Histopathological and Cytopathological Determination of Alterations and Immune Response in Spleen Tissue of *Oreochromis Niloticus* Exposed to Sub-Lethal Concentration of Diazinon

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Abstract

In this study, it was aimed to determine duration based histopathological, cytopathological and immunological alterations in spleen structure of *Oreochromis niloticus* samples exposed to sublethal concentration of diazinon standart with light microscope and TEM. At the first step of the study, the fish were exposed to 280 µg/L (LC_{50}/10) Diazinon for 21 days. Afterwards, the histopathological, cytopathological alterations and immune response in the spleen tissue were investigated by light microscope and TEM at 7th, 14th and 21st days. In the histopathological results, melanomacrophage centers with hemosiderin accumulation, cloudy swelling, pyknosis, hypertrophy and necrosis were detected in the spleen tissue. The severity of these alterations increased with the duration of the exposure. Mitochondrial deformation (cristolysis), pyknotic nucleus, vacuolation, increased phagositic activity of melanomacrophage, nuclear deformation, high amount of melanin and hemosiderin accumulation were detected in the cytopathological investigation of spleen tissue. According to those results it could be assumed that diazinon caused histopathological and cytopathological alterations in the spleen tissue of O. niloticus and also had immunotoxic effects.

Introduction

Pesticides, which are widely used in the world, play an important role in increasing agricultural productivity. However, as a result of unconscious pesticide use, human, air, water, soil and wild life are adversely affected, resistance occures in the target organisms, natural balance is disrupted by killing of beneficial organisms and phytotoxicity is seen in plants (Yıldırım 2008). Pesticides used in agricultural areas, reach fresh water sources via washing of pesticide treated soil surfaces with rain or irrigation water, or via contaminated solid and liquid particles in the atmosphere (Cengiz 2006). The organisms living in this polluted aquatic environments are exposed to these pesticides and effected acutely or chronically (Leight and Van Dolah 1999). Therefore, we should know the possible effects of these pollutants which contaminate the natural environments on organisms living in those environments.

Organic phosphorus (OP) insectisides constitute the most important and common class of insecticides used since the mid-1940s (Gültekin et al. 2001). The primary target of this group of insecticides in the organism is acetylcholine esterase enzymes (AChE) (Hazarika 2003; Kaur and Sendhu 2008) and they irreversibly inhibit AChE (Giordano et al. 2007). Diazinon (DZN) [O,O-diethyl-O-(2-isopropyl-6-methylpyrimidine-4-pyrimidinyl) phosphorothioate] is one of the most widely used OP insecticide and is used to protect many crops from a wide range of hymenopteran and hemipteran insects (Abass et al. 2011). After agricultural application, DZN may easily wash away and reach the surface water reservoirs, thus affecting a wide range of nontarget aquatic animals, including invertebrates and fish (Rauf and Arain 2013). There are many studies in the literature that DZN is highly toxic to aquatic organisms, including fish, and has various toxic effects on these organisms (Aydın and Köprücü, 2005; Üner et al., 2006; Rauf and Arain, 2013). The 96 h LC_{50} value of DZN for *Oreochromis niloticus* was reported as 2800 µg/L by El-Sherif et al. (2009).

Fish are one of the first preferred organisms in ecotoxicological studies because they play an important role in the food network, accumulate toxic compounds and respond to mutagens even at low doses (Çavaş and Ergene Gözükara 2005). *O. niloticus* (Linnaeus, 1758) belongs to one of the most important groups of fish known as good biological models due to their suitability for maintenance in culture conditions, their high capacity of reproduction, strong resistance to pollution and various diseases (Almedia 2002). The use of fish biomarkers in experimental studies has an increasing value to determine the effects of a contamination and enable early identification of aquatic environmental problems (Van der Oost et al. 2003).

The immune system in fish consists of organs, cellular structures and humoral (liquid) factors. Organs that make up the immune system are also known as lymphoid organs (Dönmez 2016). In the fish species, spleen is one of the primary lymphoid organ (Ocak 2006) and also the most important organ in terms of formation of blood cells and storage of antigens (Lawrence and Hemingway 2003). It also relates to the defense mechanism against diseases and pollutants in fish (Sundaresan 2014). There are studies in the lierature that pesticides cause toxicological alterations in the spleen of fish species. Banaee et al. (2011) reported that DZN caused increase in the size of spleen in *Cyprinus carpio*. They also reported that this pesticide increased the frequency of melanomacrophage and ellipsoid cells in the spleen. In another study, it has been found that methiocarb and endosulfan causes pathological lesions in the spleen of rainbow trout (Altınok and Çapkın 2007). Also, there are many studies in the literature that pesticides induce immune responses in fish spleen (Li et al. 2013; Ma et al. 2014; Ma and Li 2015; Díaz-Resendiz et al. 2019). According to these results, it can be suggested that altered structure and function of fish spleen can be used as biomarkers of general environmental degradation and the toxic effects of chemicals.

Monitoring only chemical accumulation in an ecosystem is not sufficient to assess its effects on organisms, populations and communities (Velmurugan 2011). In terms of sub-lethal levels of a chemical, the response of the organism to contamination can only be assessed by measuring its biological, physiological and biochemical parameters (Lagadic et al. 2000). Cellular biomarkers, including histopathological and cytopathological effects, provide an important link between the biochemical effects of an organism's internal chemistry and responses occuring in the individuals or populations. Such responses typically occur before behavioral changes and are more sensitive than growth or reproductive parameters. Also, they provide more information about the health status of an organism as integrative parameters than the assessment of a single biochemical response (Segner and Braunbeck 1998). Therefore, histological examination, as an indicator of exposure to pollutants, is a useful method for evaluating the degree of pollution, especially for subletal and chronic effects (Bernet et al. 1999). Transmission Electron Microscope (TEM) is also a very useful tool for determining the early stages of the sub-lethal effects of pollutants, since it allows the examination of tissue at cellular and even organelle levels (Velmurugan 2011). In this context, the histopathological and cytopathological examinations carried out in the species to determine the contamination in the aquatic ecosystems can provide us useful data about the health status of these ecosystems.

Although there are studies about histopathological and immunological effects of DZN on some fish species, there are no studies about the cytopathological effects of this pesticide on the immune response in the spleen of *O. niloticus* in the literature. Therefore, in this study, it is aimed to determine sub-chronic duration based possible histopathological and cytopathological alterations in spleen structure and immune response of *O. niloticus* samples exposed to certain concentration of DZN standart with light microscope and TEM.

Materials And Methods

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

O. niloticus samples were obtained from the breeding ponds of Faculty of Fisheries of Çukurova University, Adana, Turkey. Fish were anesthetized with phenoxiethanol (200 mg/l) and brought to the Hydrobiology and Aquatic Toxicology Research Laboratory, Biology Department, Science Faculty, Dicle University, Diyarbakır, Turkey.

O. niloticus samples were taken into 6 glass aquariums (40×35×40 cm) which contained dechlorinated tap water and were airated with central vantilation system in a climate-conditioning cabinet specially made for fish. There were 25 *O. niloticus* specimens in each aquariums. The fish were acclimated to laboratory conditions for 15 days before experiments. During this period and experiments, the fish were kept under an artificial light regime (14 hrs light: 10 hrs dark) at 26±1 °C temperature and fed with commercial pellets daily. Approximately 50% of each aquarium's water was subsitoted by dechlorinated tap water daily.

The Experimental Design

The analytical standard of DZN (PESTANAL® from SIGMA-ALDRICH®, CAS Number: 333-41-5) was used for the study. In order to avoid fish deaths and cause pathological lesions at the same time, 1/10 of previously reported LC50 value of DZN (El-Sherif et al. 2009) for *O. niloticus* was chosen as sublethal concentration for test concentarion after dissolving in the acetone and water and applied according to per liter of water in the aquarium. The test design was static renewal (APHA 1998). 15 samples in each of three replicates were used per groups. Test duration was 21 days. Test groups were control (Group I), aceton control (Group II) and exposure (280 µg/L DZN standart) (Group III) groups. Only one group's features of three replicates were given in Table 1. For the acetone control group the concentration of acetone in the exposure group solution was applied per liter. The chemical parameters of medium water were given in Table 2. Our study was approved by the Experimental Animals Local Ethics Committee of Dicle University (Protocol number: 2013/43) and during the experiments, EU Directive 2010/63/EU for animal experiments guidelines was followed for the study.

Table 1
The experimental groups of the study

Group Number	Groups	Number of individuals	Test Duration	*Sex of the individuals
I	Control	15	21 days	8F/7M
II	Asetone Control	15	21 days	6F/9M
III	280 µg/L DZN exposure (LC_{50}/10)	15	21 days	7F/8M
*F: Female, M: Male				

Table 2
The chemical parameters of medium water

Chemical Parameters	
pH	7.94±0.505
Dissolved oxygen	7.5±0.38 mg/L
Total chlorine	42.6 mg/L
Total hardness	287±2.35 mg/L CaCO3
Mg	36 mg/L
Electrical conductivity	7.94 Mmho/cm
NO3-N	2.1 mg/L
NO2-N	0.002 mg/L

The Histopathological Analysis

At 7th, 14th and 21st days of the experiment 5 fish were taken randomly from each exposure and control groups. The fish were immeddiately sacrificed by decapitation. Spleen samples were taken and immediately fixed with 10% formalin solution for 24 hrs at +25 ºC. After fixation, samples were washed under the tap water for 1 night for removing the fixative from the tissue. Afterwise, the samples were dehidrated with increasing grade of ethanol (30, 50, 70, 80, 90, 96 and 100%). Then, the samples were cleared with xylene and embedded in parafin. 5 µm sections were cut by microtome (LEICA). After cutting, the sections were stained with Hematoxylin-Eosin (Gurr 1972) and examined with a light microscope (Nikon NIS-Elements ECLIPS SE80i). The alterations were photographed with the camera (Nikon Digital SIGHT-DS2MV) on the microscope.

The Cytopathological Analysis

As in the histopathological preparation of the samples, at 7th, 14th and 21st days of the experiment 5 fish were taken randomly from each exposure and control groups and then sacrificed with decapitation.

The removed spleen samples were fixed with 2,5% glutaraldehyde solution for 24 hrs at +4 ºC. After fixation with glutaraldehyde solution, the samples were washed with phosphate buffer (pH: 7,4 and 0,1 M), postfixed with osmium tetroxide, dehidrated with increasing grade of ethanol (50, 70, 85, 90, 96 and 100%), maintained in propylene oxide for 30 mn at 25 ºC and propylene oxide-araldit (resin) mixture (1/1 v:v, for 2 hrs at 25 ºC), embedded in resin and incubated for 48 hrs at 60 ºC for polymerization. After preparation 70-110 nm sections were cut with ultramicrotome (LEICA) and taken on copper grids. The grids with the sections were stained with uranyl acetate and lead. Then, the samples were examined with TEM (Jeol /JEM-1010) in Dicle University Science and Technology Application and Research Center (DUBTAM), Diyarbakır, Turkey and the cytopathological alterations were photographed at 80kV (GATAN/782 ES500W Erlangshen CCD Camera).

Results

The Histopathological Results

No histopathological changes were observed in the spleen tissue of *O. niloticus* samples in the control and acetone control groups (Group I and II) for 21 days (Fig. 1a and 1b). Mild cloudy swelling, pyknosis and hypertrophy was recorded in the spleen tissue of fish exposed to 280 µg/L DZN concentration for 7 days. A few melanomacrophage centers (MMCs) with hemosiderin accumulation were detected in the tissues (Fig. 1c). At the 14th day of the exposure the severety of histopathological alterations were increased in parallel. During this period, necrotic areas began to appear in the spleen tissue of the fish. The number of cells with pyknotic nucleus increased and cloudy swelling became evident (Fig. 1d). The severety of hypertrophy and the number of MMCs with hemosiderin accumulation were also increased. The most prominent histopathological changes in the spleen tissue at the end of 21 days were the spread of necrosis and cloudy swelling throughout the tissue. The number of pyknotic nuclei and MMCs with hemosiderin accumulation were the highest (Fig. 1e). Semiquantitative scoring of histopathological lesions in spleen tissues of *O. niloticus* were given in Table 3.

Table 3
Semiquantitative scoring of histopathological lesions in spleen tissue of O. niloticus

LESIONS	TEST DURATION	GROUPS*		
		I	II	III
Cloudy swelling	7 days	–	–	+
	14 days	–	–	++
	21 days	–	–	+++
Pyknosis	7 days	–	–	+
	14 days	–	–	++
	21 days	–	–	+++
Hypertrophy	7 days	–	–	+
	14 days	–	–	++
	21 days	–	–	–
Number of MMCs	7 days	–	–	+
	14 days	–	–	++
	21 days	–	–	+++
Haemosiderin accumulation	7 days	–	–	+
	14 days	–	–	++
	21 days	–	–	+++
Necrosis	7 days	–	–	–
	14 days	–	–	+
	21 days	–	–	+++
*(–) none, (+) mild, (++) moderate, (+++) severe.				

The Cytopathological Results

No cytopathological changes were observed in the spleen tissue of *O. niloticus* samples in the control (Fig. 2a, 2b, 2c, 2d and 2e) and acetone control (Fig. 3a, 3b, 3c, 3d and 3e) groups (Group I and II) for 21 days. Mitochondrial deformation (cristolysis), pyknotic nucleus and vacuolation were observed in the spleen endothelial cells of fish exposed to 280 µg/L DZN concentration for 7 days (Fig. 4a and 4c). At the same time, nuclear deformation was detected in some reticulocytes and lymphocytes (Fig. 4b). Also, cellular debris and melanomacrophage (MMa) that phagocytizing degenerated erythrocyte were seen in the spleen tissue at 7th day of the exposure (Fig. 4d). At the 14th day, the cell debris because of necrosis

became evident. Melanin and heamosiderin accumulation in the necrotic areas were detected (Fig. 5a, 5b and 5c). The vacuolation in the endothelial cell of spleen tissue was also seen at the end of 14th day (Fig. 5d). The most severe pathological alteration in the spleen tissue of exposed fish at the 21st day was necrosis. The necrotic areas were seen widely troughout the tissue and the accumulation of heamosiderin in these areas was remarkable (Fig. 6a, 6b and 6c). Degenerated lymphocyte with vacuolation was also detected in the spleen tissue at 21st day (Fig. 6d).

Discussion

Fish under the influence of environmental pollution try to adapt through immune system activity or nonspecific defense systems. The immune system in fish consists of organs, cellular structures and humoral (liquid) factors. The organs that make up the immune system are also known as lymphoid organs. Lymphoid organs in fish are divided into two groups as primary and secondary. The primary lymphoid organs are the thymus, kidneys, and spleen, while the secondary lymphoid organs are the intestines, physical barriers (skin, gill), and liver (Zapata et al. 2006; Ocak 2006; Uribe et al. 2011). Recent studies show that histological findings of these organs are biomarker parameters for determining the physiological stress in fish caused by water quality changes (Osman et al. 2010; Liebe et al. 2013).

Pathological changes in fish spleen such as proliferation of white pulp, decrease in lymphocyte count, increase in spleen size, hemosiderosis and increase in melanomacrophage centers are generally considered to be the result of environmental contamination (Garcia Abiado et al. 2004; David and Kartheek 2015). At the 7th and 14th days of our study cloudy swelling and hypertrophy (Fig. 1c and 1d) were seen in the exposed tissue which could be lead to increased size of spleen. The increase in spleen cell size-number and the corresponding increase in spleen weight usually reflects xenobiotic-induced changes in the immune system and a proliferative response to xenobiotics (Guo and White 2010). Acute cell swelling is considered as a response to cell membrane damage caused by lipid peroxidation, direct binding of xenobiotics (such as pesticides) to the cell membrane, damage to ion channels, and the addition of transmembrane pore-forming complexes to the cell membrane (Miller and Zachary 2017). As seen in the results the severity of histopathological alterations increased with the duration of the exposure (Table 3). In this context, on the 21st day of the study, the most prominent histopathological change in the spleen tissue was necrosis spreading throughout the tissue (Fig. 1e). There are studies in the literature that pesticides cause necrotic alterations in the spleen tissues of fish species (Capkin et al. 2010; Karim et al. 2016; Farhan et al. 2021). In accordence with these findings the diffuse necrosis throughout the spleen tissue detected at the 21st day of our study. The number of pyknotic nucleus increased in parallel with the duration of the study (Table 3). Pyknosis is suggested as the irreversible condensation of chromatin and nuclei observed in both apoptotic and necrotic cell death (Hou et al. 2016). In this context, pyknosis could be considered as an early sign of necrotic cell death in the tissues exposed to environmental toxicant such as pesticides.

Macrophages which are one of the cellular factors of the immune system are indicator cells of innate immunity in fish and other vertebrates (Dönmez 2016). Macrophages in poikilotherm organisms form

clusters with pigment cells called pigmented macrophages or melanomacrophage centers (MMCs) (Satizabal 2013). Because of their pigmentation, MMa can be distinguished from macrophages in histological examinations, and they can also be observed widely in many organs of poikilotherm vertebrates (Kranz 1989; Ferreira 2011). According to the results of our study the number and size of MMCs were increased with the duration of the experiment (Table 3). The number, size and pigment contents of macrophages change in poor health conditions of fish, under stress and in response to environmental contaminants. The size, number and histopathological appearance of MMCs and the levels of macrophage activities such as chemotaxis, phagocytosis, pinocytosis and chemiluminescence are considered as important parameters among immunological biomarkers, especially in determining the effect of environmental pollutants and in bacteriological infections (Van der Oost et al. 2003; Faccioli et al. 2014; Ledic-Neto et al. 2014). There are studies in the literature that confirms these findings. It was determined that there was an increase in the number of MMCs in *Carassius auratus* where phenylhydrazine, a substance used in the paint and pharmaceutical industry, was applied (Herraez and Zapata 1986). In the microscopic examination of spleen tissues of 7 different fish species in the Gulf of Mexico, it has been accepted that the presence of more than 40 MMCs per mm^2 area can be associated with the presence of contamination in the hypoxic environment or sediment (Fournie et al. 2001). There are also studies showing that the metric properties (number, size and percentage of tissue invasion) of MMCs differ in regions where pollutants are concentrated (Rabitto et al. 2005; Suresh 2009; Ali et al. 2014).

In the cytopathological results, At the 7th and 21st days of our study the phagocytic activity of MMa was recorded associated with increased number of MMCs (Fig. 4d and 6c). The increase in the number of MMCs is generally thought to occur due to increased phagocytic activity within the cellular defense system to remove cellular debris (Ghosh and Homechaudhuri 2012; Ledic-Neto et al. 2014). In parallel with the phagocytic activity of MMa, it was seen that at the 14th and 21st days of the study there were remarkable hemosiderin and melanin accumulation in the spleen tissue (Fig. 5a, 5b, 5c, 6a and 6b). In our study the main source of hemosiderin accumulation was thought to be because of increased damaged erythrocyte destruction by MMa (Fig. 4d). Hemosiderin pigment is the most important pigment in the intracellular storage of iron during the degradation of hemoglobin and serves as an intermediate step in the recycling of iron. The pathological condition caused by the high level accumulation of hemosiderin is called hemosiderosis which is associated with increased erythrocyte destruction in the spleen (David and Kartheek 2015). Toxicology studies show that the disruptive action of different insecticides on the erythropoietic tissue such as kidney and spleen may decrease erythrocyte number and hemoglobin content as an anemic sign, and even lead to death of fish (Karim et al. 2016; Marteja and Modina 2021). Catabolism of damaged erythrocytes and iron retention in MMCs are two possible metabolisms of increased hemosiderin-iron accumulation in fish spleen (Agius and Roberts 2003). In addition, in cases where erythrocyte phagocytosis is increased, it has been determined that MMCs containing hemosiderin-iron in the spleen increase in parallel (Dönmez 2016).

The second important pigment is melanin which is thought to be derived from different exogenous sources or formed inside the cell. It is thought that melanin has an important role in neutralizing the free radicals, cations and toxic agents that occur during the destruction of phagocytized cell membranes and plays a role in the production of antimicrobial compounds such as hydrogen peroxide (Solano 2014). It has been also reported that an increase in the amount of MMCs containing melanin is a specific indication of chronic inflammation (Haaparanta et al. 1996; Jansson 2002). It is generally thought that the mechanism that causes inflammation in the tissues is necrosis (Yang et al. 2015). These findings could be the explanation of high amount of melanin accumulation in the spleen tissue of *O. niloticus* exposed to DZN at 14th day of the study where necrosis was detected (Fig. 5a).

The other important cytopathological alteration caused by DZN exposure at the 7th day of the study was mitochondrial deformation (cristolysis) in the spleen endothelial cells of *O. niloticus* (Fig. 4a). Mitochondria are vital in eukaryotic organisms as they play a central role in biological fuel production, ATP production by phosphorylation, and programmed cell death (apoptosis) (Bras et al. 2005; Voet et al. 2006; Heath-Engel and Shore 2006). Mitochondrial energy production could be impaired in pathological conditions (oxidizing chemicals, ischemia, hypoxia, calcium and other chemical agents) leading structural disorders in mitochondria which cause cellular damage. As a consquence of this impairment, necrotic cell death; because of ATP depletion, ion disorganization, mitochondrial-cellular swelling, activation of degrading enzymes, plasma membrane failure and cell lysis, may occur (Nieminen 2003). According to these findings, mitochondrial deformation (cristolysis) detected in the early days of our study (day 7) could be another cause of necrosis detected in the spleen tissue in the subsequent days of the study (days 14 and 21). Thus, there are studies in the literature reporting that DZN and other pesticides causes mitachondrial deformation in the tissues of some fish species (Samanta et al. 2018; Jindal and Sharma 2019; Díaz-Resendiz et al. 2020). Furthermore, cristolysis could be suggested as an important cytopathological feature for decreased metabolic activity of the cell since the enzymes linked to oxidative phosphorylation are located on inner mitochondrial membrane (Modica-Napolitano and Singh 2002).

The pyknotic nucleus recorded in spleen endothelial cells of fish at the 7th day of the study could be considered as an early sign of wide-spread necrosis at the 14th and 21st days of the study. Unlike apoptosis, in necrosis the cell nuclei condense into smaller chromatin clusters with irregular and scattered morphologies that can later be dissolved (Fujikawa et al. 2000; Bortul et al. 2001; Niquet et al. 2003). It was also suggested that necrotic pyknosis was likely to be initiated by the detachment of chromatin from the nuclear envelope (anucleolytic pyknosis) (Fujikawa et al. 2010; Hou et al. 2016). In this case, the nuclear envelope shrinks slightly, causing the chromatin to detach from the nuclear envelope. Subsequently, the nuclear envelope and chromatin are further condensed together, causing both structures to collapse (Sohn et al. 1998; Fujikawa et al. 2010; Hou et al. 2016). At the 7th day of our study the detachment of nuclear envelope and condensed nucleus are clearly seen in parallel with this finding (Fig. 4a). In this context, according the results gained from our study, it could be assumed that DZN caused cell death in the spleen tissue of *O. niloticus* because of necrosis rather than apoptosis.

It was previously reported in many studies that DZN caused vacuolar changes in the fish tissues (Banaee et al. 2013; Banik et al. 2016; Omar-Ali and Petrie-Hanson 2019). In accordence with these findings vacuolation was detected in the spleen tissue of *O. niloticus* at 7th, 14th and 21st days of our study (Fig. 4a, 4c, 5d and 6d). Vacuolization in eukaryotic cells can be temporary or irreversible. Irreversible vacuolization can cause cell death in the presence of a cytotoxic substance. The death of the cell could be because of the endoplasmic reticulum (ER), endosomal-lysosomal system and Golgi apparatus affected by irreversible vacuolization (Shubin et al. 2016). At the 21st day of the study the vacuolation was detected in the lymphocyte of *O. niloticus* (Fig. 6d). This result is especially important because it was known that OPs, including DZN, have immunotoxic effects on some fish species (Al-Ghanim 2012; Ahmadi et al. 2014; El-Bouhy et al. 2016; Díaz-Resendiz et al. 2019). In some studies it was suggested that the immunotoxicity of OPs was because of their disruptive effect on leukocyte cholinergic system (Toledo-Ibarra et al. 2016; Díaz-Resendiz et al. 2019). However, another study reported that some OPs deregulated lysozyme activity in immune cells of fish species (Li et al. 2013). The later finding is more relevant with our results in the manner of the cause of vacuolation in the spleen endothelial cells and lymphocytes of *O. niloticus* exposed to DZN.

Nuclear deformation was another cytopathological alteration detected at the 7th day of the study in the spleen tissue of *O. niloticus* exposed to DZN. Micronucleus, deformed nucleus and nuclear shift were some of the chemical induced nuclear deformations reported previous studies (Ali et al. 2008; Anbumani and Mary 2011). Nuclear abnormalities such as lobbed, blebbed, notched nuclei and binucleated cells have been shown in some studies as an indicator of genotoxicity (Da Silva Souz and Fontanetti 2006). Nuclear deformation and abnomalities were reported in some previous studies in fish species exposed to OPs and other pesticide groups (Muranli and Guner 2011; Kumar 2012; Khatun et al. 2021). In contrast to our study, in previous studies, it was detected that with the duration of the exposure such abnormalities were increased in parallel (Khatun et al. 2021). It was also reported positive correlation between dosage level and number of nuclear abnormalities (Ruiz de Arcaute et al. 2016; Khan et al. 2021). According to the results gained from our study, it could be suggested that DZN had genotoxic effect on the spleen tissue f *O. niloticus*.

Conclusions

The results of this study indicated that sublethal concentration of DZN caused histopathological and cytopathological alterations in the spleen tissue of *O. niloticus*. The severity of these alterations increased with the duration of the exposure. The proliferation of MMCs, increased phagositic activity of MMa, vacuolation and nuclear deformation in the lymphocytes could be sign of immunotoxic and genotoxic effects of DZN. Whereas hemosiderin accumulation in the spleen tissue could indicate disruptive effect of DZN on erythrocytes, accumulation of melanin might show inflammatory features of this pollutant. Also, it was seen that DZN caused pathological changes in the mitochondria of endothelial cells of spleen tissue which lead to cell necrosis. It was observed that DZN induced necrosis rather than apoptosis in the spleen tissue. The histopathological and cytopathological evaluation of changes in fish

spleen can also be suggested as suitable biomarkers in determining the immunotoxicological effect of an environmental pollutant.

Declarations

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Ethics Approval: This study was approved by the Experimental Animals Local Ethics Committee of Dicle University (Protocol number: 2013/43) and during the experiments, EU Directive 2010/63/EU for animal experiments guidelines was followed.

Availability of data and materials: The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Consent to Participate: Not applicable.

Consent to Publish: Not applicable.

References

1. Abass A, Kudi AC, Moodi AJ (2011) Spontaneous reactivation and aging kinetics of acetylcholinesterase inhibited by dichlorvos and diazinon. J Toxicol Sci 36:237–241. https://doi.org/10.2131/jts.36.237
2. Agius C, Roberts RJ (2003) Melano-macrophage centres and their role in fish pathology. J Fish Dis 26:499–509. https://doi.org/10.1046/j.1365-2761.2003.00485.x
3. Ahmadi K, Mirvaghefei A, Banaee M, Vosoghei A (2014) Effects of long-term diazinon exposure on some immunological and haematological parameters in rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792). Toxicol Environ Health Sci 6(1):1–7. https://doi.org/10.1007/s13530-014-0181-1
4. Al-Ghanim KA (2012) Acute toxicity and effects of sub-lethal malathion exposure on biochemical and haematological parameters of *Oreochromis niloticus*. Sci Res Essays 7(6):1674–1680. https://doi.org/10.5897/SRE12.039
5. Ali AO, Hohn C, Allen PJ, Ford L, Dail MB, Pruett S, Petrie-Hanson L (2014) The effects of oil exposure on peripheral blood leukocytes and splenic melano-macrophage centers of Gulf of Mexico fishes. Mar Pollut Bull 79:87–93. https://doi.org/10.1016/j.marpolbul.2013.12.036

6. Ali FK, El-Shehawi AM, Seehy MA (2008) Micronucleus test in fish genome: A sensitive monitor for water pollution. Afr j biotechno 7:606–612
7. Almedia JA, Diniz YS, Marques SFG, Faine LA, Ribas BO, Burneiko RC, Novelli ELB (2002) The use of the oxidative stress responses as biomarkers in nile tilapia (*Oreochromis niloticus*) exposed to in vivo cadmium contamination. Environ Int 27(8):673–679. https://doi.org/10.1016/s0160-4120(01)00127-1
8. Altinok I, Capkin E (2007) Histopathology of rainbow trout exposed to sublethal concentrations of methiocarb or endosulfan. Toxicol Pathol 35:405–410. https://doi.org/10.1080/01926230701230353
9. American Public Health Association (APHA) (1998) Standard Methods for the Examination of Water and Wastewater, American Water Works Association, Water Pollution Control Federation, 20th Edition
10. Anbumani S, Mary NM (2011) Nuclear and cytoplasmic abnormalities in the fish *Catla catla* (Hamilton) exposed to chemicals and ionizing Radiation. Res J Environ Sci 5:867–877. https://doi.org/10.3923/rjes.2011.867.877
11. Aydın R, Köprücü K (2005) Acute toxicity of diazinon on the common carp (*Cyprinus carpio* L.) embryos and larvae. Pestic Biochem Phys 82:220–225. https://doi.org/10.1016/j.pestbp.2005.03.001
12. Banaee M, Mirvaghefı AR, Mojazi Amiri B, Rafiee GR, Nematdost B (2011) Hematological and histopathological effects of diazinon poisoning in common carp (*Cyprinus carpio*). J Fisheries (Iranian J Nat Resources) 64(1):1–12
13. Banaee M, Sureda A, Mirvaghefı AR, Ahmadi K (2013) Biochemical and histological changes in the liver tissue of rainbow trout (*Oncorhynchus mykiss*) exposed to sub-lethal concentrations of diazinon. Fish Physiol Biochem. 39(3):489-501. https://doi.org/0.1007/s10695-012-9714-1
14. Banik U, Rahman MM, Khanam T, Mollah MFA (2016) Histopathological changes in the gonads, liver, and kidney of *Glossogobius giuris* exposed to sub-lethal concentration of diazinon. Progress agric 27(4):530–538. https://doi.org/10.3329/pa.v27i4.32143
15. Bernet D, Schmidt H, Meier W, Burkhardt-Holm P, Wahli T (1999) Histopathology in fish: proposal for a protocol to assess aquatic pollution. J Fish Dis 22(1):25–35. https://doi.org/10.1046/j.1365-2761.1999.00134.x
16. Bortul R, Zweyer M, Billi AM, Tabellini G, Ochs RL, Bareggi R, Cocco L, Martelli AM (2001) Nuclear changes in necrotic HL-60 cells. J Cell Biochem Suppl 36:19–31. https://doi.org/10.1002/jcb.1073
17. Bras M, Queenan B, Susin SA (2005) Programmed cell death via mitochondria: different modes of dying. Biochem (Mosc) 70:231–239. https://doi.org/10.1007/s10541-005-0105-4
18. Capkin E, Terzi E, Boran H, Yandi I, Altinok I (2010) Effects of some pesticides on the vital organs of juvenile rainbow trout (Oncorhynchus mykiss). Tissue Cell 42:376–382. https://doi.org/10.1016/j.tice.2010.10.001
19. Cavas S, Ergene-Gözükara S (2005) Induction of micronuclei and nuclear abnormalities in *Oreochromis niloticus* following exposure to petroleum refinery and chromium processing plant

effluents. Aquat Toxicol 74:264–271. https://doi.org/10.1016/j.aquatox.2005.06.001

20. Cengiz EI (2006) Gill and kidney histopathology in the freshwater fish *Cyprinus carpio* after acute exposure to delamethrin. Environ Toxicol Pharm 22(2):200–204. https://doi.org/10.1016/j.etap.2006.03.006
21. da Silva Souza T, Fontanetti CS (2006) Micronucleus test and observation of nuclear alterations in erythrocytes of Nile tilapia exposed to waters affected by refinery effluent. Mutat Res 605:87–93. https://doi.org/10.1016/j.mrgentox.2006.02.010
22. David M, Kartheek RM (2015) Histopathological alterations in spleen of freshwater fish *Cyprinus carpio* exposed to sublethal concentration of sodium cyanide. Open Vet J 5(1):1–5
23. Díaz-Resendiz KJG, Bernal-Ortega JA, Covantes-Rosales CE, Ortiz-Lazareno PC, Toledo-Ibarra GA, Ventura-Ramon GH, Girón-Pérez MI (2020) In-vitro effect of diazoxon, a metabolite of diazinon, on proliferation, signal transduction, and death induction in mononuclear cells of Nile tilapia fish (*Oreochromis niloticus*). Fish Shellfish Immunol 105:8–15. https://doi.org/10.1016/j.fsi.2020.07.001
24. Díaz-Resendiz KJG, Ortiz-Lazareno PC, Covantes-Rosales CE, Trujillo-Lepe AM, Toledo-Ibarra GA, Ventura-Ramón GH, Girón-Pérez MI (2019) Effect of diazinon, an organophosphate pesticide, on signal transduction and death induction in mononuclear cells of Nile tilapia fish (*Oreochromis niloticus*). Fish Shellfish Immunol 89:12–17. https://doi.org/10.1016/j.fsi.2019.03.036
25. Dönmez AE (2016) Melanomacrophage centers in fishes. EgeJFAS 33(1):81–87
26. El-Bouhy ZM, El-Nobi G, Reda RM, Ibrahim RE (2016) Effect of insecticide "chlorpyrifos" on immune response of *Oreochromis niloticus*, Zagazig Vet. J 44:196–204. https://doi.org/10.21608/zvjz.2016.7872
27. El-Sherif MS, Ahmed MT, El-Danasoury MA, El-Nwish NHK (2009) Evaluation of diazinon toxicity on nile tilapia fish (*O. niloticus*). J Fish Aquat Sci 4(4):169–177. https://doi.org/10.3923/jfas.2009.169.177
28. Faccioli CK, Chedid RA, Bombonato MTS, Vicentini CA, Vicentin IBF (2014) Morphology and histochemistry of the liver of Carnivorous fish *Hemisorubim platyrhynchos*. Int J Morphol 32(2):715–720. https://doi.org/10.4067/S0717-95022014000200055
29. Farhan M, Wajid A, Hussain T, Jabeen F, Ishaque U, Iftikhar M, Daim MA, Noureen A (2021) Investigation of oxidative stress enzymes and histological alterations in tilapia exposed to chlorpyrifos. Environ Sci Pollut Res 28:13105–13111. https://doi.org/10.1007/s11356-020-11528-y
30. Ferreira CMH (2011) Can fish liver melanomacrophages be modulated by xenoestrogenic and xenoandrogenic pollutants? Experimental studies on the influences of temperature, sex, and ethynylestradiol, using the platyfish as the model organism. Master of Science Thesis, Universidade de Porto, Institute of Biomedical Sciences Abel Salazar, 53 p
31. Fujikawa DG, Shinmei SS, Cai B (2000) Kainic acid-induced seizures produce necrotic, not apoptotic, neurons with internucleosomal DNA cleavage: implications for programmed cell death mechanisms. Neuroscience 98:41–53. https://doi.org/10.1016/s0306-4522(00)00085-3

32. Fujikawa DG, Zhao S, Ke X, Shinmei SS, Allen SG (2010) Mild as well as severe insults produce necrotic, not apoptotic, cells: evidence from 60-min seizures. Neurosci Lett 469:333–337. https://doi.org/10.1016/j.neulet.2009.12.022
33. Garcia-Abiado MA, Mbahinzireki G, Rinchard J, Lee KJ, Dabrowski K (2004) Effect of diets structure in tilapia, Oreochromis sp., reared in a recirculating system. J Fish Dis 27:359–368. https://doi.org/10.1111/j.1365-2761.2004.00551.x
34. Ghosh R, Homechaudhuri S (2012) Transmission electron microscopic study of renal haemopoietic tissues of *Channa punctatus* (Bloch) experimentally infected with two species of aeromonads. Turk J Zool 36(6):767–774. https://doi.org/10.3906/zoo-1112-7
35. Giordano G, Afsharinejad Z, Guizetti M, Vitalone A, Kavanagh JT, Costa GL (2007) Organophosphorus insecticides chlorphyrifos and diazinon and oxidative stress in neuronal cells in a genetic model of glutathione deficiency. Toxicol Appl Pharmacol 219:181–189. https://doi.org/10.1016/j.taap.2006.09.016
36. Guo TL, White KL (2010) Methods to Assess Immunotoxicity. In: McQueen CA (ed) Comprehensive Toxicology. 2nd Edition, Elsevier, pp 567-590. https://doi.org/10.1016/B978-0-08-046884-6.00633-3
37. Gurr E (1972) Biological Staining Methods. Kent Printers. Tonbridge. Pp. 143
38. Gültekin F, Delibas N, Yasar S, Kılınç I (2001) In vivo changes in antioxidant systems and protective role of melatonin and a combination of Vitamin C and Vitamin E on oxidative damage in erythrocytes induced by chlorpyrifos-ethyl in Rat. Arch Toxicol 75:88–96. https://doi.org/10.1007/s002040100219
39. Haaparanta A, Valtonen ET, Hoffmann R, Holmes J (1996) Do macrophage centres in freshwater fishes reflect the differences in water quality? Aquat Toxicol 34:253–272. https://doi.org/10.1016/0166-445X(95)00042-3
40. Hazarika A, Sankar AN, Hajare S, Kataria M, Malik JK (2003) Influence of malathion pretreatment on toxicity of anilofos in male rats: a biochemical ınteraction study. Toxicology 185:1–8. https://doi.org/10.1016/s0300-483x(02)00574-7
41. Heath-Engel HM, Shore GC (2006) Mitochondrial membrane dynamics, cristae remodeling and apoptosis. Biochim Biophys Acta 1763:549–560. https://doi.org/10.1016/j.bbamcr.2006.02.006
42. Herraez MP, Zapata AG (1986) Structure and function of the melanomacrophage centres of the goldfish *Carassius auratus*. Vet Immunol Immunopathol 12(1–4):117–126. https://doi.org/10.1016/0165-2427(86)90116-9
43. Hou L, Liu K, Li Y, Ma S, Ji X, Liu L (2016) Necrotic pyknosis is a morphologically and biochemically distinct event from apoptotic pyknosis. J Cell Sci 129:3084–3090. https://doi.org/10.1242/jcs.184374
44. Jansson E (2002) Bacterial kidney disease in salmonid fish. Doctoral thesis, Swedish University of Agricultural Sciences Uppsala, 52 p
45. Jindal R, Sharma R (2019) Neurotoxic responses in brain of *Catla catla* exposed to cypermethrin: A semiquantitative multibiomarker evaluation. Ecol Indic 106:105485.

https://doi.org/10.1016/j.ecolind.2019.105485

46. Karim A, Ahmad N, Ali W (2016) Histopathological changes in spleen and kidney of silver carp (*Hypophthalmicthys molitrix*) after acute exposure to deltamethrin. Biol (Pakistan) 62(1):139–144
47. Kaur R, Sandhu HS (2008) In vivo changes in antioxidant system and protective role of selenium in chlorpyrifos-induced subcronic toxicity in *Bubalus bubalis*. Environ Toxicol Pharmacol 26:45–48. https://doi.org/10.1016/j.etap.2008.01.004
48. Khan N, Sultan A, Ali A, Jan S, Khan W, Rahman I, Usman HS, Khan Z, Khan A (2021) Fish as bioindicator: ecological risk assessment of insecticide to aquatic organism particularly *Ctenopharyngodon idella*. J Geoscience Environ Prot 9:42–54. https://doi.org/10.4236/gep.2021.92003
49. Khatun M, Mostakim GM, Moniruzzaman M, Rahman UO, Sadiqul Islam M (2021) Distortion of micronuclei and other peripheral erythrocytes caused by fenitrothion and their recovery assembla in zebrafish. Toxicol Rep 8:415–421. https://doi.org/10.1016/j.toxrep.2021.02.019
50. Kranz H (1989) Changes in splenic melano-macrophage centres of dab *Limanda limanda* during and after infection with ulcer disease. Dis Aquat Org 6:167–173
51. Kumar SP (2012) Micronucleus assay: A Sensitive indicator for aquatic pollution. Int J Biosci 1:32–37
52. Lagadic L, Caquet T, Amiard JC, Ramade F (2000) Use of biomarkers for environmental quality assessment. Technique et documentation, Laviosier, Paris, pp 55–157
53. Lawrence AJ, Hemingway KL (2003) Effects of Pollution on Fish.UK.144–153
54. Ledic-Neto J, Dotta G, Garcia P, Brum A, Gonçalves TEL, Martins ML (2014) Haematology and melanomacrophage centers of Nile tilapia fed supplemented diet with propolis. Acta Sci Biol Sci 36(3):263–269. https://doi.org/10.4025/actascibiolsci.v36i3.22024
55. Leight AK, Van Dolah RF (1999) Acute toxicity of the insecticides endosulfan, chlorpyrifos and malathion to the epibenthic estuarine amphipod *Gammarus palustris* (Bousfield). Environ Toxicol Chem 18(5):958–964. https://doi.org/10.1002/etc.5620180521
56. Li XY, Liu L, Zhang YN, Fang Q, Li YY, Li YL (2013) Toxic effects of chlorpyrifos on lysozyme activities, the contents of complement C3 and IgM, and IgM and complement C3 expressions in common carp (*Cyprinus carpio* L.). Chemosphere 93(2):428–433. https://doi.org/10.1016/j.chemosphere.2013.05.023
57. Liebe S, Tomotake MEM, Ribeiro CAO (2013) Fish histopathology as biomarker to evaluate water quality. Ecotoxicol Environ Contam 8(2):09–15. https://doi.org/10.5132/eec.2013.02.002
58. Ma JG, Li XY (2015) Transcription alteration of immunologic parameters and histopathological damage in common carp (*Cyprinus carpio* L.) caused by paraquat. J Biochem Mol Toxicol 29(1):21–28. https://doi.org/10.1002/jbt.21602
59. Ma JG, Li YY, Niu DC, Li Y, Li XY (2014) Immunological effects of paraquat on common carp, *Cyprinus carpio* L. Fish Shellfish Immunol 37(1):166–172. https://doi.org/10.1016/j.fsi.2014.01.015

60. Marteja JC, Modina RMR (2021) Melanomacrophage centers in nile tilapia (*Oreochromis niloticus* L.) as biomarker for carbamate exposure. J Environ Sci Manage 24(1):25–35. https://doi.org/10.47125/jesam/2021_1/03
61. Miller MA, Zachary JF (2017) Mechanisms and morphology of cellular injury, adaptation, and death. Pathologic Basis of Veterinary Disease 19:2–43. https://doi.org/10.1016/B978-0-323-35775-3.00001-1
62. Modica-Napolitano JS, Singh K (2002) Mitochondria as targets for detection and treatment of cancer. Expert Rev Mol Med 4(9):1–19. https://doi.org/10.1017/S1462399402004453
63. Muranli FDG, Güner U (2011) Induction of micronuclei and nuclear abnormalities in erythrocytes of mosquito fish (*Gambusia affinis*) following exposure to the pyrethroid insecticide lambdacyhalothrin. Mutat Res Genetic Toxicol Environ Mutagen 726:104–108. https://doi.org/10.1016/j.mrgentox.2011.05.004
64. Nieminen AL (2003) Apoptosis and necrosis in health and disease: Role of mitochondria. Int Rev Cytol 224:29–55. https://doi.org/10.1016/S0074-7696(05)24002-0
65. Niquet J, Baldwin RA, Allen SG, Fujikawa DG, Wasterlain CG (2003) Hypoxic neuronal necrosis: protein synthesis-independent activation of a cell death program. Proc Natl Acad Sci 100:2825–2830. https://doi.org/10.1073/pnas.0530113100
66. Ocak F (2006) Lenfoid organs and the properties of immune system in fish (in Turkish with English abstract). J Fac Veterinary Med Erciyes Univ 3(1):61–66
67. Omar-Ali A, Petrie-Hanson L (2019) Histopathological changes induced by chronic, sub-lethal Diazinon exposure in Alligator gar (*Atractosteus spatula*) Tissues. Int J Sci Res Environ Sci Toxicol 4(1):1–10. https://doi.org/10.15226/2572-3162/4/1/00128
68. Osman AGM, Abd-El Reheem AM, AbuelFadl KY, GadEl- Rab AG (2010) Enzymatic and histopathologic biomarkers as indicators of aquatic pollution in fishes. Nat Sci 2(11):1302–1311. https://doi.org/10.4236/ns.2010.211158
69. Rabitto IS, Alves Costa JRM, Silva de Assis HC, Pelletier E, Akaishi FM, Anjos A, Randi MAF, Oliveira Ribeiro CA (2005) Effects of dietary Pb(II) and tributyltin on neotropical fish, *Hoplias malabaricus*: histopathological and biochemical finding. Ecotoxicol Environ Saf 60:147–115. https://doi.org/10.1016/j.ecoenv.2004.03.002
70. Rauf A, Arain N (2013) Acute toxicity of diazinon and its effects on hematological parameters in the Indian carp, *Cirrhinus mrigala* (Hamilton). Turk J Vet Anim Sci 37:535–540. https://doi.org/10.3906/vet-1212-39
71. Ruiz de Arcaute C, Soloneski S, Larramendy ML (2016) Toxic and genotoxic effects of the 2,4-Dichlorophenoxyacetic Acid (2,4-D)-based herbicide on the neotropical fish *Cnesterodon decemmaculatus*. Ecotoxicol Environ Sa 128:222–229. https://doi.org/10.1016/j.ecoenv.2016.02.027
72. Samanta P, Kumari P, Pal S, Mukherjee AK, Ghosh AR (2018) Histopathological and ultrastructural alterations in some organs of *Oreochromis niloticus* exposed to glyphosate-based herbicide, Excel

Mera 71. J Microsc Ultrastruct 6(1):35–43. https://doi.org/10.4103/JMAU.JMAU_8_18

73. Satizabal LD (2013) Melano-macrophage characterization and their possible role in the goldfish (*Carassius auratus*) antibody affinity maturation. Master of Science Thesis, University of Alberta Department of Biological Science, Canada, pp 121
74. Segner H, Braunbeck T (1998) Cellular response profile to chemical stress. In: Schuurmann G, Markert B (eds) Ecotoxicology: Ecological Fundamentals, Chemical Exposure, and Biological Effects. Wley-Liss, New York, USA, pp 521–569
75. Shubin AV, Demidyuk IV, Komissarov AA, Rafieva LM, Kostrov SV (2016) Cytoplasmic vacuolization in cell death and survival. Oncotarget 7(34):55863–55889. https://doi.org/10.18632/oncotarget.10150
76. Sohn S, Kim EY, Gwag BJ (1998) Glutamate neurotoxicity in mouse cortical neurons: atypical necrosis with DNA ladders and chromatin condensation. Neurosci Lett 240:147–150. https://doi.org/10.1016/s0304-3940(97)00936-1
77. Solano F (2014) Melanins: skin pigments and much more-types, structural models, biological functions, and formation routes. New J Sci 1–28. https://doi.org/10.1155/2014/498276
78. Sundaresan M (2014) Ultrastructure of spleen in the freshwater fish, *Tilapia mossambica* (Peters). Eur Acad Res 2(2):2894–2908
79. Suresh N (2009) Effect of cadmium chloride on liver, spleen and kidney melanomacrophage centres in *Tilapia mossambica*. J Environ Biol 30(4):505–508
80. Toledo-Ibarra GA, Díaz-Resendiz KJG, Pavón-Romero L, Rojas-García AE, Medina-Díaz IM, Girón-Pérez MI (2016) Effects of diazinon on the lymphocytic cholinergic system of Nile tilapia fish (*Oreochromis niloticus*). Vet Immunol Immunopathol 176:58–63. https://doi.org/10.1016/j.vetimm.2016.05.010
81. Uribe C, Folch H, Enriquez R, Moran G (2011) Innate and adaptive immunity in teleost fish: a review. Vet Med 56(10):486–503
82. Üner N, Özcan Oruc E, Sevgiler Y, Şahin N, Durmaz H, Usta D (2006) Effects of diazinon on acetylcholinesterase activity and lipid peroxidation in the brain of *Oreochromis niloticus*. Environ Toxicol Pharmacol 21:241–245. https://doi.org/10.1016/j.etap.2005.08.007
83. Van der Oost R, Beyer J, Vermeulen NPE (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ Toxicol Pharmacol 13:57–149. https://doi.org/10.1016/S1382-6689(02)00126-6
84. Velmurugan B (2011) Identification of potential biomarkers for chlorpyrifos and cypermethrin exposed to fish *Anabas testudineus* (Bloch) using histological, biochemical, haematological, ultrastructural and molecular assays. PhD Thesis, Madras University, Chennai, India
85. Voet D, Voet JG, Pratt CW (2006) Electron transport and oxidative phosphorylation. In: Voet D, Voet JG, Pratt CW (eds) Fundamentals of Biochemistry, 2nd edn. John Wiley and Sons, New York, pp 492–528
86. Yang Y, Jiang G, Zhang P, Fan J (2015) Programmed cell death and its role in inflammation. Mil Med Res 2(1):1–12. https://doi.org/10.1186/s40779-015-0039-0

87. Yıldırım E (2008) Tarımsal Zararlılarla Mücadele Yöntemleri ve İlaçlar. Atatürk Üniversitesi Ziraat Fakültesi Ofset Tesisi, Erzurum
88. Zapata A, Diez B, Cejalvo T, Gutierrez-de Frias C, Corte A (2006) Ontogeny of the immune system of fish. Fish Shellfish Immunol 20:126–136. https://doi.org/10.1016/j.fsi.2004.09.005
89. Zapata A, Diez B, Cejalvo T, Gutierrez-de Frias C, Corte A (2006) Ontogeny of the immune system of fish. Fish Shellfish Immunol 20:126-136. https://doi.org/10.1016/j.fsi.2004.09.005

Figures

Figure 1

The histopathological changes induced by 280 µg/L DZN in the spleen tissue of O. nilotcus for 21 days: **a)** Spleen tissue of control group (Group I) at 21st day: White pulp (WP), Red pulp (RP), Melanomacrophage centers (MMCs); **b)** Spleen tissue of acetone control group (Group II) at 21st day: White pulp (WP), Red pulp (RP), Melanomacrophage centers with hemosiderin accumulation (MMCs); **c)** The histopathological alterations in the spleen tissue of O. niloticus exposed to 280 µg/L DZN at 7th day: Hypertrophy (Ht), Pyknotic nucleus (PN), Melanomacrophage centers with hemosiderin accumulation (MMCs), Cloudy swelling (CS); **d)** The histopathological alterations in the spleen tissue of O. niloticus exposed to 280 µg/L DZN at 14th day: Hypertrophy (Ht), Pyknotic nucleus (PN), Melanomacrophage centers with hemosiderin accumulation (MMCs), Cloudy swelling (CS), Necrosis (N); **e)** The histopathological alterations in the spleen tissue of O. niloticus exposed to 280 µg/L DZN at 21st day: Hypertrophy (Ht), Pyknotic nucleus (PN), Melanomacrophage centers with hemosiderin accumulation (MMCs), Necrosis (N) , H&E, ×400.

Figure 2

The spleen cells of O. niloticus in control group (Group I) at 21st day: **a)** Megakaryocyte: Nucleus (Nu); **b)** Melanomacrophage: Nucleus (Nu), Melanin (Me), Mitochondri (Mt); **c)** Endothelial cell: Nucleus (Nu); Erythrocyte (Er): **d)** Monocyte: Nucleus (Nu): **e)** Lymphocyte; Nucleus (Nu).

Figure 3

The spleen cells of O. niloticus in acetone control group (Group II) at 21st day: **a)** Megakaryocyte: Nucleus (Nu); **b)** Melanomacrophage: Nucleus (Nu), Melanin (Me); **c)** Endothelial cell: Nucleus (Nu); Mitochondri (Mt); **d)** Monocyte: Nucleus (Nu): **e)** Lymphocyte; Nucleus (Nu).

Figure 4

The cytopathological alterations induced by 280 µg/L DZN in the spleen tissue of O. nilotcus for 7 days: **a)** Cytoparhological alterations in the spleen endothelial cell of O. niloticus: Mitochondrial deformation (cristolysis) (MD), Pyknotic nucleus (PN), Vacuolation (V); **b)** Nuclear deformation in reticulocyte and lymphocyte: Nuclear deformation (ND); **c)** cytoparhological alterations in the spleen cells of O. niloticus: Pyknotic nucleus (PN), Vacuolation (V); **d)** cellular debris and melanomacrophage phagocytizing degenerated erythrocyte : Melanomacrophage (MMa), Degenerated erythrocyte (DEr), Cellular debris (CDb).

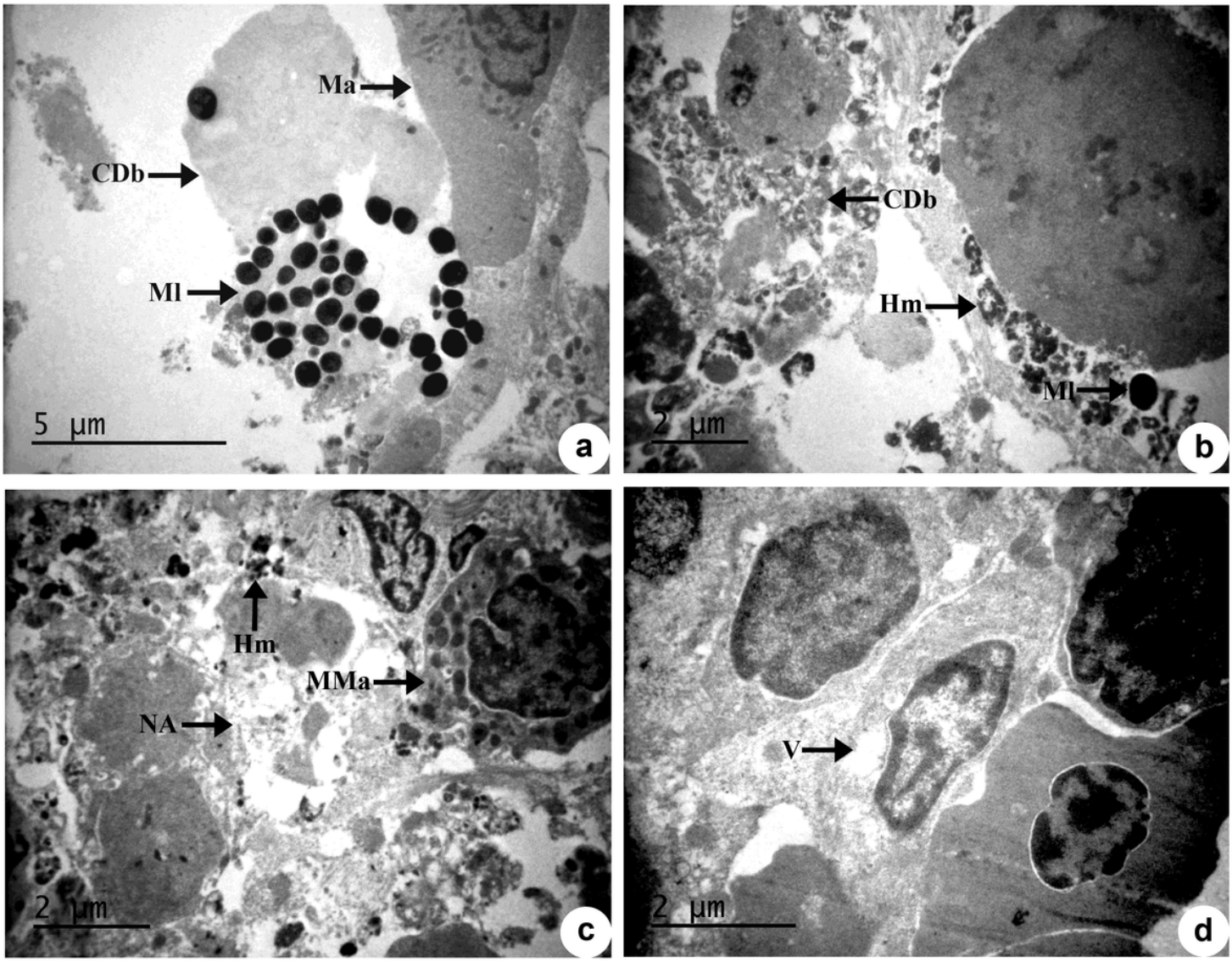

Figure 5

The cytopathological alterations induced by 280 µg/L DZN in the spleen tissue of O. nilotcus for 14 days: **a)** The cell debris and melanin accumulation in the spleen tissue of O. nilotcus: Melanomacrophage (MMa), Cellular debris (CDb), Melanin pigments (Ml): **b)** The cell debris because of necrosis and hemosiderin accumulation in the spleen tissue of O. nilotcus: Cellular debris (CDb), Melanin pigment (Ml), Hemosiderin accumulation (Hm); **c)** hemosiderin accumulation and necrotic area in the spleen tissue of O. niloticus: Hemosiderin accumulation (Hm), Melanomacrophage (MMa), Necrotic area (NA); **d)** Vacuolation in the spleen endothelial cell of O. niloticus: Vacuolation (V).

Figure 6

The cytopathological alterations induced by 280 µg/L DZN in the spleen tissue of O. nilotcus for 21 days: **a)** Necrosis with hemosiderin accumulation in the spleen tissue of O. niloticus; Necrotic area (NA), Hemosiderin accumulation (Hm); **b)** The cell debris because of necrosis and hemosiderin accumulation in the spleen tissue of O. nilotcus: Cellular debris (CDb), Hemosiderin accumulation (Hm); **c)** Melanomacrophage phagocytizing necrotic cell debris: Melanomacrophage (MMa), Cellular debris (CDb); **d)** Vacuolation in the spleen lymphocyte: Vacuolation (V).