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RESEARCH ARTICLE

Impact of acute fonofos exposure on skeletal muscle of zebrafish: Histopathological and biometric analyses

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Abstract – It is widely known that pesticides generally do not show target specificity, and off-target species are strikingly affected by these chemicals. In the current work, histological changes in skeletal muscles of zebrafish (*Danio rerio*) caused by fonofos, an acetylcholinesterase (AChE) inhibitor organophosphate insecticide, were examined. Zebrafish were treated with 1 mg/L, 2 mg/L and 4 mg/L of fonofos for 96 hours. Skeletal muscle samples were removed from the pectoral region and embedded in paraffin. Sections were stained with Mayer's Hematoxylin and Eosin, Gomori's Trichrome and Periodic Acid Schiff techniques. Histopathological alterations were investigated by light microscopy. Fibrosis, intramyofibrillar vacuoles, disintegrated myofibrils, splitting of myofibers, atrophic and disappeared fibers, histoarchitectural loss, necrosis and progressive decrement in glycogen content were noted. Muscle fiber diameter measurements were also performed. Statistical analysis showed that measured fiber diameters of all fonofos exposed groups were significantly different from the control group, and they decreased in a concentration-dependent manner. These results suggested that fonofos caused significant myoarchitectural impairments in non-target freshwater zebrafish.

Keywords: Organophosphate / insecticide / skeletal muscle / histopathology / *Danio rerio*

1 Introduction

Worldwide pesticide usage is still a big dilemma since it serves many advantages on agricultural crop productivity while it also causes considerable hazards to the health of farmers, off-target vertebrate species, and the environment. Although correctly used pesticides prevent up to 40% of crop loss (Richardson, 1998), it is also noted that they gave rise to annually at least 20.000 workers' death (Rahman, 2013). Pesticides are at the top of the most hazardous environmental pollutants list with metals and other organic compounds (Scott and Sloman, 2004). Numerous works reveal that vertebrates are strikingly affected by pesticide exposure (Kwon *et al.*, 2004; Bonfanti *et al.*, 2018; Chen *et al.*, 2019; Al-Ghanim *et al.*, 2020; Aliomrani *et al.*, 2021). It is estimated that less than 0.1% of pesticides applied to crop fields reach the primary target, with the remainder polluting the soil, water resources and air inhabited by various non-target species (Pimentel and Levitan, 1986; Arias-Estévez *et al.*, 2008).

Insecticides have one of the biggest market shares among pesticides. They are used to control approximately 9.000 species of insects and mites responsible for 14% of crop loss worldwide (Pimentel, 2009; Zhang *et al.*, 2011). Their chemical structure is substantially organophosphate (OP), and OPs are considered the most toxic pesticides to vertebrates (Shadnia *et al.*, 2005; Lukaszewicz-Hussain, 2010). Their mode of action is based on acetylcholinesterase (AChE) inhibition. When the AChE is inhibited, the neurotransmitter acetylcholine (ACh) can not be broken down in nerve synapses and muscular junctions. This case causes accumulation of ACh at these sites that lead to persistent stimulation of the muscle that is resulted in excessive stimulation and even death (Roex *et al.*, 2003).

Several studies are available in the literature revealing OP insecticides adversely affect aquatic organisms via diverse pathways (reviewed in Sidhu *et al.*, 2019). OP insecticides induced AChE inhibition (Üner *et al.*, 2006), histopathology (Pugazhvendan *et al.*, 2009), neurotoxicity (Sandoval-Herrera *et al.*, 2019), behavioral changes (Singh *et al.*, 2009), oxidative stress (Monteiro *et al.*, 2006), developmental toxicity (Pamanji *et al.*, 2015) and genotoxicity (Kumar *et al.*, 2010) in various fish species.

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Fonofos (*O*-ethyl *S*-phenyl ethylphosphonodithioate), with the trade name Dyfonate, is an OP insecticide applied to soil to prevent various worms that damage mainly corn, and also sugarcane, peanuts and tobacco (Mahajan *et al.*, 2006; US EPA, 2008). Our knowledge regarding the adverse effects of fonofos is very limited.

Zebrafish (*Danio rerio*) is a unique vertebrate model for its various features. Firstly, its small size presents many advantages such as easy husbandry, reduced housing space, required low quantities of experimental solutions and low volume of toxic waste (Hill *et al.*, 2002; 2005). Scientists have well studied the morphological, biochemical, and physiological processes of all life stages of zebrafish from zygote to adult (Hill *et al.*, 2005). These benefits make zebrafish an excellent material for toxicological researches.

Skeletal muscles play essential roles such as contraction, moving, body posture, balance, protecting internal organs and glycogen storage. Recent studies have also proved that fish muscle is an immunologically active organ (Valenzuela *et al.*, 2017). Besides, it is an important source of human nutrition. Skeletal muscle is known to be the target tissue of various xenobiotics (Gupta *et al.*, 2014). The present study aimed to reveal whether fonofos causes qualitative and quantitative alterations on the skeletal muscle of zebrafish.

2 Materıals and methods

2.1 Animal husbandry

Adult zebrafish (3–4 months old) were maintained in glass aquaria with dechlorinated aged tap water at 26 ± 2 °C. Fish were kept under natural photoperiod for two weeks before the experiment, and they were fed with *Artemia* sp. twice a day.

2.2 Experimental design

Fonofos (99.5%) (CAS No: 944-22-9) and dimethyl sulfoxide (DMSO) (≥99.5%) were purchased from Sigma-Aldrich. The stock solution was prepared by dissolving fonofos in 0.1% DMSO. Treatment concentrations were diluted from the stock solution. A solvent control (0.1% DMSO) and three exposure groups (1 mg/L, 2 mg/L and 4 mg/L) were prepared. Five fish were used for each group. A static test system was conducted; fish were not fed, and the test solutions were not renewed for 96 h.

2.3 Histopathology and biometry

Fish were euthanized in 250 mg/L of tricaine methanesulfonate (MS-222) solution (Wang *et al.*, 2020). Skeletal muscle samples were removed from the pectoral region. They were fixed in Bouin's fluid for 24 h at room temperature, dehydrated in ethanol, treated with xylol and embedded in paraffin. 5 μm-thick serial sections were stained with Mayer's Hematoxylin and Eosin (H&E) and Gomori Trichrome (GT) to observe general muscle histology, and they were also stained with Periodic Acid-Schiff (PAS) to detect glycogen content. Images were taken with Zeiss Axio Scope A1 equipped with Zeiss Axiocam ERc5s.

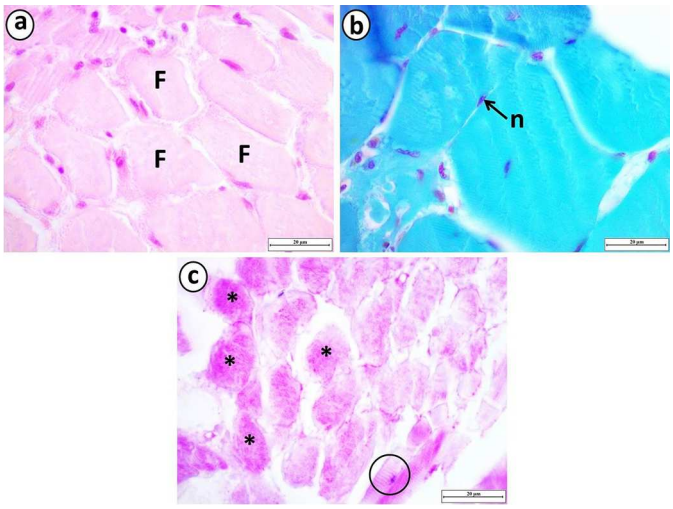

Fig. 1. Normal histological structure of the skeletal muscle of the control samples. (a) H&E. (b) GT. (c) PAS. F: muscle fiber; n: nucleus; *: glycogen storages; Ellipse: cross-striations.

Biometric measurements were performed with oblique or transversally sectioned fibers, and diameters were measured according to the minimum diameter method of morphometry (MDM) to detect any changes in myofiber diameters described by Mars and Gregory (2014). 1800 fibers were analyzed for each experimental group. 20 diameters were measured in three random fields of 10 sections from three randomly selected individuals. Leica DM500 microscope with the software program Leica Application Suite (LAS) V4.9 were used for the measurements.

2.4 Statistics

Statistical analyses were performed with SPSS (Version 20). After the priority Shapiro-Wilk normality and Levene homogeneity tests, Kruskal-Wallis H was conducted to compare skeletal myofiber sizes between the control and the treatment groups. The significance level was set at $p < 0.001$.

3 Results

Normal skeletal muscle histology was observed in the solvent control samples. The striated appearance of myofibers and peripherally located nuclei were observed. Glycogen storages were noticed with magenta color in PAS-stained sections (Fig. 1).

No mortality was detected during the experiment. Some of the exposed individuals exhibited erratic swimming movements and spinal curvature. Light microscopic examinations revealed that fonofos exposure gave rise to distinct lesions in the muscle tissues of zebrafish specimens in the experimental groups. These lesions were majorly similar in all three experimental samples; however, the severity of the lesions was in a concentration-dependent manner. General histoarchitecture was significantly broken down following the exposure to ascending concentrations of fonofos. 1 mg/L of fonofos treated group showed fibrosis (Fig. 2a,c), intramyofibrillar vacuoles,

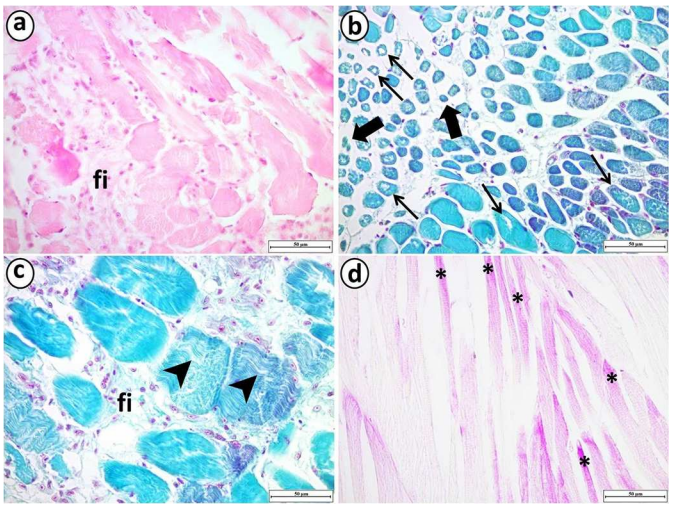

Fig. 2. Skeletal muscle sections of 1 mg/L of fonofos treated group. (a) Fibrosis (fi), H&E. (b) Intramyofibrillar vacuoles (thin arrows) and atrophic fibers (thick arrows), GT. (c) Fibrosis (fi) and disintegrated myofibrils (arrowheads), GT. (d) Decrease in glycogen storage (asterisks), PAS.

atrophic fibers (Fig. 2b), disintegrated myofibrils (Fig. 2c) and mild decrease in glycogen storage (Fig. 2d).

2 mg/L of fonofos exposed group samples exhibited general deformation of tissue integrity. Fibrous tissue formation and intramyofibrillar vacuoles (Fig. 3a) were common. While some myofibers were atrophic, some disappeared fibers were also noticed (Fig. 3b). Disintegrated myofibrils and splitting of muscle fibers were observed with distinct gaps among the fibers (Fig. 3c). PAS-stained sections proved progressive decrement in glycogen content (Fig. 3d).

4 mg/L of fonofos treated group showed a severe histoarchitectural loss. Prominent fiber splitting, atrophic fibers and disintegration of myofibrils were noted (Fig. 4a). Intramyofibrillar vacuoles (Fig. 4b,c) and necrosis (Fig. 4c) was noticed. PAS staining technique showed a significant decrease in muscular glycogen in the highest fonofos concentration group (Fig. 4d).

Mean values and standard errors of measured fiber diameters were given in Table 1. Statistical analyses showed that the fiber diameters of all treatment groups were significantly different from the control (Tab. 1). The diameters (Fig. 5a) decreased in a concentration-dependent manner (Fig. 5b) indicating fonofos-induced gradual muscular atrophy.

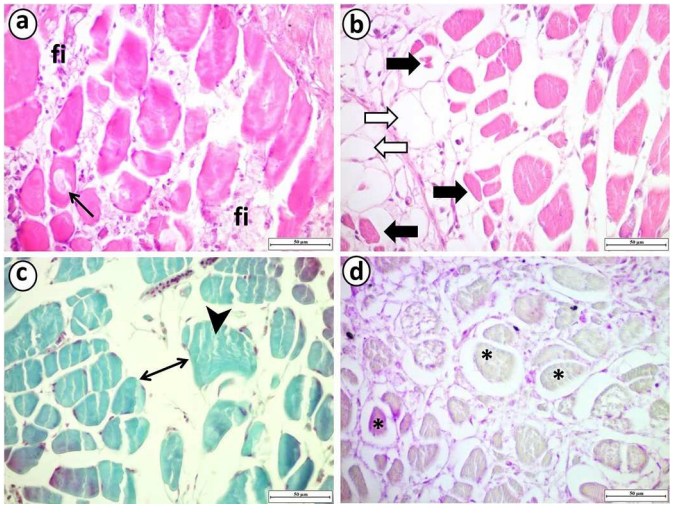

Fig. 3. Skeletal muscle sections of 2 mg/L of fonofos treated group. (a) Fibrosis (fi) and intramyofibrillar vacuole formation (arrow), H&E. (b) Deformation of general tissue integrity with atrophic myofibers (black arrows) and disappearance of some fibers (white arrows), H&E. (c) Splitting of muscle fibers (double-headed arrow) and disintegrated myofibrils (arrowhead), GT. (d) Progressive decrement in glycogen storage (asterisks), PAS.

4 Discussion

Pesticides have gained considerable notoriety as responsible for adverse effects on various non-target species (Lushchak *et al.*, 2018). The current work showed that fonofos induced myotoxicity by causing striking histopathological alterations in the skeletal muscle of zebrafish. Muscular tissue was sensitive to fonofos treatment at the sublethal concentrations.

There are relatively few studies about muscle histopathology in fishes. Skeletal muscle toxicity caused by environmental contaminants available in the literature generally brought about similar lesions in fish. Two different freshwater fish species from pesticide polluted Lake Qarun were investigated and vacuolar degeneration in muscle bundles, atrophy, splitting of muscle fibers, and necrosis was observed in *Tilapia zillii*. In *Solea vulgaris*, degeneration in muscle bundles, vacuolar degeneration and edema between muscle bundles were noted (Mohamed, 2009). The skeletal muscles of *Carassius gibelio*, caught from Buyuk Menderes River polluted by several sources such as industrial and domestic wastes, fertilizers and pesticides, showed intermyofibrillar edema, vacuolization, dissociation of connective tissue, muscular atrophy and myofiber necrosi (Adalı and Koca, 2016).

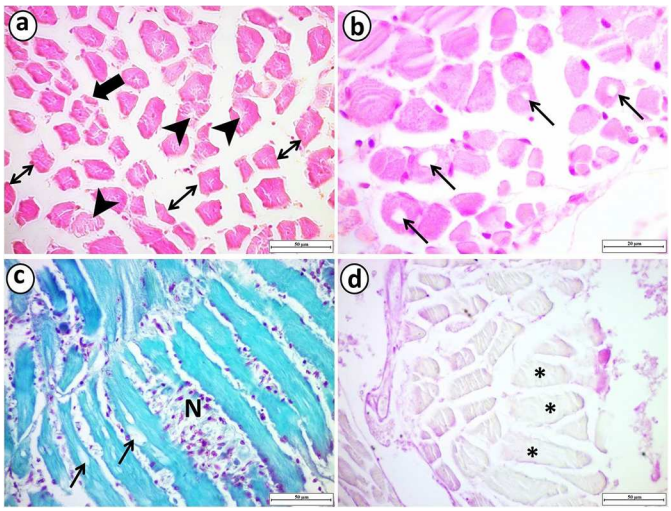

Fig. 4. Skeletal muscle sections of 4 mg/L of fonofos treated group. (a) Atrophic fiber (arrow), disintegrated myofibrils (arrowheads) and splitting of muscle fibers (double-headed arrow) accompanying with histoarchitectural loss, H&E. (b) Intramyofibrillar vacuoles (arrows), H&E. (c) Intramyofibrillar vacuoles (arrows) and necrosis (N), GT. (d) Severe decrement in glycogen content (asterisks), PAS.

Table 1. Measurements of skeletal muscle fiber diameters (μm) of zebrafish following exposure to fonofos for 96 h. Values indicate mean and standard error of measurements. An asterisk (*) indicates significant difference compared to the control ($p < 0.001$).

	Control	1 mg/L	2 mg/L	4 mg/L
Fiber diameter (μm)	26.49 ± 0.21	24.65 ± 0.20*	22.83 ± 0.19*	19.14 ± 0.13*

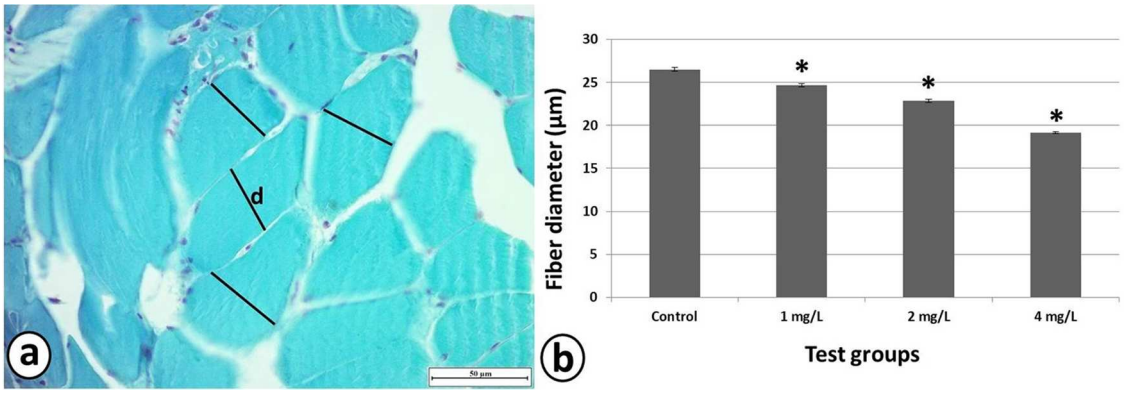

Fig. 5. (a) Examples of fiber diameter (d) measurements of the control group (b) alterations in muscle fiber diameter measurements following 96 h fonofos exposed groups compared to the control.

Skeletal muscles are the targets of many chemicals due to their large portion in total body weight, high metabolic activity and various binding sites (receptors, neurotransmitters, enzymes, *etc*.); moreover, cholinergic and noncholinergic components of muscles are regulated by OPs (Gupta *et al.*, 2014). Previous studies focused on skeletal muscle histopathology of fish treated with OP insecticides. Muscle tissues of diazinon exposed *Tilapia nilotica* exhibited ultrastructural changes such as swelling of the sarcoplasmic reticulum, loss of myofibrils extending over the entire length of the sarcomere, destruction in muscle structure, and abundance of phagocytic lysosomes (Sakr and Gabr, 1992). In the present work, fonofos-induced lesions as fibrosis, intramyofibrillar vacuoles, disintegrated myofibrils, atrophic myofibers, splitting of the fibers, histoarchitectural loss and necrosis were observed. Measurements also revealed progressive decrement in myofiber size. However, no significant histopathological alterations were reported in the muscles of *Oreochromis niloticus* exposed to fenitrothion (Benli and Özkul, 2010). Chlorpyriphos treatment brought about reduced myotome size,

hypertrophy and vacuole formations in the myocytes of *Xenopus laevis* larvae (Colombo *et al.*, 2005). In higher vertebrates, myopathic alterations were also stated following OP intoxication in the diaphragm, gastrocnemius and psoas muscles of rats (Karalliedde and Senanayake, 1989). Cisson and Wilson (1982) noted muscular atrophy in birds exposed to tricresyl phosphate and parathion. Fenthion induced edema, inflammation and necrosis in diaphragm tissues of rats (Büyükokuroğlu *et al.*, 2008). It was revealed that OP intoxication induced atrophy and muscular necrosis in humans (Fukuhara *et al.*, 1977). It was announced that skeletal muscle toxicity induced by AChE inhibitors might be related to excess free radical generation, lipid peroxidation, depletion of high-energy phosphates, high cytosolic Ca^{2+} degrees, mitochondrial damage, necrosis, apoptosis, and myopathy (Gupta *et al.*, 2009).

On the other hand, PAS-stained sections suggested that acute fonofos treatment brought about muscular glycogen decrement in zebrafish. Glycogen decrement was reported as a common effect of pesticide exposure related to energy demands to eliminate the chemical and rapid catabolism of glycogen storages after toxic stress (Begum and Vijayaraghavan, 1999; Becker *et al.*, 2009). Organochlorine pesticide endosulfan exposure gave rise to decreased muscle glycogen content through glycogenolysis in *Anguilla anguilla* (Gimeno *et al.*, 1995). Moreover, a decrease in glycogen levels was noted in muscles of *A. anguilla* following lindane treatment (Ferrando and Andreu, 1991). It was revealed that glyphosate reduced muscle glycogen content in *Leporius obtusidens* (Glusczak *et al.*, 2006). Clomazone also caused glycogen decrement in the muscle tissue of *Rhamdia quelen* (Crestani *et al.*, 2006). Begum and Vijayaraghavan (1999) observed a gradual decrement in muscular glycogen content of *Clarias batrachus* exposed to Rogor insecticide. However, Sastry and Siddiqui (1984) noted increased glycogen content following quinalphos treatment in *Channa punctatus*. The chemical formulation of the pesticide and interspecies variations may alter the physiological responses to chemical stress.

The results indicated that besides their agricultural benefits, OP insecticides give serious harm to freshwater fish and threaten their lives. It is clear that 'benefit' must be thought of as a whole phenomenon consisting of both the human population's food requirements and all other species' health. So that eco-friendly techniques for agricultural developments are required to sustain the fate of the biosphere.

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