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Phagocytosis and ROS production as biomarkers in Nile tilapia (*Oreochromis niloticus*) leukocytes by exposure to organophosphorus pesticides

C.E. Covantes-Rosales[a], A.M. Trujillo-Lepe[a], K.J.G. Díaz-Reséndiz[a], G.A. Toledo-Ibarra[a,b], G.H. Ventura-Ramón[a,b], P.C. Ortiz-Lazareno[c], M.I. Girón-Pérez[a,b,*]

[a] Universidad Autónoma de Nayarit, Secretaría de Investigación y Posgrado, Laboratorio de Inmunotoxicología, Boulevard Tepic-Xalisco s/n, Cd. de la Cultura Amado Nervo, C.P. 63000, Tepic, Nayarit, Mexico

[b] Centro Nayarita de Innovación y Transferencia de Tecnología A.C., Laboratorio Nacional para la Investigación en Inocuidad Alimentaria-Unidad Nayarit, Calle Tres s/n, Cd Industrial, Tepic, Nayarit, Mexico

[c] Centro de Investigación Biomédica de Occidente (CIBO), Instituto Mexicano del Seguro Social (IMSS), Guadalajara Jalisco, Mexico

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ABSTRACT

Organophosphorus pesticides (OPs) are broad-spectrum insecticides. One of the commonly used OPs is diazinon (DZN). The aim of this study was to evaluate the immunotoxic effect of DZN on phagocytic parameters of blood leukocytes using the teleost fish *Oreochromis niloticus* as a study model. For this purpose, fish were exposed *in vivo* to 0.97, 1.95 and 3.97 mg/L of DZN for 6 and 24 h. Our results indicated that phagocytic active cells decreased in fish exposed *in vivo* to 0.97 and 1.95 mg/L of DZN for 6 and 24 h. Regarding ROS production, H_2O_2 and O_2^- levels were higher on fish exposed to 1.95 mg/L for 6 and 24 h, while H_2O_2 production increased at 0.97 mg/L for 24 h. From this we can conclude that phagocytic parameters are sensitive to assess the effect of acute intoxication with organophosphorus pesticides on Nile tilapia.

1. Introduction

Organophosphorus pesticides (OPs) are a group of broad-spectrum insecticides widely used in agricultural activities for the control of pests [1]. OPs are a group of great interest in ecotoxicological studies since they represent approximately 50% of the worldwide use of pesticides [2].

Diazinon (DZN) is one of the OPs commonly used in several countries (0.0-diethyl-0-[2-isopropyl-6-methyl-pyrimidin-yl]phosphorothioate) [3–7], which is used as an insecticide in agricultural and domestic activities [8]. The mechanism of action of this type of pesticides is through the inhibition of the enzyme acetylcholinesterase (AChE), which causes an excessive accumulation of the neurotransmitter acetylcholine (ACh) in the nerve terminals, leading to uncontrolled nerve impulses and death of the target insects. However, neurotoxic effects are not exclusive to target insects, and OPs can affect organisms that are accidentally exposed to these substances (non-target organisms) [9].

Aquatic organisms are particularly sensitive to pesticides [10], due to the fact that aquatic organisms, as fishes, are in direct contact with pesticides diluted in the water, consequently they are exposed to these toxic substances by dermal contact, inhalation or orally [11]. In this sense, teleost fish are of great interest for studies on ecotoxicology, since they are the most abundant group of vertebrates on the planet. Furthermore, fish are ideal bioindicator organisms for the evaluation of pollutants in aquatic bodies around the world [12–15].

In addition to the classic neurotoxic effects of OPs, these substances also affect other organs and systems. One of the physiological systems frequently altered by contaminants is the immune system. Therefore, the various parameters of the immune response can be used as biomarkers of immunotoxicity due to exposure to OPs [16]. Cellular immune mechanisms are useful tools to evaluate immunotoxic effects; thus, the phagocytic capacity and reactive oxygen species (ROS) production could be excellent biomarkers. Phagocytosis is a highly-conserved, innate, primordial, and non-specific defense mechanism that contributes to innate immunity through the ingestion and elimination of pathogens [17,18]. Phagocyte capacity has been described as an indicator of the health status of fish, as well as a sensitive biomarker of environmental exposure to pesticides [19,20]. Likewise, the respiratory burst is an oxygen-dependent, non-specific, microbicide defense mechanism of phagocytic cells. During the respiratory burst phenomenon cells produce cytotoxic compounds called ROS, such as superoxide anion (O_2^-), singlet oxygen (O_2), hydrogen peroxide (H_2O_2), and

* Corresponding author. Universidad Autónoma de Nayarit, Secretaría de Investigación y Posgrado, Laboratorio de Inmunotoxicología, Boulevard Tepic-Xalisco s/n, Cd. de la Cultura, C.P. 63000, Tepic, Nayarit, Mexico.
E-mail address: ivan_giron@hotmail.com (M.I. Girón-Pérez).

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hydroxyl radicals (OH^-) [22].

In normal conditions, the immune system of fish is efficient in antigen clearance. However, the presence of pesticides can negatively affect the immune capacity of these organisms [23,24]. Nile tilapia (*Oreochromis niloticus*) is a teleost fish with an ecological and economic importance that is distributed worldwide in tropical and subtropical waters. Therefore, the aim of this study is to determine the effect of *in vivo* exposure to DZN on the phagocytic and respiratory burst capacity in blood leukocyte from Nile tilapia fish, as potential biomarkers of immunotoxicity induced by OPs.

2. Materials and methods

2.1. Organism

Male Nile tilapia fish (273 ± 43 g and 20 ± 3 cm) were obtained from a local commercial aquaculture farm. The fish were maintained in a 400 L tank. During the 4-week acclimation period, the fish were given commercial feed at a day-rate of 3% their body weight. Water temperature was maintained at 26 ± 2 °C and dissolved oxygen values were 6.0 mg/L.

2.2. Experimental design

Previous to the exposition bioassays, fish were acclimated in a 30 L glass aquarium for 24 h (1 fish/tank). In order to decrease stress, temperature and aeration were kept constant. In addition, organisms were not fed during this period in order to avoid prandial effects and to prevent deposition of stool during the bioassay. After the acclimation period, fish (n = 10) were exposed *in vivo* to 0.97, 1.95 and 3.91 mg/L (1/8, 1/4 and 1/2 of the previously reported CL_{50} value at 96 h) [24] of a commercial formulation of DZN (25% active ingredient), for 6 and 24 h. The bioassays were performed statically (with no water replacement). A control group was established with organisms that were maintained in the same conditions but without pesticide.

2.3. Sample preparation

Total leukocytes were isolated from blood collected by cardiac puncture (3 mL). For this, the blood was placed in Histopaque-1077 (3 mL) and allowed to settle by gravity (40 min). After settling, total leukocytes were collected from the upper phase. Subsequently, they were washed with PBS (pH 7.3) and recovered by centrifugation (3500 rpm/5min/4 °C) and resuspended in PBS (1 mL). Viability was evaluated using the 4% trypan blue exclusion method.

2.4. Assessment of phagocytosis by flow cytometry

Phagocytosis was studied using fluorescent latex beads and flow cytometry as described in Ref. [21] with some modifications. Cell suspensions (2×10^6 leukocytes) were mixed with 200 µL of RPMI medium with Fluoresbrite® YG carboxylate microspheres (Polysciences Inc., Warrington, USA), 1 mm in diameter, at a cell/bead ratio of 1:20 per well and incubated for 24 h at 28 °C (air/CO_2: 95/5%). Wells containing cell suspension without beads were used as negative controls. Adherent cells were loosened by trypsinization for 5 min using 50 µL of trypsin-EDTA per well. To remove non–ingested beads, the cell suspension was placed on top of a solution consisting of 1.5 mL of PBS (pH 7.3), with 3% (w/v) bovine serum albumin and 4.5% (w/v) D-glucose, centrifuged at 100 x g for 10 min at 4 °C, washed once with 1 mL PBS and resuspended in 500 µL PBS prior to flow cytometry analyses. Using flow cytometry, cells were analyzed for forward scatter (FSC) and sideward scatter (SSC) patterns, representing the size and granularity of the cells, respectively; and for green bead fluorescence (detected with 530/30 nm bandpass filter; FL1). 10,000 events were recorded for each sample. Results obtained were represented as a percentage of phagocytic cells in total blood leukocytes, mononuclear cells and polymorphonuclear cells.

2.5. Assessment of respiratory burst by flow cytometry

Two fluorescent probes were used to determine the respiratory burst: Dihydrorhodamine 123 (DHR), which is oxidized by H_2O_2 to fluorescent rhodamine (RHO) [25] and Dihydroethidium (DHE), which reacts with O_2^-, to form a fluorescent red product (ethidium) [26]. Briefly, leukocytes isolated from blood (2×10^6) were suspended on 200 µL of RPMI medium, transferred to 1.5 mL polystyrene tubes and incubated at room temperature (10 min). After incubation, DHR (5 µM) or DHE (5 µM) were added to determine H_2O_2 or O_2^- levels, respectively. The samples were mixed and incubated by gentle tilting for 15 min. in darkness. Prior to flow cytometry analyses, the cells were suspended in 500 µL PBS. The fluorescence of RHO was detected with the FL1 filter (530/30 nm bandpass filter), while ethidium fluorescence was detected with the FL2 filter (610/20 nm bandpass filter). 10,000 events were recorded for each sample. The obtained data were represented in average fluorescence intensity (MFI) for both probes in all treatments. ROS production in total blood leukocytes, mononuclear cells, and polymorphonuclear cells was calculated.

2.6. Statistical analysis

The data were analyzed using the non-parametric Kruskal-Wallis one-way ANOVA, with *post-hoc* Dunn test using Sigma Plot statistical software®, With a level of significance of $p < 0.05$.

3. Results

3.1. Identification of leukocyte populations by flow cytometry

The total leukocyte population, mononuclear cells (MNC) and polymorphonuclear cells (PMNC) was identified by flow cytometry, based on cell size (FSC-A) and granularity (SSC-A), as shown in Fig. 1.

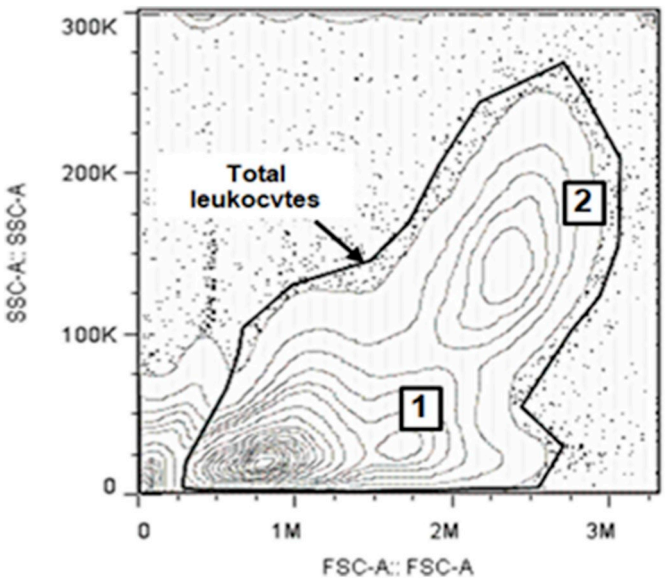

Fig. 1. Identification of leukocytes populations isolated from blood samples of Nile tilapia (*Oreochromis niloticus*); Representative graph of the size and granularity (FSC-A/SSC-A), showing the populations of the total leukocytes, MNC (1) and PMNC (2).

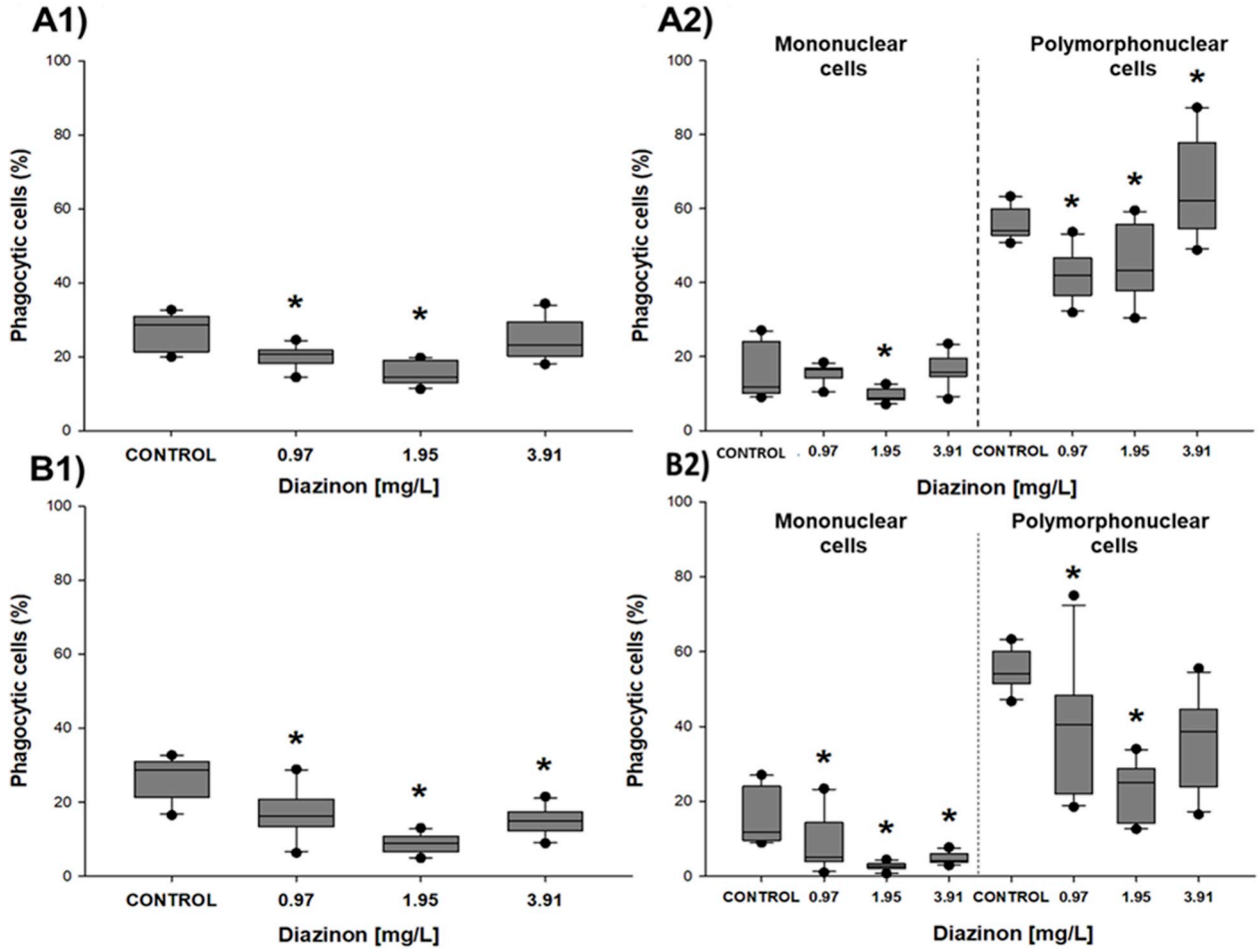

Fig. 2. Percentage of blood leukocytes that phagocytize fluorescent beads of *O. niloticus* exposed *in vivo* to 0.97, 1.95 and 3.97 mg/L of DZN. Percentage of phagocytosis of total leukocytes from fish exposed for 6 and 24 h (figure 2-A1 and 2-B1). Percentage of phagocytosis of MNC and PMNC from fish exposed for 6 and 24 h (figure 2-A2 and 2-B2). The Kruskal-Wallis test, with the Dunn post-hoc was used for the statistical analysis of the control and exposed groups (*: $p < 0.05$).

3.2. Phagocytic capacity of total leukocytes of Nile tilapia exposed to DZN

Our results indicate that *in vivo* exposure to DZN at 0.97 and 1.95 mg/L for 6 h reduces the phagocytic capacity of total leukocytes (Fig. 2-A1). When the data were analyzed by cellular population, it was evident that the phagocytic capacity was significantly decreased in MNC after exposure to 1.95 mg/L DZN. In the PMNC population, exposure to 1.95 and 0.94 mg/L also decreased the phagocytic capacity, but at 3.95 mg/L this parameter actually increased (Fig. 2-A2). When the effect of DZN was analyzed on total leukocytes from fish exposed for 24 h, we observed that the phagocytic capacity decreased in all experimental groups compared to the controls (Fig. 2-B1). Similar results were obtained when the effect of DZN was analyzed in the different populations, except in PMNC from fish exposed to 3.91 mg/L (Fig. 2-B2).

3.3. ROS production in leukocytes of Nile tilapia exposed to DZN

3.3.1. H_2O_2 production

The results indicate that *in vivo* exposure to DZN (1.95 mg/L) for 6 h significantly increased H_2O_2 levels compared to the control group (Fig. 3-A1). Additionally, H_2O_2 production was analyzed in two different cell populations (MNC and PMNC). The concentration of 1.95 mg/L of the pesticide induces a significant increase in both types of cell populations; moreover, 0.97 mg/L also increased the production of H_2O_2 in MNC (Fig. 3-A2).

When fish were exposed to 1.95 and 0.97 mg/L DZN for 24 h, H_2O_2 production was also increased in total leukocytes isolated from the exposed fish (Fig. 3-B1). Once leucocyte populations were analyzed separately, only the 1.95 mg/L concentration caused an increase in H_2O_2 levels in MNC, whereas 0.97 and 1.95 mg/L induced an increase in H_2O_2 production in PMNC (Fig. 3-B2).

3.3.2. O_2^- production

The results indicate that *in vivo* exposure to 1.95 mg/L of DZN, for 6 h, induced a significant increase of this oxygen radical in total leukocytes, MNC and PMNC; while exposure to 0.97 and 3.97 mg/L did not alter O_2^- production (Fig. 4-A1 and 4-A2). In addition, on fish exposed to 1.95 mg/L for 24 h, the production of O_2^- increased significantly on total leukocytes (Fig. 4-B1), Notably, MNC and PMNC populations did not show an increase in the production of this oxygen radical (Fig. 4-B2).

4. Discussion

Aquatic ecosystems are final deposits for various contaminants [27], including OPs, which exert a variety of toxic effects. Even though neurotoxic effects are among the most studied for this type of contaminant [28], there is evidence of their immunotoxic effect [29]. Specifically, there are reports of the development of an altered immune response in fish from ecosystems contaminated by pesticides [30]. Thus, it is imperative to evaluate the correct biomarkers in order to determine the impact of these contaminants on the aquatic organisms [31]. In the present work, we evaluated phagocytic capacity and ROS

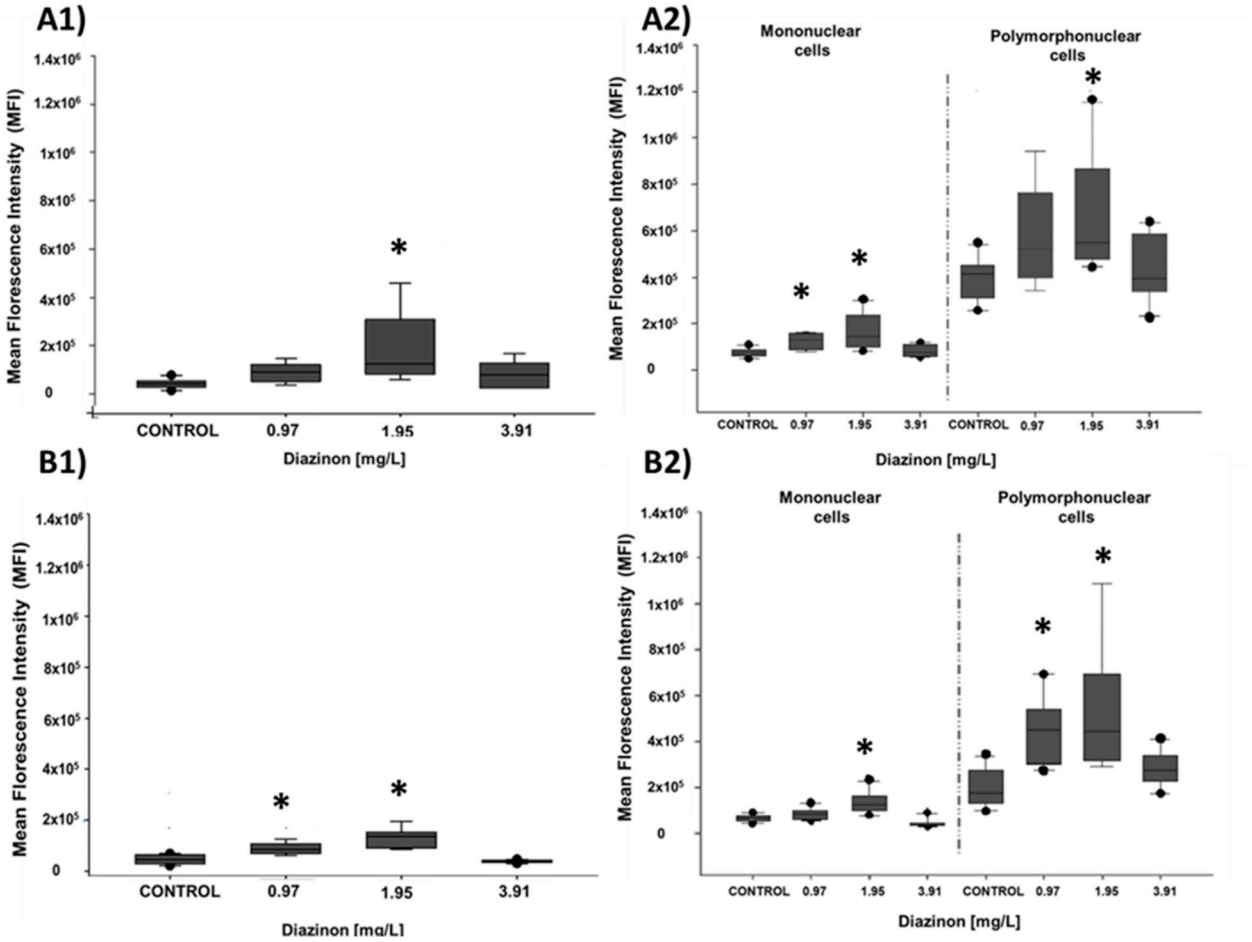

Fig. 3. Determination of H_2O_2 production in leukocytes isolated from blood of Nile tilapia exposed *in vivo* to 0.97, 1.95 and 3.97 mg/L of DZN. H_2O_2 production in total leukocytes from fish exposed for 6 and 24 h (figure 3-A1 and 3-B1). H_2O_2 production of MNC and PMNC from fish exposed for 6 and 24 h (figure 3-A2 and 3-B2). Statistical analyses for all the experimental groups were performed by the Kruskal-Wallis test, followed by the Dunn post-hoc test (*: $p < 0.05$).

production (innate immunity parameters) as biomarkers for DZN exposition.

A decrease in the phagocytic capacity has been reported in various species of fish exposed to different OPs. In this study, it is reported that acute *in vivo* DZN exposure results in a decrease of the phagocytic capacity in total leukocytes, and also in blood MNC and PMNC. Nonetheless, the effect of DZN was neither dose- or time-dependent (Fig. 2). These results are in agreement with other studies performed with diverse OPs in Nile tilapia and other species of fish. Specifically, studies using Nile tilapia as an animal model show that acute and chronic exposure to chlorpyriphos affects leukocyte phagocytic capacity [30,32,33]. There are other studies showing similar results using other species of fish as bioindicators. For example, in the common carp (*Cyprinus carpio carpio*), it was demonstrated that exposure to malathion (0.5 and 1.0 mg/L) significantly reduces the phagocytic capacity [34]. In line with this, malathion (10 mg/L) reduces the phagocytic capacity of Murray cod fish (*Maccullochella peelii*) by 15% [19]. Thus, the previous studies and ours confirm that OPs alter the phagocytic capacity in fish, supporting the idea that this parameter can be used as an OP-susceptibility biomarker.

Furthermore, there are other important biomarkers for the evaluation of the toxic effects of OPs, such as ROS concentration. Even though ROS can act as a microbicide, their overproduction can disturb biomolecules, including lipids, proteins, and nucleic acids, generating oxidative damage and oxidative stress [35–37]. In the present study, the increase in H_2O_2 and O_2^- levels in total leukocytes, MNC and PMNC of fish exposed to DZN *in vivo* was evident (Figs. 3 and 4). Notably, the effect of DZN was neither dose- nor time-dependent; specifically, the highest H_2O_2 and O_2^- production was detected in fish exposed to 1.95 mg/L. These results could be related to the oxidative damage detected in spleen, liver, and gills of Nile tilapia exposed to DZN *in vivo*, in a previous study from our research group [38].

In a normal scenario, ROS production/detoxification is a process regulated enzymatically and non-enzymatically. OPs such as DZN have shown the ability to alter antioxidant enzymes and increase damage to biomolecules [39], as has been reported in teleost fish such as mosquito fish (*Gambusia affinis*) [40], and the common carp (*Cyprinus carpio*) [9]. Additionally, when organophosphorus pesticides such as DZN are metabolized, ROS can be produced by activation of CYP-450 [35]. Moreover, the metabolic processing of DZN produces metabolites that could be conjugated with GSH and other phase II enzymes [22,41]. This is very important given that poisonings with high doses of pesticides can deplete GSH [42] and cause oxidative stress, although the toxic compound does not itself induce an increase of ROS [43]. Therefore, in studies like the present research, where organisms are exposed *in vivo* to the pesticide, the energetic and enzymatic cost during the biotransformation of the parent compound could be a ROS-inducing factor. However, it has been reported that exposure to OPs induces oxidative stress as a direct immunotoxicity mechanism [1,23,35].

ROS production in immune cells is regulated by the NADPH-oxidase family of enzymes or Nox, specifically the Nox-2 isoform, which is located in the cell membrane of phagocytic cells and in the phagosome membrane. Some studies have shown that OPs can increase the presence of these oxidant molecules by the alteration of the electron

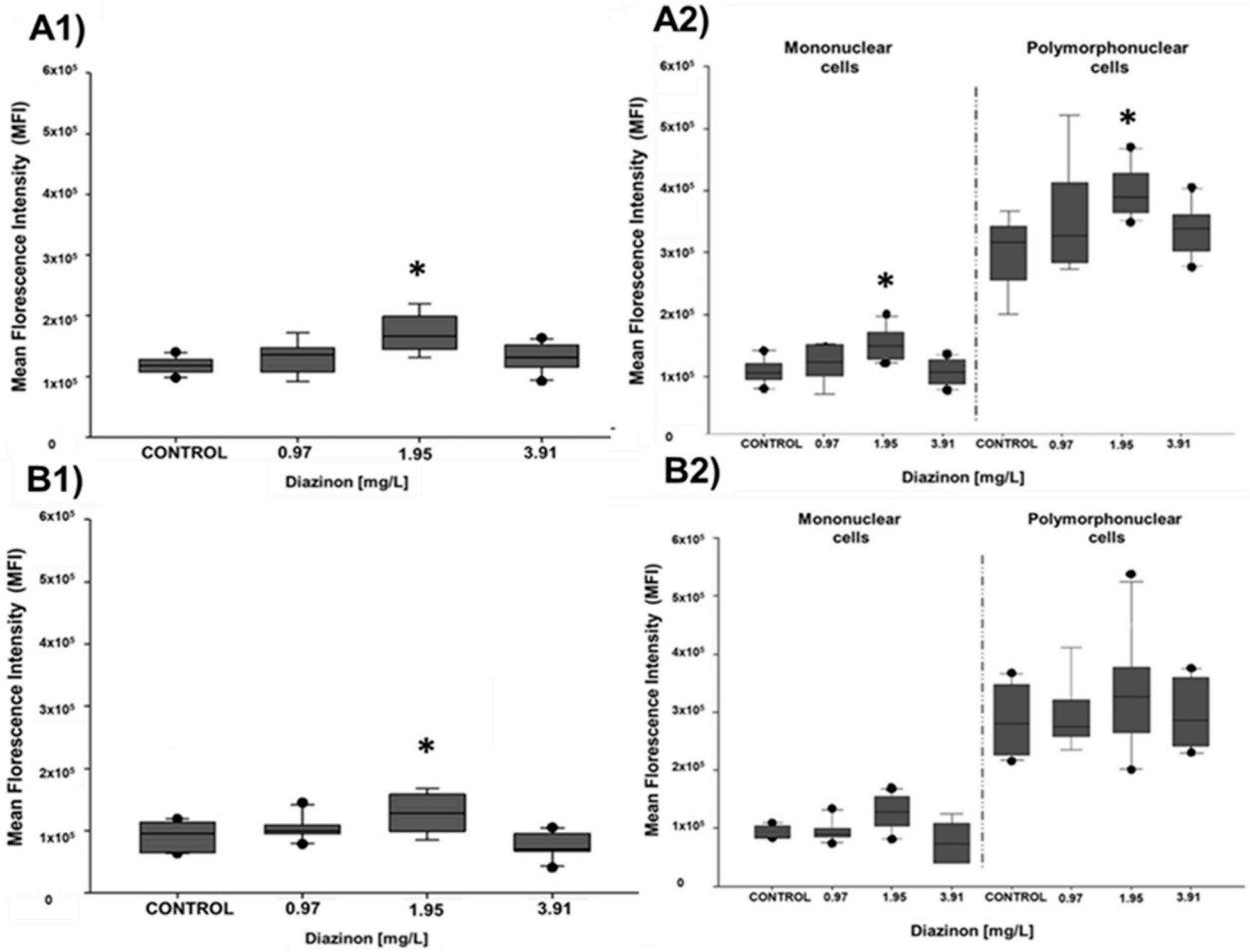

Fig. 4. Determination of O_2^- production in leukocytes isolated from blood of Nile tilapia exposed *in vivo* to 0.97, 1.95 and 3.97 mg/L of DZN. O_2^- production in total leukocytes from fish exposed for 6 and 24 h (Fig. 3-A1 and B1). O_2^- production of MNC and PMNC from fish exposed for 6 and 24 h (Fig. 3A2 and B2). Statistical analyses for all the experimental groups were performed by the Kruskal-Wallis test, followed by the Dunn post-hoc test (*: $p < 0.05$).

transport chain (such as those present in the Nox enzyme family), resulting in an increase of O_2^- that leads to the generation of other free radicals [44]. The Nox family of enzymes is one of the main targets of pesticide interaction, thus, phagocytic cell populations can be affected by these xenobiotics. However, the expression of this enzyme family is not restricted to professional phagocytic cells, and other isoforms (Nox 1, 3, 4 and 5) have been detected in non-phagocytic immune cells and other cell types, although no a lesser extent. This explains the sharp difference in ROS production between MNC and PMNC, in the exposed and control groups [45,46,59].

Another described mechanism is that OPs induce immunotoxicity indirectly, by the alteration of the non-neuronal cholinergic system present in leukocytes, which has been described in both superior (mammals) and inferior (fish) vertebrates [23,47–51,56]. Specifically, it has been demonstrated that *in vivo* exposure to DZN induces AChE inhibition and a subsequent increase of ACh concentrations in lymphoid tissue (spleen) of Nile tilapia; this causes the over-activation of leukocyte cholinergic receptors (AChR) [47,60]. AChR over-activation results in an increase of calcium flow [52], which in turn induces the increase of ROS production, given that both of these processes are highly coordinated in these cell types [53]. The rise in intracellular calcium generates mitochondrial stress that promotes the production of ROS in this organelle [54].

In this way, AChR over-activation in leukocytes has a direct impact on cell function, which was demonstrated by the use of selective AChR agonists and antagonists [55,56]. It was demonstrated that the acetylcholine nicotinic receptor α7 (nAChR-α7) causes a decrease in phagocytosis. In this sense, nicotine exposure decreases neutrophil phagocytic and chemotactic capacity [57].

The information published so far indicates that OPs have potential effects on antimicrobial processes such as migration, phagocytosis, ROS production, and causes alterations in the pro-inflammatory cytokine production (TNF-α IL-1β, IL-6, and IL-18) [1]. Thus, immunological alterations caused by OPs, such as DZN, reduce the microbicide capacity and increase the susceptibility to pathogens [58]. This study supports the notion that DZN interferes with primordial defense mechanisms in teleost fish, parameters that can be used as immunotoxicity biomarkers in OP exposure.

5. Conclusion

In vivo exposure to DZN caused a decrease in the phagocytic capacity and an increase of ROS production in Nile tilapia fish leukocytes. In consequence, it could increase fish susceptibility to pathogen aggressions. These phagocytic biomarkers could be used to evaluate immunotoxic pesticide effects on farmed and wild fish such as Nile tilapia.

Disclosure of interest

There is no conflict, and the authors declare that they have no direct relationship with the previously mentioned commercial entities or any other related.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.10.002.

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