



The responses of cholinergic system in the brain tissue of Van Fish (*Alburnus tarichi*) exposed to antifungal tebuconazole compound toxicity

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ABSTRACT

Today, fungicide toxicity is quite common in aquatic ecosystems, and this situation adversely affects marine organisms. For this reason, it is essential to determine the effects of fungicides on aquatic organisms and to try to prevent organisms from being exposed to these toxic chemicals. In this study, changes in cholinergic system enzymes and (malondialdehyde) MDA levels as a result of exposure to acute fungicide toxicity in Van fish (*Alburnus tarichi*, Gldenstdt 1814) were investigated. Brain tissue was taken from Van fish exposed to 2.5 M Tebuconazole used in agriculture by sampling at 24, 48, 72, and 96 hours. Brain tissue acetylcholinesterase (AChE), butyrylcholinesterase (BChE) activities, and MDA levels were measured in this context. In the study, AChE (0.965 ± 0.03 , 0.575 ± 0.01) and BChE (0.421 ± 0.02 , 0.291 ± 0.01) activities decreased in Van fish brain tissue due to exposure to Tebuconazole, but MDA (0.099 ± 0.01 , 0.192 ± 0.01) level increased ($p < 0.05$).

Keywords: Van Fish (*Alburnus tarichi*, Gldenstdt 1814), Fungicide, Tebuconazole, AChE, BChE, MDA

Introduction

One of the most critical factors threatening human, animal, and environmental health is pesticide residues. In chemical control, pesticides should not harm the environment and human health while protecting plants from diseases. However, due to the unconscious use of pesticides against pests, many problems such as deterioration of the natural balance, environmental pollution, and resistance of pests arise. Therefore, attention should be paid to the biological, toxic, and physical properties of fungicides. In the studies, it was determined that tebuconazole from the triazole group does not disappear in a short time due to its long half-life (Batta, 2005). In a study investigating its residue in soil and water 120 days after spraying with tebuconazole used in agriculture active ingredient fungicide, it was determined that the residual amounts of the said fungicide were still high (Nasr et al. 2003). Systemically effective tebuconazole also prevents the synthesis of ergosterol in fungi and can cause toxic effects on organisms in the aquatic environment for a long time (Bayer Crop Science Limited, 2005; Yeltekin et al. 2018). In other studies investigating the effects of tebuconazole, it was determined that tebuconazole residues persist for a long time and cause toxicity in living tissues (Siek and Paszko 2021).

Reactive oxygen species (ROS) originating from environmental pollutants cause structural and functional changes in the cells of aquatic organisms and can also cause changes in biochemical parameters (Parvez and Raisuddin, 2005). It is stated that in the presence of oxidative stress, the tissue and cell membranes of fish can be easily oxidized due to their high polyunsaturated fatty acid content (Mendes, 2009). Lipids in the membranes of intracellular organelles are highly susceptible to free radical damage. Lipid peroxidation, which occurs when free radicals react with lipids, can have highly damaging effects. Lipid peroxidation leads to the production of large quantities of toxic by-products. These produced by-products act as second messengers and exert their products in a region far from where they were produced. Damage from lipid peroxidation is highly detrimental to cell function (Devasagayam et al. 2003).

In general, terms, biomarkers are indicators of multiple toxic interactions such as physiological, biochemical, immunological, and histopathological effects caused by certain environmental pressures. Enzymes as biomarkers are usually associated with the first level of organization and can be considered an 'early warning sign. In this context, the enzymes to be regarded as biomarkers are esterases and oxidative stress enzymes. Cholinesterase enzymes are enzymes found in many tissues, body fluids, and plasma. They are divided into AChE and BChE according to their sensitivity to the inhibitor and

substrate specificity. AChE enzyme is the main cholinesterase enzyme found in muscle, brain, and erythrocyte membrane. ACh is an enzyme that catalyzes various choline decomposition reactions, such as butyrylcholine and acetylthiocholiniodide. AChE and butyrylcholinesterase are the most well known cholinesterase enzymes. One AChE enzyme molecule hydrolyzes 4×10^5 ACh molecules per minute, and its 150 ms turnover time makes it the most effective hydrolytic enzyme. After the release of acetylcholine from the cholinergic synapses, the nerve transmissions are terminated due to its breakdown with the help of cholinesterases (Fetoui et al. 2010; Uçar et al. 2021; Yeltekin et al. 2020). They are among the acetylcholinesterase inhibitors with compounds such as pesticides and nerve gases. The AChE enzyme, which has a very high activity, breaks down approximately 25,000 acetylcholine (ACh) molecules per second. Chemicals inactivate the hydroxyl group of the serine amino acid in the enzyme's active site by phosphorylating it. As a result, the increase in acetylcholine in the cholinergic nerve junctions causes the smooth muscles to contract and the glands to secrete. The inhibitory effect on AChE activity shows that it also affects critical vital processes such as energy metabolism in nerve cells (Akdeniz, 2010). Therefore, studies on chemicals that cause cholinesterase inhibition are essential in terms of ecotoxicology. In addition to the studies on the activities of cholinesterases in serum or plasma, the relationship between brain acetylcholinesterase (AChE) inhibition and mortality is a very important point of view. Therefore, this study planned to investigate the effects of tebuconazole, which is widely used around Lake Van, on cholinergic enzymes (AChE, BChE) and malondialdehyde (MDA) in Van fish brain tissue.

Material and Methods

Fish

In the study, 80 Van fish of about 85-90 grams and 20-25 cm in length were used. The fish to be used in the study was obtained from Van Lake after obtaining the permission of the date 06.09.2018 and 08 number Van Yuzuncu Yil University Animal Research Ministry of Agriculture and the local ethics committee for animal experiments. After the fish were randomly distributed to 300 L water tanks, tebuconazole was applied after a one-week adaptation period. In the study, the water was constantly ventilated with oxygen stones, and the fish were fed twice a day, and the normal light process was applied. The tebuconazole concentration (2.5 M) to be administered Lutnicka et al. (2016). After the fish were kept in the anesthesia environment, they were separated into cranial incision tissues. Fish were sampled from both the concentration group and the control group at 24, 48, 72 and 96 hours.

Measuring AChE/BChE Enzyme Activity

In order to prepare for analysis, each tissue was homogenized for 5 minutes in a homogenizer by adjusting the pH to 7.4 in KH_2PO_4 buffer at 1/10 w/w. The obtained homogenates were centrifuged at 3000 rpm for 15 minutes. The obtained supernatant was used to determine the amount of MDA with acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzyme activities. AChE and BChE enzymes were determined spectrophotometrically according to the Ellman method. In the Ellman method, the thiol ester acetylthiocholine is used instead of the oxy ester acetylcholine as substrate. According to the principle of the Ellman method, acetylthiocholine is hydrolyzed by acetylcholinesterase, and the thiocholine released as a result of hydrolysis is combined with the Ellman reagent DTNB [5,5'-dithio-bis-(2-nitrobenzoic acid)] reacts. As a result of the reaction, yellow-colored chromophore TNB (5-thio-2-nitrobenzoic acid) is formed. The rate of formation (intensity of color) of this yellow compound formed at the end of the reaction is determined by measuring the absorbance at 412 nm (Ellman et al. 1961). The intensity of this yellow color is directly proportional to the AChE/BChE enzyme activity.

Measuring Lipid Peroxidation (MDA)

Homogenization of brain tissues was done according to Mis et al. (2018). This method was described by Placer et al. (1966) is based on the reaction of malondialdehyde (MDA), one of the aldehyde products of lipid peroxidation, with thiobarbituric acid (TBA). The resulting MDA forms a pink complex with TBA. The absorbance of this solution is measured at 532 nm with a spectrophotometer to determine the degree of lipid peroxidation.

Statistical Analysis

The one-way analysis of variance (ANOVA) and Duncan tests were performed to test statistically significant differences between the experimental groups using SPSS Software (version SPSS18.0). Statistical decisions were made with a significance level of $p < 0.05$.

Results and Discussion

Oxidative stress occurs as a reaction to the stress caused by the effects of chemicals such as fungicides and pesticides, which damages the enzyme systems of all living things. It is of great importance to evaluate the oxidative stress parameter and the activity of neurotransmitter enzymes after exposure to a therapeutic agent or synthetic chemical compounds. The effect of oxidative stress biomarkers obtained in this study on its function is summarized in Figure 1, Figure 2 and Figure 3. The study's findings, the brain tissue AChE enzyme levels of Van fish decreased as the exposure time increased at the same tebuconazole concentration. It was observed that there was a significant decrease, especially at the 96th hour. These observed differences were also found to be statistically significant ($p < 0.05$) (Fig.1).

According to the results obtained in the study, brain tissue BChE enzyme level of Van fish exposed to tebuconazole decreases as time progresses. It shows a statistically significant decrease especially after the 48th hour ($p < 0.05$) (Fig. 2).

Malondialdehyde, one of the most important markers of oxidative stress, gave significant responses after tebuconazole application in the brain tissue of Van Fish. According to the results obtained, it was observed that the level of lipid peroxidation increased immediately after tebuconazole exposure started. It was determined that the MDA level increased as the exposure time increased. These observed differences were also found to be statistically significant ($p < 0.05$) (Fig. 3)

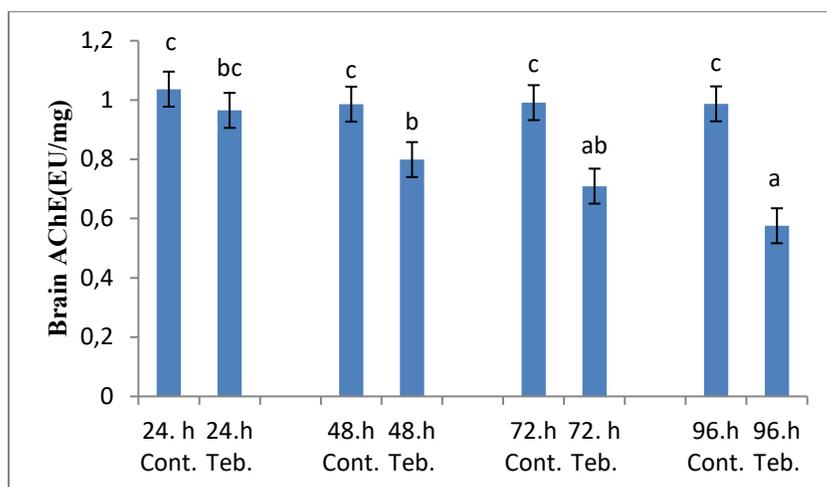


Figure 1. Van Fish brain tissue AChE activity exposed to tebuconazole

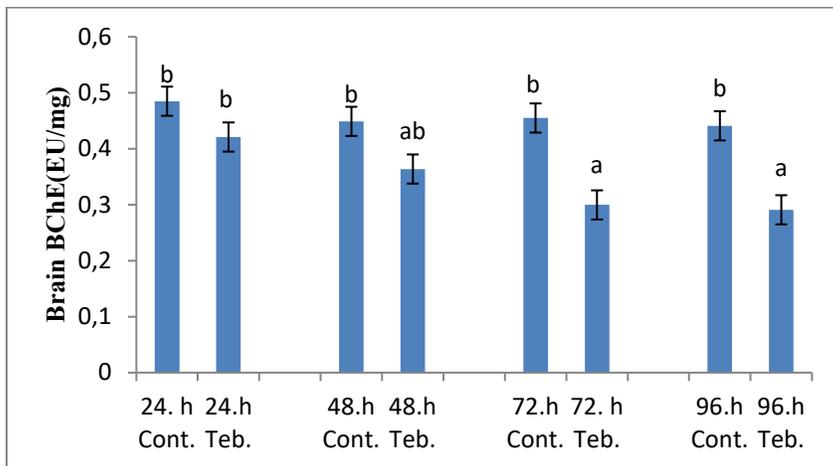


Figure 2. Van Fish brain tissue BChE activity exposed to tebuconazole

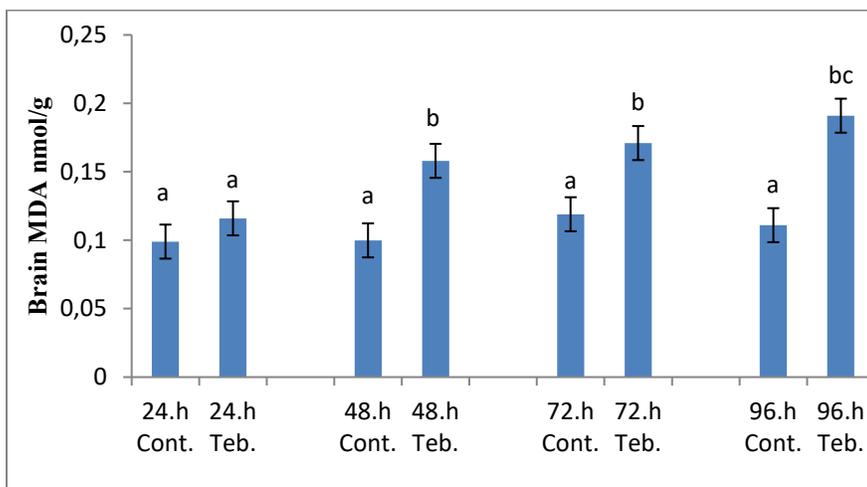


Figure 3. Van Fish brain tissue MDA level exposed to tebuconazole

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. Triconazole 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. This study determined whether exposure to Tebuconazole would cause changes in Van Fish brain tissue AChE, BChE, and MDA levels. According to the findings, it was determined that Tebuconazole, an azole compound, decreased AChE and BChE levels by increasing oxidative stress in the brain tissue of Van fish. As the exposure time to the applied tebuconazole increases, the free radicals formed to increase and start the destruction in metabolism. As a result, AChE

and BChE enzyme systems may be damaged, and their secretion may decrease. Similarly, in other studies with pesticides, AChE and BChE levels were found to decrease (Atamanalp et al. 2021). Santana et al. (2021) conducted a study examining the enzyme change by applying toxicity to fish with pesticides, herbicides and fungicides. In this study, it was determined that AChE and BChE levels of fish decreased in all three pesticide, fungicide and herbicide applications and even caused inhibition in some of them. It was determined that if the pesticide used was an organophosphate compound, it completely inhibited AChE and BChE enzymes, but the activation was significantly reduced in other pesticides and fungicides (Alak et al. 2019a, Alak et al. 2019b; Ramírez-Santana et al. 2020). It was determined that the oxidative stress levels in the larvae increased with the fungicide triconazole compound applied to zebrafish larvae (Souders et al. 2020).

Again, a study was conducted in rats with tebuconazole fungicide. This study determined that Tebuconazole caused oxidative stress in tissues and triggered apoptosis (Nong et al. 2020). As with organophosphates, it has been determined that other pesticides- fungicide can inhibit the AChE enzyme. AChE is frequently used as toxic indicators of fungicide. The amount of neurotransmitter acetylcholine in sympathetic synapses, neuromuscular junctions and central nervous system. It has been reported that the inhibition of this enzyme, which regulates animals and humans, greatly affects (Gluszczak et al., 2007). Acetylcholinesterase is an enzyme that controls impulse transmission by hydrolyzing acetylcholine in cholinergic synapses and terminating its function. Accumulation of acetylcholine as a result of enzyme inhibition causes excessive presynaptic stimulation, the continuation of the event results in paralysis and death (Sepici-Dincel et al., 2009).

It is known that free radicals increasing with pesticides and oxidative stress increasing with these reduce antioxidant enzymes. As a result, lipid peroxide formation (LPO) increases. Increasing LPO causes an increase in MDA, damaging the tissue cells and membrane structure (Fetouni et al. 2010). LPO formed in the membrane structure affects the permeability of the cell membrane and causes the disruption of intracellular balances (Gao et al. 2020). Our study determined that the brain tissue malondialdehyde level increased over time with tebuconazole application. This shows that the toxicity of azole compounds creates oxidative stress and increases the formation of free radicals. Our study determined that the brain tissue malondialdehyde level increased over time with tebuconazole application. This shows that the toxicity of azole compounds creates oxidative stress and increases the formation of free radicals. The free radicals formed can destroy the brain tissue, especially the lipid structure. In other studies, it was determined that exposure to fungicide increased the level of LPO (Das et al. 2020). Again, a study was conducted in which rainbow trout was exposed to azole compounds. In the study, oxidative stress and neurotoxic effects in fish were investigated. As a result, it was determined that azole compounds significantly increased oxidative stress and MDA levels (Rossi et al. 2020). Bartu et al. conducted a study investigating the inhibition of azole compounds on AChE and BChE. In the study, they revealed that AChE and BChE inhibitors are competitive inhibitors with enzyme kinetic experiments. Azole compounds have been reported to increase oxidative stress, increase MDA and inhibit the activity of AChE, and AChE measurement has been shown to be useful as a good biomarker. From this study and other studies, it is understood that ROS has an important role in fish tissue fungicide azole toxicity (Rafael et al. 2021).

Conclusion

Understanding this balance is essential to assess the complexity of toxicological effects in tissues. For aquatic toxicology, AChE and BChE enzymes are indicators that can be very effective in toxicology studies. Therefore, it is essential to investigate tissue-specific toxicity in elucidating toxic metabolism. In this study, the acute toxicity mechanism caused by tebuconazole fungicide in Van fish brain tissue was tried to be clarified by the approaching multi-biomarker (AChE, BChE activity, and MDA) parameters. When the data were interpreted, it was concluded that oxidative stress induced by tebuconazole fungicide in the brain tissue due to acute administration causes oxidative damage in the structural and functional activities of the cell by affecting the cholinergic system, inhibiting enzyme activities, and causing lipid peroxidation. Hence, to identify robust cause-effect relationships between fungicides and fish ChEs, future studies should turn their focus on filling the gaps found here.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived conflict of interests.

Ethics committee approval: This study was conducted by Van Yüzüncü Yıl University Animal Experiments Ethics Committee (Ethics approval no: date 06.09.2018 and 08 number)

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