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Effect of diazinon, an organophosphate pesticide, on signal transduction and death induction in mononuclear cells of Nile tilapia fish (*Oreochromis niloticus*)

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ABSTRACT

Diazinon (DZN) is an organophosphate pesticide characterized by inhibiting the enzyme acetylcholinesterase (AChE) (E.C. 3.1.1.7), affecting the nervous system. There is currently enough evidence proving this pesticide also affects the immune response; however, the immunotoxicity mechanisms through which these substances exerts toxic effects remain unclear. For that reason, this work evaluated the effect of diazinon on the intracellular calcium flux, ERK1/2 phosphorylation (pERK1/2), apoptosis, senescence, and mitochondrial membrane potential ($\Delta\Psi m$) in spleen mononuclear cells (SMNC) of Nile tilapia, a teleost fish of commercial and ecological relevance. The results obtained indicate that diazinon causes significant damage in all evaluated parameters, which play an essential role in intracytoplasmic signaling of immune cells, suggesting these signal pathways could be related with the immunotoxicity mechanism of these type of pesticides.

1. Introduction

Immune response in teleost fish can be deregulated by pollutants existing in aquatic ecosystems as organophosphate pesticides (OPs), which are substances widely used to control urban and agricultural pests. However, the inadequate handling of OPs leads to toxic effects on the health of non-target organisms [1]. Diazinon (DZN) (O,O-Diethyl O-[4-methyl-6-(propan-2-yl)pyrimidin-2-yl] phosphorothioate) is an OP that has been used in agriculture and homes for several decades because it is a broad-spectrum insecticide. For instance, it is estimated that in the USA, around 2,722 t of DZN are used in agriculture every year. Although its use has been restricted in some countries, many others still use DZN in domestic activities. Therefore, there exists a clear concern regarding the potential risk to human health as well as the ecological damage OPs can cause [2–4].

The main action mechanism of DZN is the inhibition of enzyme acetylcholinesterase (AChE) (E.C. 3.1.1.7) activity, leading to the accumulation of neurotransmitter acetylcholine (ACh). In addition to the neurotoxic effect caused by DZN, different studies show that this pesticide also exerts toxic effects on the immune system [1,5–8]. Our research group has used Nile tilapia (*Oreochromis niloticus*), a worldwide commercially important fish species, as a model organism to evaluate the immunotoxic effects of DZN. The experimental results obtained in this model have proven that acute exposure to DZN significantly alters several cell and humoral parameters of the immune response, such as: IgM concentration, cell proliferation, respiratory burst, and phagocytosis [7,9–11]. Likewise, other research groups have demonstrated that some OPs (diazinon, chlorpyrifos, phosalone, and malathion) deregulate lysozyme activity, phagocytic function, and complement C3, IL-1β, IL-1R, and IFN-γ mRNA expression levels [8,12–15]. However, the immunotoxicity mechanisms of these substances are not completely elucidated. Previous studies carried out by our research group have demonstrated extra-neuronal cholinergic activity in lymphocytes of Nile tilapia and its inhibition as a consequence of exposure to DZN. In this way, it is suggested that the leukocyte cholinergic system could be targeted by OPs in the immunotoxicity phenomenon [16–18].

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Nevertheless, the alterations related to signal transduction and cell death mechanisms in leukocytes have not been studied. Therefore, the aim of this work was to evaluate the effect of DZN on intracellular Ca^{2+} flux, ERK1/2 phosphorylation, apoptosis, senescence, and mitochondrial membrane potential in mononuclear cells of Nile tilapia fish.

2. Material and methods

2.1. Organisms

Male Nile tilapia fish (*O. niloticus*) were obtained at a local farm (273 ± 43 g and 20 ± 3 cm). The fish were acclimatized to laboratory conditions in 400 L tanks at 28 ± 2 °C and constant air flow, under optimal conditions (pH 8.0, 6.0 mg/L dissolved oxygen, and 85% oxygen saturation) for 4 weeks. The fish were daily fed with commercial feed Winfish®, corresponding to 3% of their weight.

2.2. Experimental groups

Once the acclimation process was finished, fish (n = 10) were exposed to 0.97, 1.95, and 3.91 mg/L (1/8, 1/4, and 1/2 of the previously reported CL50 value at 96 h) [11] of a commercial formulation of diazinon (25% active ingredient) for 6 and 24 h. The bioassays were performed statically (with no water replacement). Then, each organism was euthanized by cooling in ice water (a method that was approved by the Institutional Animal Care at the University of Oregon) [19] and the spleen was immediately dissected. The control group (unexposed fish) was maintained under the same conditions but without pesticide exposure.

2.3. Isolation of spleen mononuclear cells from fish

The spleen was manually disaggregated with soft movements using a syringe plunger. Spleen mononuclear cells (SMNC) were separated by density gradient (5 mL cell suspension in 2.5 mL Histopaque-1077) at 1000 × *g* for 20 min at 20 °C. Later, the SMNC were recovered at 1900 × *g* for 15 min and the cellular pellet was reconstituted in 1 mL RPMI-1640 medium.

Cell populations were characterized through flow cytometry according by size (forward scatter, FSC) and cellular complexity (side scatter, SSC) in a BD Accuri C6 flow cytometer (BD Becton Dickinson, San Jose, CA). Once the study cell population was established, a cell count was performed.

2.4. Assessment of intracellular Ca^{2+} flux

Fluo-4 NW Calcium Assay kit (Molecular Probes™) was used to asses intracellular Ca^{2+} flux. Briefly, 1 x 10^6 cells/mL were centrifuged at 1900 × *g* for 7 min; cellular pellet was washed with PBS (pH 7.3), resuspended in 300 μL buffer C (1X HBSS, 20 mM HEPES) and incubated at 28 °C for 10 min. Then, 300 mL buffer A (Fluo-4 NW) were added to the cell suspension and incubated at 28 °C for 30 min. Finally, the samples were incubated at room temperature for 30 min and analyzed for 10 min in the flow cytometer using a 488-nm excitation laser. As stimulus to increase intracellular Ca^{2+} flux, ionomycin (48 ng/mL) combined with phorbol myristate acetate (PMA) (48 ng/mL) was used, which was added after a minute and a half of reading in the flow cytometer. The stimulus was used for the purpose of evaluating the intracellular response presented by the control cells and exposed to the pesticide.

2.5. Assessment of pERK1/2

PE mouse anti-ERK1/2 (pT202/pY204) was used to determine pERK, which was obtained from BD Biosciences Pharmingen (Franklin Lakes, NJ, USA). For measurement of pERK1/2 in SMNC from Nile tilapia, flow cytometry was used according to the procedure by Chow et al., [20]. Briefly, SMNC (2 x 10^6 cells/mL) freshly extracted were centrifuged at 1900 × *g* for 7 min. Then, 100 μL fixation buffer (BD Cytofix™) were added to the cellular pellet and the solution was gently agitated in vortex before incubation at room temperature in the dark for 15 min. Cells were washed with stain buffer (FBS, BD Pharmingen™), resuspended in 100 μL permeabilization buffer III (BD Phoslow™), and incubated at 4 °C for 20 min. Afterward, 5 μL of pERK1/2 antibody were added to the cell suspension, followed by incubation at 4 °C for 30 min. Finally, cells were washed and resuspended in 400 μL FBS for analysis in flow cytometer using a 488-nm excitation laser. 10,000 events were collected.

2.6. Determination of viability and cellular death

Determination of apoptosis was performed using an Annexin V-FITC kit (BD Pharmingen™). Briefly, 1 x 10^6 cells/mL were centrifuged at 1900 × *g* for 7 min and washed with cold PBS (pH 7.3). The cellular pellet was added 100 μL binding Annexin V buffer 1X, 5 μL Annexin V-FITC and 5 μL propidium iodate (PI). The mix was incubated at room temperature in the dark for 15 min. Finally, cells were resuspended in 400 μL binding Annexin V buffer 1X and analyzed in flow cytometer using a 488-nm excitation laser. We collected 10,000 events in the flow cytometer. Cells that were considered viable were FITC Annexin V and PI negative; cells that were in early apoptosis were FITC Annexin V positive and PI negative; cells that were in late apoptosis were both FITC Annexin V and PI positive; and cells that were in necrosis were Annexin V-FITC negative and PI positive. The results were represented as percentage of viable, apoptotic (early and late), and necrotic cells [21].

2.7. Determination of senescent cells

Cell senescence was determined through β-galactosidase activity (SA-β-gal), using 5-Dodecanoylaminofluorescein di-β-D-Galactopyranoside (C_{12}FDG, Invitrogen) as substrate [22]. Firstly, 1 x 10^6 cells/mL were washed with PBS (pH 7.3) at 1900 × *g* for 7 min, the cellular pellet was added bafilomycin-A1 (100 nM, Sigma) and incubated at 28 °C and 5% CO_2 for 1 h. After the incubation period, C_{12}FDG (10 μM) was added and the mixture was incubated for 1 h. Cells were washed with cold PBS and finally, 200 μL PBS were added to cellular pellet. Using an excitation laser of 488 nm, 10,000 events were collected in the flow cytometer.

2.8. Determination of mitochondrial membrane potential

The determination of mitochondrial membrane potential (ΔΨm) was performed using a cationic cyanine dye, 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) (Sigma-Aldrich) [21]. Cells (1 x 10^6/mL) were centrifuged at 1900 g for 7 min, then 500 μL PBS and 0.5 μL $DiOC_6(3)$ (20 nM) were added to cellular pellet. The samples were incubated at room temperature for 15 min. Finally, they were analyzed in the flow cytometer (10,000 events) using an excitation laser of 488 nm.

2.9. Statistical analysis

Data were processed with FlowJo v10 while the statistical analysis was completed in SigmaPlot® v10.0. We determined the normality and homogeneity of the data variances through the Kolmogorov-Smirnov test and Levene's test. For non-parametric data, a Kruskal-Wallis test was used followed by a multiple Tukey-type comparison. The significance level was set at $p < 0.01$.

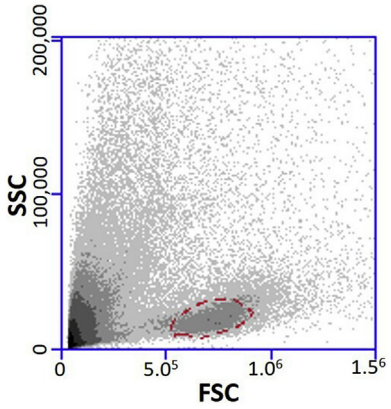

Fig. 1. Spleen mononuclear cells (SMNC) characterized by flow cytometry through size (FSC) and cellular complexity (SSC).

3. Results

3.1. Characterization of SMNC by flow cytometry

Before assessing the parameters evaluated, we identified the SMNC by forward scatter (FSC) and side scatter (SSC) in the flow cytometer (Fig. 1).

3.2. Intracellular calcium in SMNC

Results indicate that basal Ca^{2+} flux (cells without PMA + ionomycin) increased significantly ($p < 0.01$) in SMNC of organisms exposed to DZN (1.95 and 3.91 mg/L) for 6 h, compared to SMNC from unexposed fish (control) (Fig. 2a). Similar results were observed in fish exposed to DZN for 24 h at the three concentrations evaluated of the pesticide (0.97, 1.95, and 3.91 mg/L) (Fig. 2b).

In contrast to the previous results, when the Ca^{2+} flux in SMNC stimulated with PMA + ionomycin of fish exposed to DZN vs control was evaluated, a lower intracellular Ca^{2+} flux ($p < 0.01$) was observed in cells of fish exposed to 0.97 and 3.91 mg/L DZN for 6 and 24 h, as compared with cells of control fish (Fig. 3).

3.3. pERK1/2

The results of pERK1/2 in SMNC indicate this parameter was not significantly altered in fish exposed to DZN (0.97, 1.95, and 3.91 mg/L) for 6 h (Fig. 4a). However, at 24 h of exposure to the tested concentrations (0.97, 1.95, and 3.91 mg/L) of the pesticide, ERK1/2 phosphorylation significantly increased, in comparison with SMNC of unexposed fish (control) (Fig. 4b).

3.4. Viability and cellular death

The results of viability and cell death indicate that exposure to DZN (0.97, 1.95, and 3.91 mg/L) for 6 h did not lead to significant alterations in any of the three parameters evaluated (viability, apoptosis, and necrosis) (Fig. 5). However, in SMNC of fish exposed to 0.97 and 1.95 mg/L to the pesticide during 24 h, cell viability decreased significantly ($p < 0.01$) while the percentage of apoptotic cells increased ($p < 0.01$), in comparison with the cells of unexposed fish (control) (Fig. 6). On the other hand, the results indicate that DZN does not induce necrosis in SMNC of exposed organisms (Figs. 5c and 6c).

3.5. Cellular senescence

The results of cell senescence indicate that the exposure to DZN for 6 or 24 h, at the three concentrations evaluated (0.97, 1.95, and 3.91 mg/L), induces a significant increase in this parameter ($p < 0.01$), when compared with senescence values of SMNC in unexposed fish (Fig. 7).

3.6. Mitochondrial membrane potential

The results in the percentage of ΔΨm in SMNC indicate that DZN at the concentrations (0.97, 1.95 and 3.91 mg/L) and times (6 or 24 h) tested caused a significant decrease ($p < 0.01$) in ΔΨm of SMNC, as compared to SMNC of unexposed organisms (control) (Fig. 8).

4. Discussion

Although there is ample evidence on the immunotoxic effect of OPs, most of the studies have aimed to evaluate the effects of these substances on functional parameters of immune system cells, such as: proliferation, respiratory burst, phagocytosis, IgM concentration, lysozyme activity, phagocytic function, complement C3 expression at mRNA, contents of complement C3, IL-1β, IL-1R, and relative IFN-γ mRNA levels [6–15,23–26]. However, the molecular mechanisms of immunotoxicity of these substances are not completely clear, since OPs might exert a direct cytotoxic effect on lymphocytes. Alternatively, as indicated by previous results published by our research group, OPs can alter the leukocytes cholinergic system through an indirect mechanism [17–18]). In this way, the cholinergic system of this type of cells could be the main target of OPs in the molecular mechanism of immunotoxicity of these substances. To date, there are no reports in scientific literature evaluating the immunotoxic effect of DZN on intracellular signaling of Ca^{2+} and pERK1/2, which play an essential role

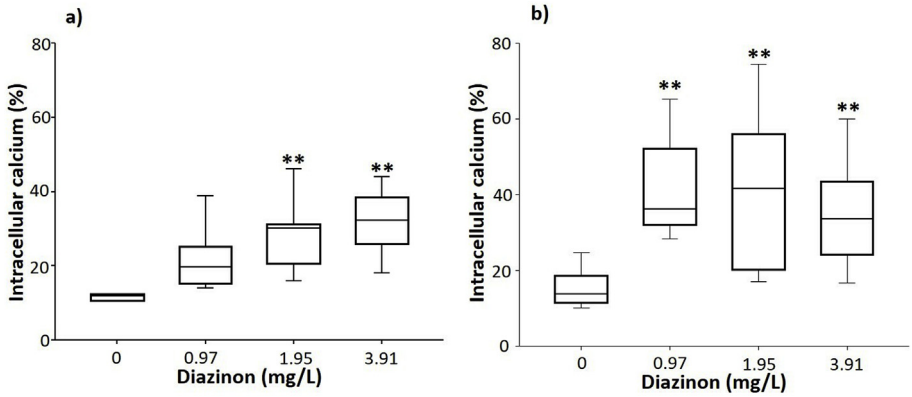

Fig. 2. Basal intracellular Ca^{2+} flux in SMNC of unexposed and exposed organisms *in vivo* to DZN (0.97, 1.95, and 3.91 mg/L) for a) 6 h and b) 24 h. Data reported as medians (horizontal bars) with 25–75% interquartile ranges (n = 10). ** indicates statistically significant differences of $p < 0.01$ with respect to controls.

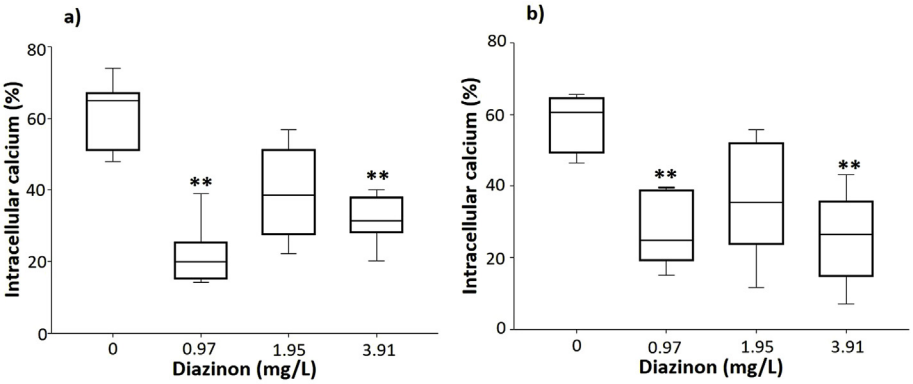

Fig. 3. Intracellular Ca^{2+} flux stimulated with PMA and ionomycin in SMNC of unexposed and exposed organisms *in vivo* to DZN (0.97, 1.95, and 3.91 mg/L) for a) 6 h and b) 24 h. Data reported as medians (horizontal bars) with 25–75% interquartile ranges (n = 10). ** indicate statistically significant differences of $p < 0.01$ with respect to control group.

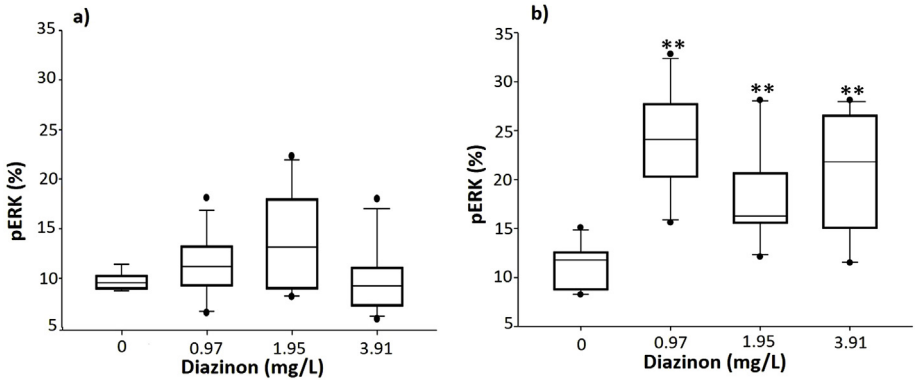

Fig. 4. PERK1/2 in SMNC of unexposed and exposed organisms *in vivo* to DZN (0.97, 1.95, and 3.91 mg/L) for a) 6 h and b) 24 h. Data reported as medians (horizontal bars) with 25–75% interquartile ranges (n = 10). ** indicate statistically significant differences of $p < 0.01$ with respect to control group.

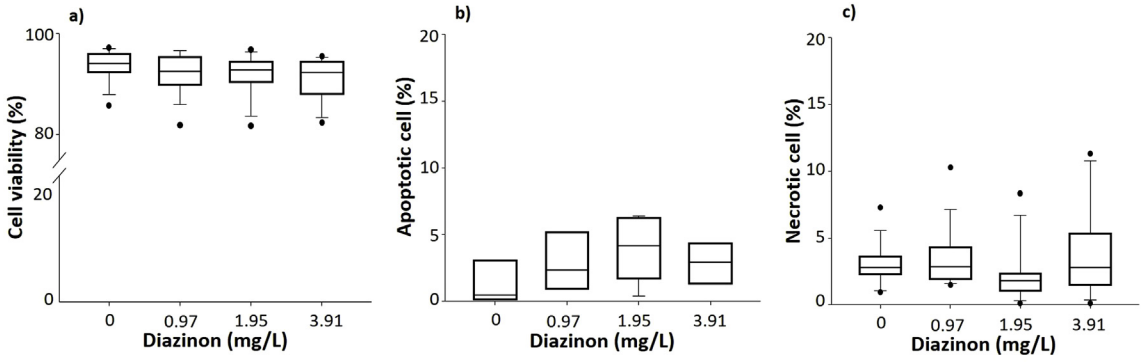

Fig. 5. Viability and death in SMNC of unexposed and exposed organisms *in vivo* to DZN (0.97, 1.95, and 3.91 mg/L) for 6 h. Percentages of a) viability, b) apoptosis, and c) necrosis are shown. Data are reported as medians (horizontal bars) with 25–75% interquartile ranges (n = 10).

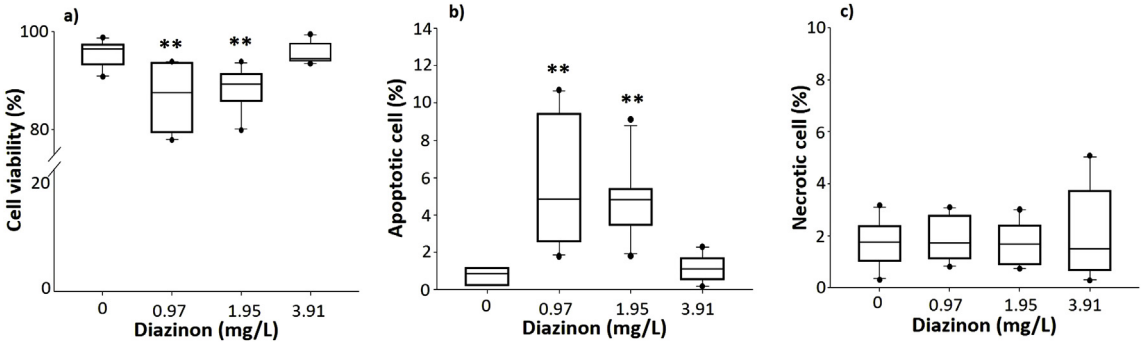

Fig. 6. Viability and death in SMNC of unexposed and exposed organisms *in vivo* to DZN (0.97, 1.95, and 3.91 mg/L) for 24 h. Percentages of a) viability, b) apoptosis, and c) necrosis are shown. Data are reported as medians (horizontal bars) with 25–75% interquartile ranges (n = 10). ** indicate statistically significant differences of $p < 0.01$ with respect to controls.

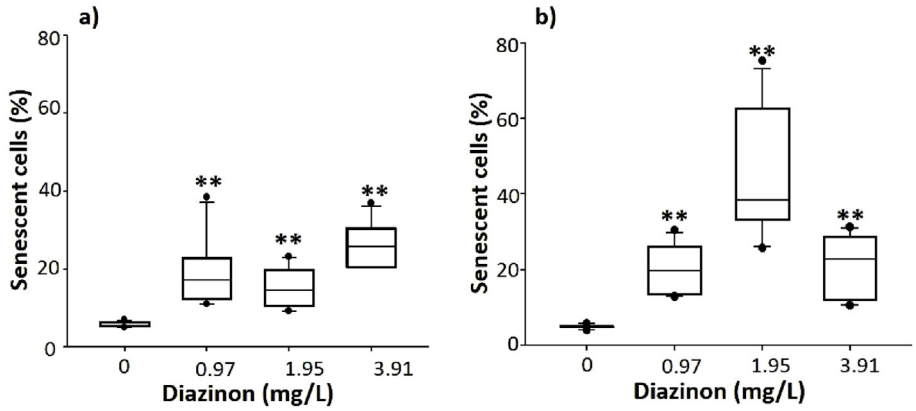

Fig. 7. Senescent cells of unexposed and exposed organisms *in vivo* to DZN (0.97, 1.95, and 3.91 ml/L) for a) 6 h and b) 24 h. Data are reported as medians (horizontal bars) with 25–75% interquartile ranges (n = 10). ** indicate statistically significant differences of $p < 0.01$ with respect to control group.

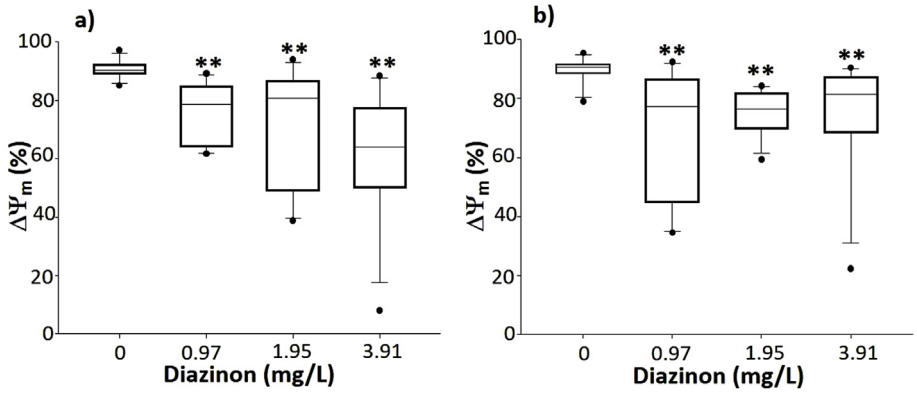

Fig. 8. ΔΨm in SMNC of control organism and those exposed *in vivo* to DZN (0.97, 1.95, and 3.91 mg/L) for a) 6 h and b) 24 h. Data are reported as medians (horizontal bars) with 25–75% interquartile ranges (n = 10). ** indicate statistically significant differences of $p < 0.01$ with respect to controls.

in intracytoplasmic signaling. Therefore, this study is the first to evaluate parameters taking part in cell signaling and antiproliferative processes (senescence and apoptosis) and assess ΔΨm in leucocytes of Nile tilapia, a freshwater fish of great ecological and commercial value worldwide that has even been used as a biomedical model.

Intracellular Ca^{2+} is one of the main components of intracytoplasmic signaling cascades, usually triggered after the stimulation of membrane receptors. In T and B lymphocytes, the increase in calcium flux, commonly promoted by exposure to antigens, leads to multiple intracellular alterations that regulate gene expression and molecule production (CD40 ligand, FasL, TNF-α, and IL1, 4, 6, 8,12) [27–29]. Then, this work demonstrates that exposure to DZN *per se* increases basal Ca^{2+} flux in SMNC but significantly reduces the capacity to respond against PMA and ionomycin stimuli (molecules typically inducing Ca^{2+} flux). This could suggest that, in organisms exposed to OPs, leukocytes might exhibit a lower capacity to respond to antigenic stimuli, which could be related to the decrease in proliferation, phagocytic capability, and cytokine and antibody production as reported by several research groups [7,11,13–15,20]. Furthermore, it is likely related to the decrease in the resistance to infections reported through bacterial challenges in fish exposed to OPs [30].

The present work proves that DZN induces an increase in pERK1/2 and the loss of ΔΨm, as well as an increase in the percentage of senescent and apoptotic cells. All these alterations are likely related to the cytosolic Ca^{2+} concentration and flux given that, according to reports, higher levels of intracellular Ca^{2+} induce dysregulation in ERK1/2 phosphorylation, molecules that can induce cell death by apoptosis or senescence, depending on their intracellular location and excessive activation. In this way, it has been reported that the overactivation of ERK in the nucleus can induce death by apoptosis while the overactivation of cytosolic ERK induces senescence [31]. In agreement with the data obtained in this work, we suggest that DZN can indirectly induce the permanent ERK1/2 phosphorylation by increasing intracellular Ca^{2+} flux, which finally leads the cell to apoptosis and senescence.

Senescence and apoptosis are closely influenced by Ca^{2+} flux, given that the latter considerably regulates transduction pathways mediated by PLCγ-IP3 [32]. Additionally, cell aging and cell death can also be related to oxidative stress and the excessive production of ROS in organisms exposed to DZN, a phenomenon that has previously been reported by our research group [9,33].

On the other hand, Ca^{2+} induces the activation of proapoptotic transcriptional factors as well as caspase-mediated signals. Furthermore, this cation modulates the activity of voltage-dependent calcium channels (VDCCs), causing to higher levels of calcium in this organelle, which leads to depolarization of the mitochondrial membrane, an increase in ROS, and the subsequent opening of the mitochondrial permeability transition pore (mPTP). This allows for cytochrome C release into the cytoplasm and, in consequence, the activation of caspase cascades, intracellular phenomena that might be related to the loss of ΔΨm reported in this study [34–36].

5. Conclusion

The results from this work demonstrate that DZN promotes the deregulation in intracellular Ca^{2+} flux and pERK1/2, leading to the depolarization of the mitochondrial membrane, and in turn triggering cell senescence and/or death by apoptosis. This suggests that DZN modulates signaling of Ca^{2+} and pERK1/2, key parameters to the survival of immune system cells. Therefore, our work provides new

understanding of the toxic effects caused by DZN in immune system cells of fish.

On the other hand, the results obtained in this research suggest that Ca^{2+} is a cell signaling molecule that plays a key role in the immunotoxicity mechanism of OPs. This finding opens new perspectives in the research for strategies to counter accidental intoxication by this type of pesticides both on humans and aquatic organisms.

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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