly increased compared to the control ($p < 0.05$). The kidney and muscle tissues indicated a significant decrease in antioxidant enzyme activities (superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px)) compared to the control ($p < 0.05$). Therefore, these data may reflect one of the molecular pathways that play a role in tebuconazole toxicity.

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INTRODUCTION

The negative effects of the unconscious and uncontrolled use of pesticides on human health and the environment should not be ignored. Environmental residues of the pesticides used create pollution in soil and water. Corruption, especially in natural water resources, has reached dimensions that will affect the continuity of water resources. Thus, water's physical, chemical, and biological properties have changed negatively (Ramírez-Santana *et al.*, 2020). Tebuconazole, which is included in the azoles group, is used as a fungicide against the fungal threat in rice fields. Systemically effective tebuconazole also prevents the synthesis of ergosterol in fungi and may cause toxic effects on organisms in the aquatic environment for a long time (Bayer Crop Science Limited, 2005).

It has been reported that azole compounds cause possible death in non-target organisms (farm animals, bees, birds, fish, and invertebrates) and cause changes in the structure of the ecosystem and the number of species in the long term (Zhang *et al.*, 2020; Yeltekin and Sağlam, 2019).

Pesticides; it is also known that it causes significant environmental pollution by spreading to the natural environment by passing into the air, soil, water, and plants. Pesticides, by creating oxidative stress in living things, cause free radicals in their structure over time. When the number of free radicals increases to a level above the level that antioxidants can tolerate, tissues begin to be damaged. Reactive oxygen metabolites are formed in the xenobiotic metabolism, phagocytic activation, various synthesis and degradation reactions, especially in mitochondrial electron transport, and the oxidative stress that develops as a result of the shift of the prooxidant/antioxidant balance in favor of prooxidants damages biomolecules with various mechanisms (Li *et al.*, 2011). Reactive oxygen metabolites are formed mainly in mitochondrial electron transport, xenobiotic metabolism, phagocytic activation, different synthesis and degradation reactions, and the oxidative stress that develops due to the shift of the prooxidant/antioxidant balance in favor of prooxidants damages biomolecules with various mechanisms. Reactive oxygen species (ROS) originating from environmental pollutants cause structural and functional changes in the cells of aquatic organisms and cause biochemical parameter changes. Increased formation of reactive oxygen metabolites, decreased antioxidant enzyme levels, and defects in DNA repair mechanisms lead to increased oxidative DNA damage. The most important feature of the antioxidant defense system is that its components work synergistically against reactive oxygen species. Therefore, all antioxidants are vital in maintaining homeostasis in living things (Cooke *et al.*, 2003). DNA, a stable molecule,

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can also undergo oxidative chemical damage, such as lipids, carbohydrates, and proteins. Studies have shown that the oxidative base modification of DNA damage is 8-hydroxy deoxyguanosine (8-OHdG). Thus, the level of 8-OHdG formed in the tissues shows us the story of DNA damage (Evans and Cooke, 2004; Cooke *et al.*, 2003).

The genes that control apoptosis, also known as programmed cell death, are known as caspases. These are cysteine proteases, and they break the peptide bond after aspartic acid. In the mitochondrial-dependent intrinsic pathway of apoptosis, Bcl-2 family proteins activate procaspase-9 due to the mitochondrial membrane being affected, while procaspase-8 is activated via receptors in the cell membrane in the receptor-dependent extrinsic pathway (Vermeulen *et al.*, 2005; Chipuk and Green, 2008). The cell is led to apoptosis by activating caspase-3 in the common pathway via caspase-9 in the intrinsic pathway and caspase-8 in the extrinsic pathway of apoptosis (Mirkes, 2002).

Lake Van is polluted day by day with environmental effects. Wheat and barley cultivation around Lake Van also causes the lake to be contaminated with pesticides. Van fish, which is a significant protein source for the region, has great economic importance. Lake Van is the second-highest soda lake globally (pH: 9,8), and the Van fish is an endemic fish species living in this lake. Therefore, this study aims to determine the level of change in antioxidant enzymes, malondialdehyde, DNA damage (8-OHdG) and apoptosis (Caspase-3) in kidney and muscle tissues at 24, 48, 72 and 96 h as a result of exposure of Van fish to tebuconazole.

MATERIALS AND METHODS

The fish

Van fish caught from Lake Van was brought alive and put into fiberglass tanks filled with 300L water. Fish were randomly divided into the concentration group and control group. 2.5 mg/L tebuconazole was applied to the concentration group aquarium (Lutnicka *et al.*, 2016). Fish were fed daily with commercially available fish food. The temperature of the water was determined as 15–17°C, dissolved oxygen: 7.1 mg/L. A light period of 12 h at night and 12 h during the day was applied. In the study, the kidney and muscle tissues of the fish were taken by applying the anesthetic substance aminobenzoate methanesulfonate (MS222, 100mg / lt) for sampling at 24, 48, 72, and 96 h.

Antioxidant enzyme analysis

SOD enzyme activity determination: Superoxide dismutase (SOD) enzyme activity was measured with

Randox -Ransod enzyme kit in an autoanalyzer at 505 nm at 37°C. Catalase enzyme activity determination (Xia *et al.*, 1995; Flohe and Ötting, 1984). CAT enzyme activity was measured at 240 nm with the UV spectrophotometric method of Aebi (1984) based on hydrogen peroxide (H₂O₂) decomposition by catalase. GSH-Px enzyme activity: Glutathione peroxidase enzyme activity was measured with Randox-Ransel enzyme kits at 340 nm in an autoanalyzer, using the ultraviolet method at 37°C (Paglia and Valentine, 1967; Flohe and Gunzler, 1984).

Analysis of malondialdehyde (MDA), DNA damage (8-OHdG), and apoptosis (Caspase-3)

Placer *et al.* (1966) of the lipid peroxidation analyzed according to the method is based on the reaction of MDA, one of the aldehyde products, with thiobarbituric acid (TBA). The resulting MDA is a pink complex with TBA. The absorbance of this solution was measured at 532 nm with a spectrophotometer, and the degree of lipid peroxidation was determined. In the study, Fish (8-OHdG) ELISA kit (Catalogue No: 201-00-0041) (SunRed brand) was used to determine 8-hydroxy-2-deoxyguanosine (8-OHdG) levels. Half an hour before starting the study, the kit materials were brought to room temperature (Standard, Standard diluent, Microelisa Strip Plate, Str-HRP-Conjugate Reagent, 30XWash solution, Biotin-(8-OHdG) Ab, Chromogen Solution A, Chromogen Solution B, Stop Solution) and the kit procedure was applied with the specified materials (Yeltekin and Oğuz, 2018). Fish (CASP3) ELISA Kit (Catalogue No: 201-00-0031) (SunRedtrade) was used for determining caspase-3 levels of fish in each tissue (Alak *et al.*, 2021).

Statistical analysis

The one-way analysis of variance (ANOVA) and Duncan tests was performed using SPSS Software (version SPSS 18.0) to test the significant statistical differences between the experimental groups. Statistical decisions were made using a significance level of $p < 0.05$. The data were expressed as the mean \pm SE.

RESULT

Antioxidant enzymes

According to the results of the study, it was observed that the levels of antioxidant enzymes in kidney and muscle tissues decreased over time after exposure to antifungal azole compounds of Van fish. It was determined that this decrease started as of the 24th h and continued at the 48th and 72nd h, especially at the 96th h, at a statistically significant level. It was determined that the antioxidant level of kidney tissue decreased significantly (Fig. 1).

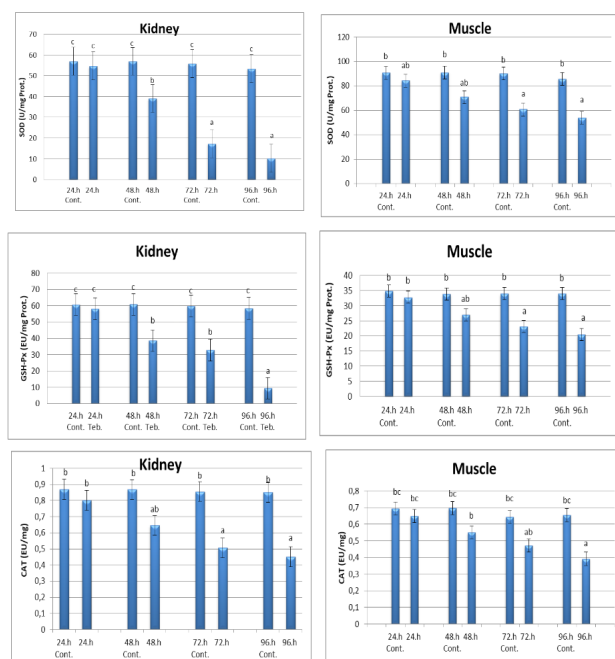


Fig. 1. The change of antioxidant enzyme levels in kidney and muscle tissues of Van fishes treated with tebuconazole. Different letters a, b, c, and d indicate a significant difference at $p < 0.05$.

Change in MDA, 8-OHdG, and Caspase-3 levels

The study observed that the MDA level in the kidney and muscle tissues increased over time after exposure to the anti-fungal azole compound of Van fish, and this increase reached statistically significant levels towards the 96th h. It was determined that there was a statistically significant increase in 8-OHdG levels in kidney and muscle tissue. It was determined that the caspase-3 level was statistically different, especially in the kidney tissue. It was observed that apoptosis in muscle tissue increased statistically significantly in the following h ($p < 0.05$) (Fig. 2).

DISCUSSION

Agricultural products developed to feed the rapidly increasing world population bring along the intensive use of fungicides. Azole fungicides are a broad chemical class used to control molds and fungal infections on plants. These chemicals are also applied to ornamentals in commercial/residential applications (Yeltekin *et al.*, 2020). Trifluoromethyl is one such triazole fungicide, but toxicity data are scarce on the potential for sublethal effects in nontarget aquatic organisms compared to other triazole fungicides. In this study, Van fish were used to determine whether exposure to 2.5mg/l tebuconazole concentration

would cause changes in antioxidants, lipid peroxidation, DNA damage, and caspase-3 levels.

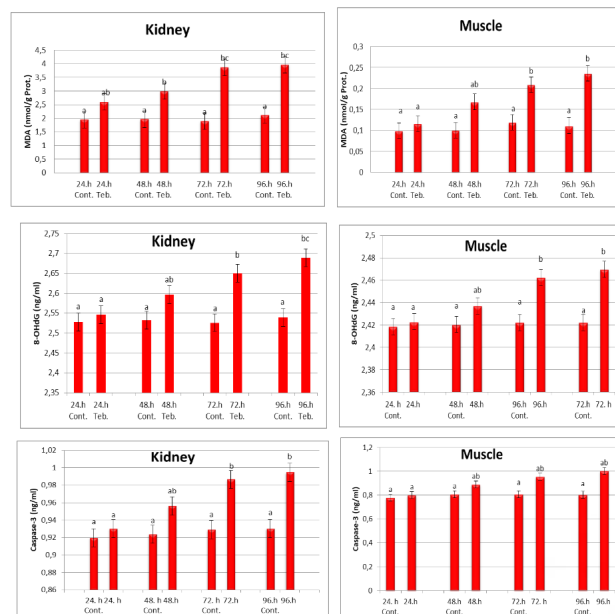


Fig. 2. Changes in caspase-3, 8-OHdG, and MDA levels in kidney and muscle tissues of Van Fish treated with tebuconazole. Different letters a, b, c, and d indicate a significant difference at $p < 0.05$.

This study determined that there was a significant increase in caspase-3 levels in kidney and muscle tissues of Van fish after exposure to tebuconazole. This suggests that exposure to tebuconazole, which is highly toxic to the aquatic environment, may be induced. This result was in agreement with previous findings. Studies have shown that azole fungicide toxicity affects the immune system in fish and stimulates apoptosis pathways in cells (Teng *et al.*, 2019). Tebuconazole exposure in rats has increased caspase-3 levels (Yua *et al.*, 2013; Alaa *et al.*, 2020).

Similarly, Xu *et al.* (2016) found that pesticide toxicity application triggered apoptosis, and accordingly, the level of caspase-3 increased. A study was conducted to investigate the oxidative stress and apoptosis caused by azole compounds. The study stated that azole compounds show subcellular effects on genes involved in ROS detoxification and reduce mitochondrial metabolism. These effects stimulate caspase enzymes by affecting apoptosis (Kuchovská *et al.*, 2021).

Azole compounds cause oxidation of lipids and destruction of the cell membrane by the oxidative stress they create. This destruction affects both the nuclear membrane and the permeability of mitochondrial membranes. The resistance of cell membranes with increased permeability

to free radicals formed by oxidative stress also decreases. Thus, both the lipid peroxidation and DNA damage rate begin to grow in apoptosis (Lima *et al.*, 2020). A study was conducted to investigate the effects of azole compounds on experimental models. In the study, it was stated that lipid peroxidation and DNA damage increased. In our research, as the exposure time to tebuconazole increases, the damage increases due to oxidative stress. Thus, an increase in lipid peroxidation and then the destruction of DNA damage (8-OHdG) was detected. It was determined that 8-OHdG and MDA levels increased as the fungicide concentration and time increased in zebrafish larvae exposed to fungicide toxicity (Wang *et al.*, 2021). A study was conducted in which the damage of azole compounds on living tissue was investigated. The study stated that it destroys the cell cycle by affecting lipid, protein, and other molecules (Liu *et al.*, 2021). Similarly, it was determined that the levels of 8-OHdG and MDA in liver tissue increased continuously after zebrafish were exposed to fungicide toxicity (Teng *et al.*, 2019).

Studies with azolic compounds using fish are scarce, making it difficult to understand the mode of action of these toxic agents at the species. In this study, the toxicological effects of tebuconazole on aquatic organisms were observed using Van fish. According to our findings, tebuconazole fungicide causes oxidative stress in Van fish's kidneys and muscle tissues. With this, as the duration of fungicide exposure increases, the levels of antioxidant enzymes (SOD, CAT, GSH-Px) decrease, and the effects of inflammation decrease. It is then possible to conclude that azole compounds have potential toxicological effects on aquatic organisms. Free radicals formed by oxidative stress disrupt the electron balance in the cell and destroy the secretory metabolism of the antioxidant enzyme system. Enzymes associated with the line defence in the antioxidant system, such as CAT, and SOD, are essential for detoxifying pollutants in aerobic organisms (Giraud *et al.*, 2017; Queiroz *et al.*, 2021). GSH-Px shows a rapid enzymatic response when exposed to azole compounds similar to a heterocyclic compound produced in the process of degradation of the tebuconazole (Vieira *et al.*, 2019). CAT and SOD are necessary antioxidant enzymes that regulate intracellular H_2O_2 made in the detoxification process (Gebicka and Krych-Madej, 2019; Queiroz *et al.*, 2021). Likewise, GSH-Px, CAT, and SOD are suitable biomarkers to compounds that exhibit the formation of nitrogen compounds, such as tebuconazole. Similarly, in a study conducted with azole fungicide in fish, it was stated that antioxidant enzymes decreased over time. This study noted that the toxicity of azole compounds in fish kidneys and muscle tissues lost the prophylactic effects of potential antioxidant enzymes (Khalaf *et al.*, 2021).

CONCLUSION

This study shows that tebuconazole has a toxic effect and creates oxidative stress in Van fish. In addition, it is observed that lipid peroxidation, DNA damage, and caspase-3 levels increase as time progresses. As a result, it is seen that the mixing of tebuconazole, wheat, and barley fungicide, which is widely used around Lake Van, with rain and irrigation waters, is harmful to the health of Van fish and the community. When the study is evaluated both in terms of Van fish and ecologically, it can be said that the adverse effects of pesticides on living things are very high.

Based on these results, the study provides a better integral and mechanistic understanding of toxicity in the kidney and muscle in tebuconazole-induced oxidative stress, DNA damage, inflammatory responses, and cell apoptosis in Van fish at environmentally relative concentrations. Therefore, this study provides a better understanding of the evidence of the biological effects of tebuconazole on aquatic studies for a better pesticide environmental risk assessment.

Data availability

All data are available from the corresponding author upon request.

Ethics approval

In accordance with the decision of the Animal Experiments Local Ethics Committee dated 25.05.2017 and numbered 05, it was decided by our committee, with the date 23.05.2017 and number 36008, that the study be carried out in the Faculty of Fisheries, Fisheries Basic Sciences Department.

Statement of conflict of interest

The authors have declared no conflict of interest.

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