Dicle Üniversitesi Veteriner Fakültesi Dergisi
https://dergipark.org.tr/tr/pub/duvetfd
Araştırma Makalesi/Research Article
ISSN:1307-9972 Dicle Üniv Vet Fak Derg 2024;17(1):57-64 e-ISSN:1308-0679
DOI: 10.47027/duvetfd.1450989

# Impact of Diazinon Standard on Histopathological and Ultrastructural Properties on Brain Tissue of *Oreochromis niloticus* (Linnaeus, 1758)

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| Geliş Tarihi/Received | Kabul Tarihi/Accepted | Yayın Tarihi/Published |
|---|---|---|
| 11.03.2024 | 21.05.2024 | 30.06.2024 |

**Abstract**

In this study, the histopathological and ultrastructural alterations in the brain tissue of *Oreochromis niloticus* (Linnaeus, 1758) exposed to a sublethal concentration of diazinon standard for 21 days were determined. For this purpose, *O. niloticus* individuals were exposed to 280 µg/L ($LC_{50}$/10) diazinon concentration for 21 days and on the 7th, 14th and 21st days of the exposure, the brain samples of these individuals were removed. After the histological and ultrastructural preparations of the brain samples, the prepared samples were evaluated with light and transmission electron microscopes (TEM). Examination of the samples indicated that a sublethal dose of diazinon induced histopathological and ultrastructural modifications in the brain tissue of *O. niloticus*. The severity of these alterations was increased with the duration of the time. The most severe histopathological alteration was necrosis determined on the 14th and 21st days of exposure. However, histopathologically the most frequent changes were cloudy swelling, hypertrophy and pycnotic nucleus in the glial cells of the brain. Ultrastructurally, mitochondrial degeneration, cristolysis, axon and dendrite deformations were seen in the tissues. These ultrastructural findings showed that the sublethal concentration of diazinon disturbed the energy metabolism of the cells which might result in neurodegenerative dysfunction of *O. niloticus* brain.

**Key Words:** Brain tissue, cloudy swelling, cristolysis, histopathological alteration, ultrastructural alteration, transmission electron microscope

**Diazinon Standardının Oreochromis niloticus (Linnaeus, 1758)'un Beyin Dokusu Üzerindeki Histopatolojik ve Ultrayapısal Etkileri**

**Öz**

Bu çalışmada, 21 gün boyunca diazinon standartının subletal bir konsantrasyonuna maruz bırakılan *Oreochromis niloticus* (Linnaeus, 1758) beyin dokusunda histopatolojik ve ultra yapısal değişiklikler belirlenmiştir. Bu amaçla, *O. niloticus* bireyleri, 21 gün boyunca 280 µg/L ($LC_{50}$/10) diazinon konsantrasyonuna maruz bırakılmış ve maruziyetin 7., 14. ve 21. günlerinde, maruz kalan canlıların beyin örnekleri alınmıştır. Beyin örneklerinin histolojik ve ultra yapısal preperasyonunun ardından, hazırlanan örnekler ışık ve geçirimli elektron mikroskopları (TEM) ile değerlendirilmiştir. Örneklerin değerlendirilmesi sonucu, subletal diazinon konsantrasyonunun *O. niloticus*'un beyin dokusunda histopatolojik ve ultra yapısal değişikliklere neden olduğu belirlenmiştir. Bu değişikliklerin şiddeti zamanla artmıştır. En şiddetli histopatolojik değişiklik, maruziyetin 14. ve 21. günlerinde belirlenen nekroz olarak tespit edilmiştir. Bununla birlikte, histopatolojik olarak en sık gözlenen değişiklikler beyin glial hücrelerinde bulutlu şişme, hipertrofi ve piknotik çekirdek olarak belirlenmiştir. Ultra yapısal olarak, hücrelerde mitokondrial dejenerasyon, kristoliz, akson ve dendritlerde deformasyon belirlenmiştir. Bu ultra yapısal bulgular, subletal diazinon konsantrasyonunun hücrelerin enerji metabolizmasını bozduğunu ve bu durumun *O. niloticus*'un beyininde nörodejeneratif işlev bozukluğuna yol açabileceğini göstermektedir.

**Anahtar Kelimeler:** Beyin dokusu, bulutlu şişme, geçirimli elektron mikroskobu, histopatolojik değişiklik, kristoliz, ultrayapısal değişiklik

## INTRODUCTION

Farmers in modern agricultural practices widely use pesticides to sustain the human population and, consequently, increase crop production. However, the indiscriminate use of pesticides leads to lethal effects on non-target organisms (1). After surface runoff, these toxic substances can enter the water system and may have dangerous effects on non-target organisms, especially fish (2). Simultaneously, these toxic chemicals can alter water quality, affecting the health of fish and other aquatic organisms (3,4).

Insecticides belonging to the OP group induce hyperactivity of muscarinic and nicotinic receptors by irreversibly inhibiting acetylcholinesterase (AChE), leading to the buildup

of acetylcholine (ACh) at cholinergic synapses and ultimately culminating in cholinergic syndrome (5). It is known that commonly used OPs cause adverse effects on organisms (6, 7). O,O-Diethyl O-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphorothioate, commonly referred to as Diazinon (DZN), stands out as a crucial and moderately persistent organophosphate insecticide utilized widely across agricultural fields, residential settings, and recreational environments (8). This pesticide penetrates water resources via rain, surface runoff and accidental spillage of DZN and accumulates in the tissues of aquatic organisms (9). Literature reveals investigations highlighting the toxicological repercussions of DZN exposure on aquatic fauna (10, 11).

Given their importance in the food web, propensity for toxic compound accumulation, and responsiveness to mutagens even at low concentrations, fish are considered valuable indicators for gauging the effects of aquatic pollution and their responses to toxic pollutants closely parallel those observed in mammals (12,13). As a result, employing fish biomarkers in experimental investigations becomes progressively significant for evaluating the consequences of pollution and facilitating the prompt identification of aquatic environmental concerns (14,15). *Oreochromis niloticus* (Linnaeus, 1758) is esteemed within the scientific community as a prime candidate for biological research, thanks to its favorable characteristics. In recent times, *O. niloticus* has been employed as a bioindicator species for biological consequences of pollutants in aquatic settings (16). In their study, El-Sherif et al. (17) determined that the $LC_{50}$ value of DZN for *O. niloticus* was 2800 µg/L.

Simply tracking the buildup of chemicals in an ecosystem does not provide adequate insight into the impacts of toxic substances on organisms, populations, and ecological communities (18). Cellular biomarkers in fish, encompassing histopathological and ultrastructural impacts, serve to bridge the gap between internal biochemical changes and the resulting responses at the individual or population level, thereby establishing a crucial connection. Serving as comprehensive indicators, they offer greater insights into the overall health condition of an organism compared to analyzing a single biochemical response (19). Many histopathological studies conducted on fish species affected by pollutants have shown that these biomarkers are effective indicators in determining water quality (20). DZN is an acetylcholinesterase inhibitor. As a result, the presence of AChE inhibitors like DZN can lead to modifications in the neurological functions and responses of non-target organisms, even at concentrations considered to be very low (21). In this context, it can be hypothesized that DZN may induce pathological changes in the brain tissue of *O. niloticus*. Within this framework, our study seeks to histopathologically and ultrastructurally analyze the alterations observed in the brain tissue of *O. niloticus* individuals exposed to the technical formulation of diazinon (DZN).

## MATERIALS AND METHODS

### Obtaining and Maintenance of *O. niloticus* Samples

Healthy adult *O. niloticus* samples were taken from the aquaculture ponds of the Faculty of Fisheries at Çukurova University, Adana, Türkiye. The anesthetized fish (200 mg/L phenoxyethanol) were taken to the Department of Biology, Hydrobiology and Aquatic Toxicology Research Laboratory of Dicle University Faculty of Science, Diyarbakır, Türkiye.

In the laboratory, for 15 days the *O. niloticus* samples were acclimated to laboratory conditions by placing them in the specially designed climate-controlled cabinet. In the cabinet, there were 6 aquariums, measuring 100x40x60 cm, each housed 25 fish. Lighting was provided using four fluorescent lamps (Daylight 36W/54), with a photoperiod of 14 hours light and 10 hours dark. The temperature was maintained at a constant 26±1°C throughout the adaptation and experimental stages using a laboratory thermostat-controlled climate system. No experiments were conducted on the organisms during the 15-day acclimation period to ensure complete adaptation to laboratory conditions. Throughout both the acclimation period and the experiments, 50% of the water in each aquarium was replaced daily with dechlorinated tap water. During the adaptation period, the fish were fed commercial pellet (Table 1) diets once a day, allowing them to feed freely (ad libitum) under controlled and monitored conditions. Compliance with EU Directive 2010/63/EU for animal experiments was ensured throughout the adaptation and experimental stages. Furthermore, our study received approval from the Experimental Animals Local Ethics Committee of Dicle University (Protocol number: 2013/43).

**Table 1.** Content of pellets

| Content | Percentages |
|---|---|
| Protein | 35% |
| Fat | 3% |
| Fiber | 5% |
| Water | 10% |
| Others (multivitamins, essential minerals, etc.) | 47% |

### The Experimental Design

The analytical standard of DZN (at 99% purity) (PESTANAL® from SIGMA-ALDRICH®, CAS Number: 333-41-5) was used. The concentration of DZN was calculated based on one-tenth of the $LC_{50}$ value (280 µg/L) (17) and per liter of water in the aquarium. The DZN standard was dissolved in acetone and diluted with distilled water to ensure that the solvent had minimal effects on the organisms.

For the sub-acute study, each group comprised 10 healthy adult individuals across 3 replicates. These groups were categorized as follows: control (Group I), acetone control (Group II), and exposure to 280 µg/L DZN standard (Group III). The experiment lasted 21 days, with the specific features of each group outlined in Table 2. The acetone control group was provided with a concentration of acetone per liter identical to that of the solution administered to the exposure group. The experimental setup utilized a static renewal method (22).

**Table 2.** The experimental groups of the study

| Group Number | Groups | Total number of individuals | Test Duration | Sex of the individualsa |
|---|---|---|---|---|
| I | Control | 10 | 21 days | 1F/9M |
| II | Acetone Control | 10 | 21 days | 5F/5M |
| III | 280 µg/L DZN exposure (LC50/10) | 10 | 21 days | 6F/4M |

a: F: Female, M: Male

### The Histopathological Analysis

At three specific time points during the experiment, namely the 7th, 14th, and 21st days, three fish were randomly sampled from both the exposure and control groups. These fish were euthanized through decapitation, and their brain samples were immediately immersed in a 10% formalin solution for 24 hours at 25°C. After fixation, the samples underwent a histological preparation process. Subsequently, the samples were treated with xylene and embedded in paraffin. The microtome (LEICA) was utilized to slice sections of 5 µm thickness, which were then stained with hematoxylin-eosin (23) and viewed under a light microscope (Nikon NIS-Elements ECLIPS SE80i). Any histopathological changes observed were documented using a camera (Nikon Digital SIGHT-DS2MV) attached to the microscope.

### The Ultrastructural Analysis

Brain samples obtained on the 7th, 14th, and 21st days were fixed in a 2.5% glutaraldehyde solution at 4°C for 24 hours. Following fixation, the samples underwent phosphate buffer (pH: 7.4, 0.1 M) rinses, osmium tetroxide postfixation, and dehydration through a gradient of ethanol concentrations (50%, 70%, 85%, 90%, 96%, and 100%). They were then treated with propylene oxide for 30 minutes at 25°C, followed by immersion in a propylene oxide-araldite resin mixture (1:1 v/v) for 2 hours at 25°C. After embedding, the samples were polymerized at 60°C for 48 hours. Ultrathin sections measuring 70-110 nm were cut using an ultramicrotome (LEICA) and placed on copper grids. The sections were stained with uranyl acetate and lead, followed by examination under a transmission electron microscope (TEM) (Jeol/JEM-1010) at the Dicle University Science and Technology Application and Research Center (DUBTAM) in Diyarbakır, Turkey. Ultrastructural changes were documented using a GATAN/782 ES500W Erlangshen CCD Camera.

## RESULTS

### The Histopathological Results

The average weight and length of the sacrificed *O. niloticus* samples were 37 gr and 135 mm, respectively. There were no histopathological alterations in the control (Figure 1a) and acetone control (Figure 1b) groups for 21 days. Also, there were no deaths in the control and experimental groups throughout the experiments. On the 7th day of 280 µg/L DZN exposure, cloudy swelling, hypertrophy and pycnotic nucleus were seen in the brain tissue (Figure 2a). Mild necrosis was also evident in the tissues. The severity of the histopathological alterations was increased on day 14 (Figure 2b). The number of pycnotic and hypertrophic cells was increased as well as cloudy swelling. The necrotic area in the tissues was expanded. The most severe changes were seen on the 21st day of the exposure. The melanomacrophage centers (MMCs) and hemolytic infiltration were first identified on this day (Figure 2c). The necrotic area was spread throughout the brain tissue. Cloudy swelling and pyknotic nucleus were also evident.

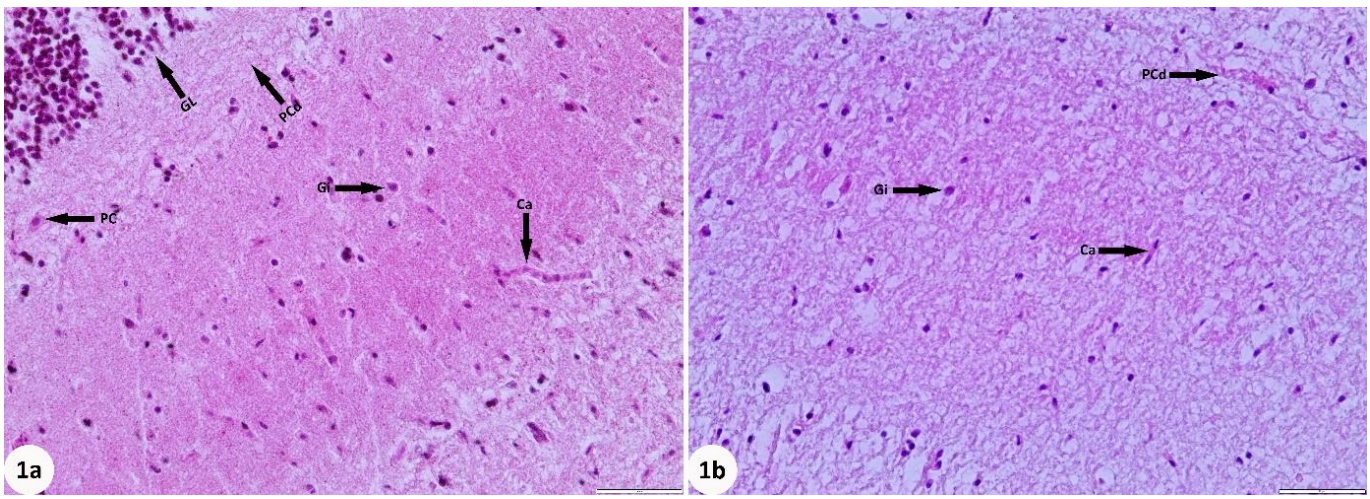


**Figure 1**. Histological brain structure of *O. niloticus* in control and acetone control groups at 21st day: **1a)** Control group: Capilarry (Ca), Glial cells (Gi), Purkinje cell (PC), Purkinje cell dendrites (PCd), Granular layer (GL); **1b)** Acetone control group: Glial cell (Gi), Purkinje cell dendrites (PCd), Capilarry (Ca).

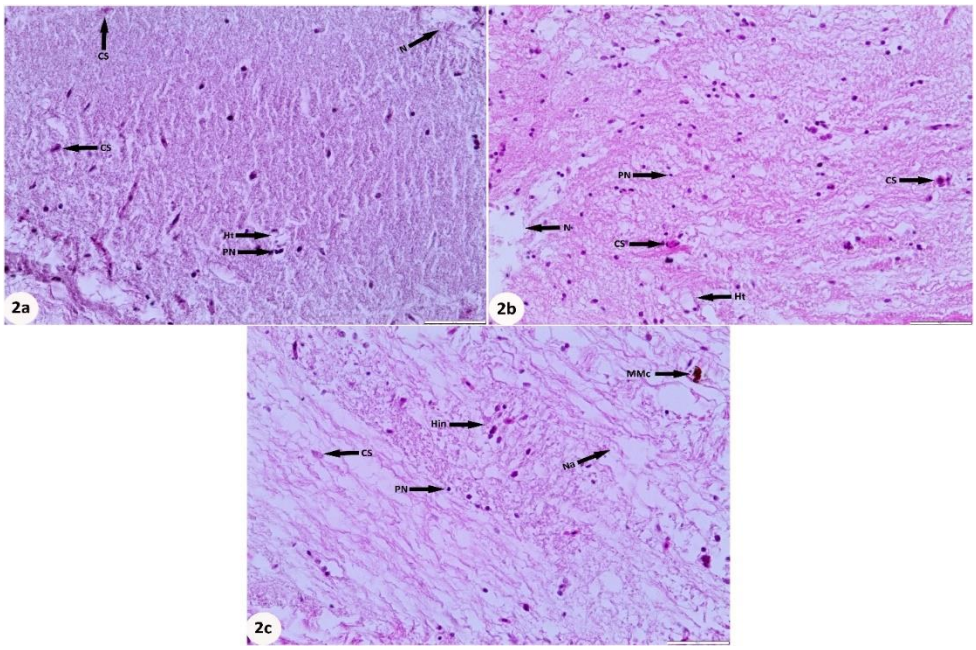


**Figure 2.** Histopathological alterations caused by DNZ in the brain tissue of *O. niloticus*: **2a)** Histopathological alterations in brain tissue of *O. nilotucus* exposed to 280 µg/L DZN for 7 days: Cloudy swelling (CS), Hyperthropy (Ht), Piycnotic nucleus (PN), Necrosis (N); **2b)** Histopathological alterations in brain tissue of *O. nilotucus* exposed to 280 µg/L DZN for 14 days: Cloudy swelling (CS), Hyperthropy (Ht), Piycnotic nucleus (PN), Necrosis (N); **2c)** Histopathological alterations in brain tissue of *O. nilotucus* exposed to 280 µg/L DZN for 21 days: Hemolytic infiltration (Hin), Melanomacrophage centers (MMCs), Cloudy swelling (CS), Piycnotic nucleus (PN), Necrotic area (NA). H&E, ×400.

### The Ultrastructural Results

There were no ultrastructural changes in the control (Figures 3a and 3b) and acetone control groups (Figures 3c and 3d) for 21 days. On day 7 of 280 µg/L DZN exposure, mitochondrial deformation, cristolysis and axon deformation were detected (Figures 4a and 4b). On the 14th day of the exposure, the severity of the ultrastructural alterations was increased. The number of deformed axons was also increased (Figures 4c and 4d). Deformed dendrite extensions and small necrotic areas were also seen. Cristolysis of mitochondria was also evident. The most severe pathological changes occurred on day 21. The necrotic area was expanded all across the brain tissue (Figures 4e and 4f). Mitochondrial deformation was also detected at 21 days of 280 µg/L DZN exposure.

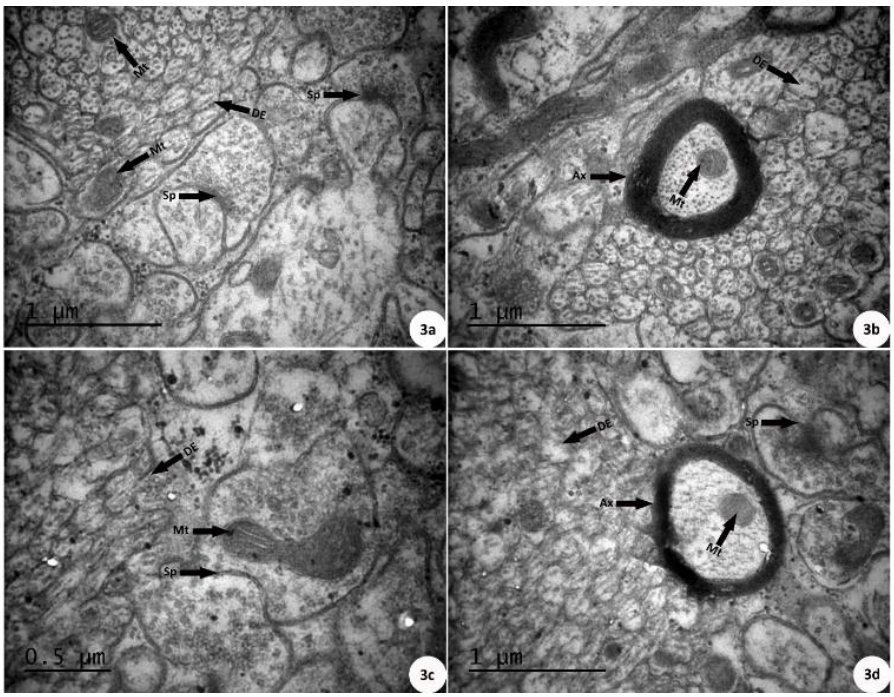


**Figure 3.** Brain ultrastructure of *O. niloticus* in control and acetone control groups at 21st day: **3a and 3b)** Control group: Sinaps (Sp), Dendtite extensions (DE), Mitochondria (Mt), Axon (Ax); **3c and 3d)** Acetone control group: Sinaps (Sp), Dendtite extensions (DE), Mitochondria (Mt), Axon (Ax).

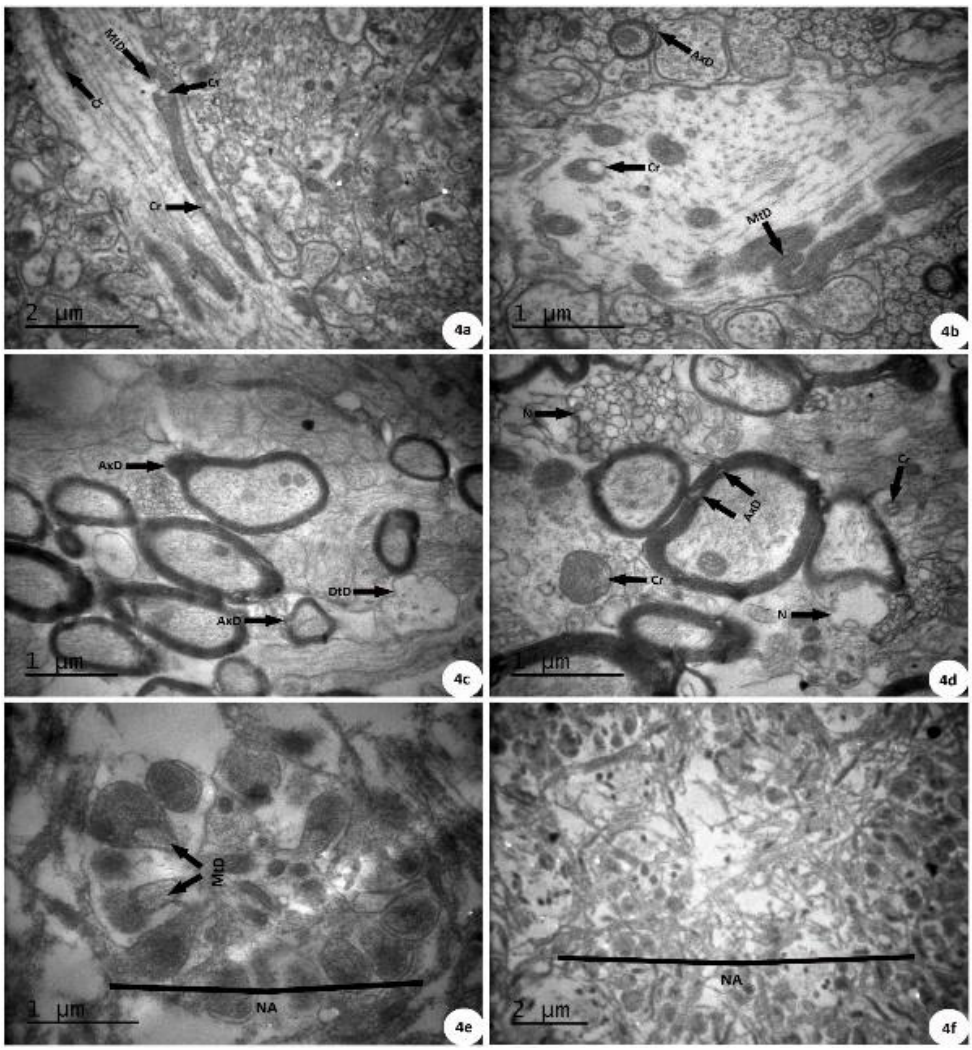


**Figure 4.** Ultrastructural alterations caused by DNZ in the brain tissue of *O. niloticus*: **4a and 4b)** Ultrastructural alterations in brain tissue of *O. nilotucus* exposed to 280 µg/L DZN for 7 days: Mitochondrial deformation (MtD), Cristolysis (Cr), Axon deformation (AxD); **4c and 4d)** Ultrastructural alterations in brain tissue of *O. nilotucus* exposed to 280 µg/L DZN for 14 days: Axon deformation (AxD), Dendrite deformation (DtD), Cristolysis (Cr), Necrosis (N); **4e and 4f)** Ultrastructural alterations in brain tissue of *O. nilotucus* exposed to 280 µg/L DZN for 21 days: Mitochondrial deformation (MtD), Necrotic area (NA).

## DISCUSSION AND CONCLUSION

Exposure of fish to toxic chemicals such as pesticides can result in the accumulation of reactive oxygen species (ROS) and free radicals in their cells, leading to oxidative stress damage (24,25). This oxidative stress can disrupt the normal cellular structure of fish, causing histopathological and ultrastructural lesions in many tissues and organs (26). Particularly, organophosphorus pesticides can induce necrosis in tissues by causing methylation and phosphorylation of proteins in cells, significantly reducing the organism's chances of recovery (27).

In organisms, the central nervous system analyzes the information coming from the sensory organs and determines the reactions of the organism to its environment (28). This situation applies to all vertebrates, including teleosts (29, 30). Many studies have reported that the brain morphology and histology of organisms are of great importance, and in this context, any pathological change that occurs in the brain of organisms has a great impact on the survival of that organism (31,32).

In our study, it could be seen that sublethal concentrations of DZN caused histopathological alterations in the brain tissue of *O. niloticus* individuals even on the earliest days of the exposure (Figure 2a). On the 7th and 14th day of the exposure cloudy swelling, hypertrophy and pycnotic nucleus in the glial cells were evident. Cloudy swelling is considered a pathological response characterized by clouding and swelling of the cell cytoplasm due to damage to the cellular membranes involved in ionic transfer (33). Cloudy swelling was reported in many studies about the effects of pesticides on fish species and organophosphates, like DZN, were among these pesticides (34,35). Another most frequent alteration was hypertrophy on days 7th and 14th (Figures 2a and 2b). It is thought that hypertrophy or cell swelling that occurs in cells exposed to a toxic chemical is caused by disruptions in ATPases that regulate cell volume or impairment of cellular energy transfer (36,37). As a result, oxidative stress resulting from lipid peroxidation leads to necrosis of cells (38,39).

Hypertrophy is also considered an indication of increased enzyme activity because of pesticide metabolization in the cell (40). On the 21st day of the DZN exposure, there was hemolytic infiltration in the brain tissue of *O. niloticus* (Figure 2c). It has been reported that hemolytic infiltration in aquatic organisms can occur to provide an alternative energy source to cells under the stress of toxicant exposure (41). MMCs seen on day 21st (Figure 2c) could be a sign of widespread necrosis of brain tissue because it is known that MMCs are associated with degenerative and necrotic lesions of the tissues (37). Also OPs were reported to increase MMCs in tissues and organs of exposed fish species (42). The number of pycnotic nuclei in the glial cells of brain tissue increased with the duration of the time (Figures 2a, 2b and 2c). In both apoptosis and necrotic cell death, cell chromatin and nuclei can irreversibly condense and shrink, causing pyknosis (43). This pathological alteration is an especially important early sign of cell death caused by pesticide exposure. Because in many studies it was shown that pesticides caused pycnosis and consequently necrosis in the tissues of exposed fish (44, 45). Necrosis determined on the 14th and 21st days of our study (Figures 2b and 2c) was also reported in other studies about DZN toxicity in various fish organs and tissues (46). Necrosis is an irreversible pathological lesion that causes loss of function of the cell and affects the activity of the affected organ (47). In this context, it can be assumed that necrosis can decrease the survival chance of an organism which exposed to toxic chemicals.

Ultra-morphological analyses are considered one of the best tools used to determine toxicant responses in cells (48). TEM, which produces a two-dimensional image of cells, is an important tool in determining morphological changes that cannot be detected by light microscopy, such as changes in the number of organelles of the cell and nuclear and organelle deformations (49). In this context, electron microscopic studies have an important place in the literature not only in determining the effect of toxicity in organisms but also in determining early morphological responses that may pose a risk to human and environmental health (50).

On days 7 and 14 of 280 µg/L DZN exposure, mitochondrial deformation, cristolysis and axon deformation were detected in the brain tissue of exposed fish (Figures 4a, 4b, 4c and 4d). Mitochondrial deformation and cristolysis indicated that DZN affected the subcellular morphology of the glial brain cells of *O. niloticus*. Mitochondria is an especially important organelle for cell viability because any disturbance occurring in this organelle can cause cell death by necrosis due to energy collapse (51). The enzymes used in the oxidative phosphorylation of the cell are located on the cristae of the inner mitochondrial membrane, and the density and number of cristae vary depending on the metabolic activity of the cell (52). Therefore, the main site of oxidative phosphorylation in cells is mitochondrial cristae (53). Accordingly, any ultrastructural deformation occurring in the mitochondrial cristae such as cristolysis could cause impairment of ATP metabolism of the cell. Furthermore, this situation can disturb the integrity of the cell causing necrosis (54,55).

The axonal deformation or degeneration detected on 7th and 14th days of DZN exposure (Figures 4b, 4c and 4d) could be a result of mitochondrial impairment in the neuron cells of the brain tissue. Because it is known that neurons are sensitive to mitochondrial impairment due to their high demand for ATP (56,57). Because ATP biosynthesis occurs on the mitochondrial cristae (58), it could be assumed that DZN exposure resulted in axonal deformation or degeneration by causing mitochondrial impairment and cristolysis. Furthermore, this situation might give rise to neurodegenerative dysfunctions in the exposed *O. niloticus* individuals. Because axonal degeneration is commonly regarded as a distinctive feature of neurodegenerative disorders (59).

In the brain, dendrites serve as primary sites for receiving, integrating, and processing a multitude of excitatory synaptic inputs, as well as some inhibitory inputs, which terminate on either the dendritic shaft or spines. The intricate morphology and size of dendrites significantly influence the connectivity patterns between neurons, as dendritic trees spread out into characteristic spatial domains to receive specialized synaptic inputs (60). Hence, dendrites are crucial for integrating these inputs and modulating the generation of action potentials (61). Therefore any disturbances in dendrite extensions could cause neurodegenerative impairments in the brain of organisms. Accordingly, it could be said that DZN caused neurodegenerative impairment in the brain of exposed *O. niloticus* individuals by causing ultra morpho-pathological alterations in the dendrite extensions of this species.

In this study, it was determined that sublethal DZN concentration (280 µg/L) caused histopathological and ultrastructural alterations in the brain tissue of *O. niloticus* exposed to the standard formulation of this pesticide for 21 days. The severity of these alterations was increased with the duration of the time. Histopathologically the most common alterations were determined as cloudy swelling, hypertrophy and pycnotic nucleus in the glial cells. These alterations could indicate that DZN disturbed the cellular membranes, and caused oxidative stress and impairment of cellular energy in the brain tissue of *O. niloticus*. Ultrastructural examination of the brain tissue showed that mitochondrial deformation was evident in the cells of brain tissue. With this finding, it could be assumed that this pathological alteration caused axon and dendrite deformation which might result in neurodegenerative impairment in the brain of exposed *O. niloticus* individuals. More detailed biochemical and molecular studies can be suggested for a better understanding of DZN toxicity on the brain of fish species in future studies.

**ACKNOWLEDGEMENTS**

This study was supported by the Scientific and Technological Research Council of Turkey (TUBITAK) [Grant number:114Z730] and also we thank Süreyya Kaçar, electron microscope technician, for her outstanding contributions to our work.

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