Fish & Shellfish Immunology

Immunotoxicity and hepatic function evaluation in Nile tilapia (*Oreochromis niloticus*) exposed to diazinon

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Abstract

The LC_{50} of the organophosphorus pesticides (OPs) diazinon to Nile tilapia (*Oreochromis niloticus*) was determined, thereafter, hepatic activity, phagocytic index, percentages of active cells, relative spleen weight, total IgM concentration and lymphoproliferation rates were compared between diazinon exposed groups (LC_{50} and ½LC_{50}) and non-exposed control group.

Experimental data show that diazinon is highly toxic for juvenile Nile tilapia ($LC_{50} = 7.830$ ppm) and presents immunotoxic properties which affect both the innate and cellular adaptive immune responses of this fish, as revealed by the fact that splenocyte proliferation and phagocytic indices were significantly decreased after acute exposure to the pesticide. However, the hepatic biochemical parameters and the total circulating IgM concentrations were not affected in this experimental model.
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1. Introduction

The immune system of aquatic organisms, such as fish, is continuously affected by periodic or unexpected changes of their environment. Adverse environmental situations may acutely or chronically stress the health of fish, altering some of their biochemical parameters and suppressing their innate and adaptive immune responses [1].

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The major xenobiotics involved as immunomodulators are pesticides (insecticides, herbicides and fungicides), organic pollutants such as polynuclear aromatic hydrocarbons (PAH) and polychlorinated biphenyls (PCB). Exposure to sub-lethal concentrations of pesticides is suspected of predisposing fish to diseases because of their immunodepressive effects [2].

Pesticides are toxic substances released into the environment in large amounts with the potential to cause adverse effects on human and wildlife populations [3]. Organophosphorus pesticides (OPs) are insecticides used world-wide, designed as irreversible acetylcholinesterase (AChE) inhibitors. They were introduced as an alternative to the persistent and more bioaccumulative organochlorine pesticides. Diazinon (0,0-diethyl 0-(6-methyl-2-{1-methylethyl}-4-pyrimidinyl)phosphorothioate) is a broad spectrum OP insecticide, effective against many pests of fruits, vegetables, tobacco, forage, field crops, pasture, grasslands and ornamental plants. After entering the water this pesticide remains stable for up to six months and accumulates in non-target tissues, provoking undesirable and unintended biochemical changes [4−7] and presents toxic effects on the immune system of wildlife organisms [8,9]. For instance, the effect of diazinon on hematological indices and enzyme activities in blood plasma has been evaluated in carp (*Cyprinus carpio* L.), indicating that the exposure to these OPs induced marked alterations in its biochemistry [10]. Moreover, other reports on fish models showed that diazinon caused changes in immunological parameters, such as population of monocytes and lymphocytes [11], macrophages [12] and antibody-producing cells [13,14].

The majority of the immunotoxicological studies has been made in higher vertebrates, i.e. mammals, and although the dynamic of pesticides always affects the aquatic environment, their possible immunotoxic and biochemical effects on aquatic organisms, such as fish, have received relatively little attention so far [3]; thus, not all of the effects of diazinon on fish are known, in spite of its very intensive use.

Tilapia (*Oreochromis* spp.) is a teleost fish with a worldwide distribution; therefore it is a good model for assessing aquatic ecosystems and for toxicological studies. A recent comparative study of five economically important taxa of tilapia showed that Nile tilapia (*Oreochromis niloticus*) presents a strong immune system which provides this variety with a good capacity to tolerate biotic and abiotic stresses [15]. However, the immune system of this fish could be significantly affected by the presence of OPs, such as diazinon, in extensively cultivated areas where rainfall and irrigation wash these substances down into rivers and streams, and subject the aquatic life to contamination.

Therefore the present study was aimed at evaluating the toxicity of the diazinon through lethal concentration 50 (LC_{50}) determinations and the effect of acute exposure to this insecticide on several biochemical and immunological parameters of Nile tilapia. Hepatic enzyme activities: alanine aminotransferase (ALT), alkaline phosphatase (ALP) and γ-glutamyl transferase (GGT), phagocytic index (PI), percentages of active cells (%AC), relative spleen weight (RSW), total IgM concentration and lymphoproliferation rates were compared between groups of tilapia exposed to diazinon (LC_{50} and ½LC_{50}) and non-exposed control group.

2. Materials and methods

2.1. Animals

The Nile tilapia were hatched, grown and maintained in 40 L glass aquariums (two fish per tank), at 28 ± 2 °C and aerated constantly, for a 10-day adaptation period before initiating the experiment. Their daily food consisted of commercial dry pellet for fish. Male animals weighing approximately 110 g were used for all experiments.

2.2. LC_{50} determination

Experiments were designed to evaluate the effect of the LC_{50} level of diazinon (Anajalsa, Mexico) on Nile tilapia. Diazinon was dissolved directly in the 40-L tanks. In a preliminary experiment, designed to determine the general range of concentration of diazinon that is lethal to tilapia in our experimental conditions, groups of 10 fish (two fish per 40 L tank, using five tanks) were exposed to diazinon. The experiment was repeated for each concentration of diazinon (0.1−12.0 ppm) [16], without water change, during a 96-h time period. The mortality rate was recorded for each concentration of diazinon and a separate group of 10 non-exposed fish was used as control.

According to this ranking assay, a second bioassay was then undertaken which consisted of the exposure of 10 fish (two fish per 40 L tank) to 7.5, 7.6, 7.8, 7.9, and 8.0 ppm of diazinon. These experiments were performed in triplicate

for each concentration of pesticide (so total count of fish was 30 per diazinon concentration) and the recorded data were analyzed using Probit method [17] with the help of EPA Probit Software 1.5 Version.

2.3. Experimental groups

Immunotoxicological tests and hepatic function evaluation were estimated by exposing 10 fish (two fish per tank) to different concentrations of diazinon (LC_{50} and $\frac{1}{2}LC_{50}$). Non-exposed control group of 10 fish was maintained under similar conditions as exposed groups, but without pesticide. Evaluation of the different immune and biochemical parameters (hepatic activity, phagocytic index, percentages of active cells, relative spleen weight, total IgM concentration and lymphoproliferation rates) was performed in triplicate for each sample.

Control and experimental fish were anesthetized with tricaine methanesulfonate (MS 222), blood was obtained by cardiac puncture and the fish were sacrificed by decapitation and subsequently carefully dissected to remove the spleen.

2.4. Hepatic function

One milliliter of peripheral blood was collected in heparin tubes from both the exposed groups (LC_{50} and $\frac{1}{2}LC_{50}$, for 96 h) and non-exposed control groups ($n = 10$), centrifuged at 2500 rpm for 20 min, and frozen at $-10\ °C$ for no more than two days before analysis. Quantitative determination of alkaline phosphatase (ALP), alanine aminotransferase (ALT) and γ-glutamyl transferase (GGT) activities was performed automatically in a Synchron zx4 Beckman Coulter analyzer, with the appropriate Beckman Coulter-Synchron® Systems, based on kinetic rate methods, using different specific substrates. The enzymes under study catalyze the change of a colorless specific substrate to a colored product, resulting in changes in absorbance which are directly proportional to the activity of the enzyme in the sample and are used by Synchron® System(s) to calculate and express the enzyme activities.

2.5. Phagocytic assays

Phagocytic functional assays were performed in vitro, using the glass adherence method: 200 mL of blood from the exposed groups (CL_{50} and $\frac{1}{2}CL_{50}$, for 96 h) and non-exposed control group ($n = 10$) were defibrinated with glass beans, placed on a glass coverslip, incubated in a moist chamber at $28\ °C$ for 20 min and washed thoroughly with Hank's buffered salt solution (HBSS) supplemented with 0.002% human albumin (HA) to eliminate the red blood cells. A mixture of 20% autologous serum and 80% of *Candida albicans* cells (equivalent to 1×10^{6}) in HBSS− HA was added to the coverslip and incubated ($28\ °C$, 40 min, in a moist chamber), washed as described earlier, incubated again with HBSS−HA ($28\ °C$, 20 min) and given a final wash. Staining was done using Wright's solution for 1 min. The %AC was quantified by counting a combined total of 100 phagocytic (neutrophils and monocytes) and nonphagocytic cells and expressing the results as percentage of positive phagocytic cells. The PI was expressed as the average number of yeast cells engulfed per cell and calculated by dividing the total number of yeast engulfed by cell count, which were in this case 100−200 phagocytic and nonphagocytic cells [18,19].

2.6. Relative spleen weight (RSW)

The spleens from fish of each group exposed to LC_{50} and $\frac{1}{2}LC_{50}$ of diazinon and non-exposed control group ($n = 10$) were lightly blotted, weighed and the RSW was expressed according to the following equation: $RSW = (SW_{(g)}/FW_{(g)})100$, where SW represents the spleen weight and FW the fish weight. The individual RSW were averaged ($n = 10$) and compared between study groups [20].

2.7. Total IgM concentration

The total IgM concentration in the plasma of the groups of tilapia exposed to diazinon (LC_{50} and $\frac{1}{2}LC_{50}$) and the non-exposed control group ($n = 10$) was measured by ELISA according to the method of Takemura (1993) [21]. Purified tilapia IgM, rabbit anti-tilapia IgM antibody (a-IgM) and a-IgM labeled with horseradish peroxidase (a-IgM HRP) were prepared in advance. Each well of a 96-well plate (Costar) was coated with 100 mL of a-IgM

(6.8 mg/mL) in 0.05 M sodium carbonate buffer, pH 9.6, and incubated for 2 h at 25 °C. Residual protein-binding sites were blocked by adding 200 mL of 1% BSA (Sigma, St. Louis, MO) dissolved in 10 mM phosphate-buffered saline (PBS), pH 7.4, containing 0.05% Tween 20 (PBS–Tween) to the wells for 60 min at 25 °C. After washing the wells three times with PBS–Tween, 100 mL of plasma sample (1:10 000) or standards (serial dilution of purified tilapia IgM) were added to each well and then incubated overnight at 4 °C. All the dilutions were made with PBS–Tween. After washing the wells three times with PBS–Tween, 100 mL of a-IgM HRP (diluted 1:10 000 in PBS–Tween) were added to the wells and incubated for 2 h at 25 °C. After three successive washes with PBS–Tween, peroxidase activity was measured by adding 100 mL of 100 mM citrate buffer, pH 4.5, containing 0.01% o-phenylenediamine dihydrochloride (Sigma, St. Louis, MO) and 0.04% H_2O_2. Following a 30-min incubation period at 25 °C, the enzymatic reaction was stopped by adding 25 mL of 4 N H_2SO_4 and the optical density of each sample was determined at 490 nm using a microplate reader OpsysMR (DYNEX Technologies). The conversion from optical density to IgM concentration was calculated using a lineal regression formula [22].

2.8. Lymphocyte proliferation test

The mononuclear cells were obtained and isolated from spleen of tilapia, from exposed groups (LC_{50} and ½LC_{50}) and non-exposed control group ($n = 10$), using Histopaque-1077. The cellular concentration was calculated and adjusted in RPMI-1640 medium supplemented with 10% fetal calf serum. One volume of cell suspension containing 1×10^6 cells was dispensed into 96-well culture plates, and stimulated with an equal volume of PMA and ionomycin (to a final concentration of 20 ng/mL PMA and 1 mg/mL of ionomycin); control cells received RPMI-1640 medium alone. The volume was finally adjusted to 160 mL/well with RPMI-1640 medium. After incubation for 72 h at 28 °C and 5% CO_2 in a humid incubator (NAPCO), cell proliferation was determined by MTT (3-(4,5-diamethyl-2-thiazolyl)2,5-diphenyl-2H-tetrazolium) based assay [23]: 5 mg/mL of MTT were added to each well and incubated at 28 °C for 4 h. The precipitated formazan was dissolved with dimethylformamide at 25% and sodium dodecyl sulfate (SDS) at 25%; the optical density was measured at 570 nm in an ELISA reader OpsysMR (DYNEX Technologies). Each sample was examined in triplicate. Stimulation index (SI) was calculated with the following equation: $SI = A_1/A_0$; Where A_1 is the absorbance of the test stimulated sample and A_0 is the absorbance of the sample without stimulation.

2.9. Statistical analysis

The mean ± S.D. was determined for each study group in a given experiment.

Data were analyzed by one-way ANOVA and Tukey's test using Sigma Stat 2.0 Software to indicate whether there was a statistical difference ($p < 0.05$ or $p < 0.001$) between study groups.

3. Results

3.1. LC_{50} of diazinon to Nile tilapia

Probit analysis of data collected on percent mortality at 96 h exposure to different concentrations of the pesticide revealed the LC_{50} value to be at 7.830 ppm. Thus all experimental groups were exposed to 7.830 ppm (LC_{50}) and 3.915 ppm (½LC_{50}) of diazinon, for 96 h (Fig. 1).

3.2. Diazinon effect on hepatic function

The activities of the enzymes under study (ALT, ALP and GGT) in Nile tilapia exposed to diazinon (7.830 ppm and 3.915 ppm) for 96 h did not show statistical differences ($p > 0.05$) when compared with non-exposed control group (Table 1).

3.3. Diazinon effect on phagocytic indices

The effect of diazinon on phagocytic cells was evaluated through the determination of the PI and %AC, in exposed groups and non-exposed control group. The PI was significantly reduced in tilapia exposed to 7.830 ppm and
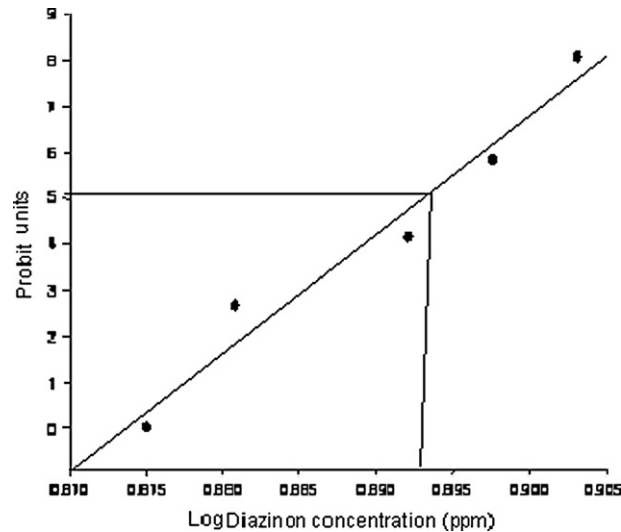
Fig. 1. Lethal concentration 50 (LC_{50}). Determination of LC_{50} of diazinon to Nile tilapia using the Probit method, where the LC_{50} value is the anti-log of the concentration when Probit unit is 5.

3.915 ppm of diazinon (PI $= 1.5 \pm 0.7$ and 1.2 ± 0.4, respectively) compared with control group (PI $= 2.9 \pm 0.6$). Likewise the %AC decreased significantly in tilapia exposed to 7.830 ppm and 3.915 ppm of these OPs (%AC $= 62.0 \pm 12.7$ and 59.8 ± 11.9, respectively) compared with non-exposed control group (%AC $= 80.6 \pm 25.6$) (Figs. 2 and 3).

3.4. Diazinon effect on relative spleen weight (RSW)

The relative spleen weight was significantly reduced ($p < 0.05$) in fish exposed to both doses of diazinon: RSW $= 0.096 \pm 0.06$ (at 7.830 ppm) and 0.1 ± 0.04 (at 3.915 ppm), compared with non-exposed control group (RSW $= 0.19 \pm 0.06$) (Fig. 4).

3.5. Diazinon effect on plasma IgM

Fish exposed to 7.830 ppm and 3.915 ppm of pesticide presented IgM concentrations of 409.5 ± 23.13 mg/mL and 405.4 ± 36.8 mg/mL, respectively, and the control group showed an IgM concentration of 392.2 ± 26.3 mg/mL (Fig. 5). The statistical analysis showed that the exposure to diazinon for 96 h did not significantly affect the concentration of circulating antibodies ($p > 0.05$).

3.6. Diazinon effect on cellular proliferation

The effect of diazinon on mitogen induced lymphoproliferation (PMA and ionomycin) was examined in splenocytes using the MTT assay. Experimental data indicate that the lymphocytes from fish exposed to 7.830 ppm and 3.915 ppm of diazinon showed a significant proliferation reduction (SI $= 0.97 \pm 0.1$ and 1.0 ± 0.4, respectively), compared with non-exposed control group (SI $= 2.13 \pm 0.7$) (Fig. 6).

Table 1
Enzymatic activity in plasma from Nile tilapia ($n = 10$) exposed to diazinon (96 h)

Hepatic parameter	Diazinon concentration		
	Control	7.830 ppm	3.915 ppm
GGT (UI/L)	27.4 ± 10.7	27.5 ± 12.9	24.0 ± 19.0
ALT (UI/L)	9.6 ± 8.6	8.2 ± 7.1	7.0 ± 5.4
ALP (UI/L)	15.5 ± 9.8	22.7 ± 4.5	17.4 ± 8.7

Data shown as mean $\pm$ S.D., and compared with one-way ANOVA. GGT: γ-glutamyl transferase; ALT: alanine aminotransferase; and ALP: alkaline phosphatase. No statistically differences were observed.
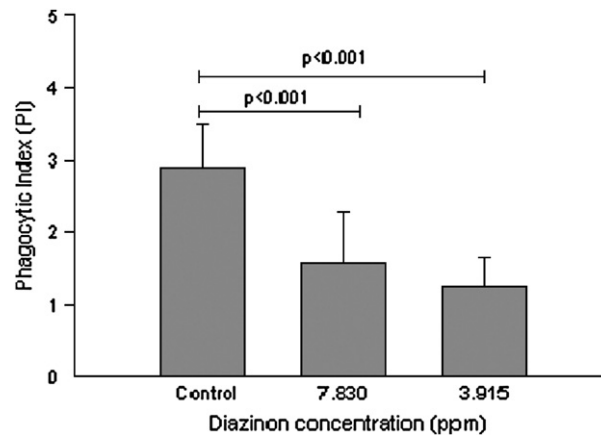
Fig. 2. Phagocytic index. Determination of phagocytosis, expressed as phagocytic index (PI) of blood cells from Nile tilapia exposed to LC_{50} and $\frac{1}{2}LC_{50}$ of diazinon during 96 h, and non-exposed fish (control). Results are expressed as mean $\pm$ SD, one-way ANOVA and Tukey test were applied.

4. Discussion

OPs are known to cause immunosuppression, however, there have been few studies involving fish. Different values of LC_{50} of diazinon have been reported (ranging from 0.1 to 12 ppm), indicating a variation in the toxic activity of this insecticide, which could be related to numerous factors such as species, stage of development, environmental conditions and exposure period. For these reasons, the present study started with the determination of the LC_{50} of diazinon to *O. niloticus* which allowed us to study its effect on this particular species [17].

The pesticides are absorbed and carried to the liver, where the metabolizing enzymes responsible for both the bioactivation and detoxification are present [24]. In serum or plasma, the measurements of the activity of ALT, ALP and GGT enzymes are used routinely as an indicator of biochemical alterations of hepatic function caused by different types of toxic agents.

The activity of the ALT was similar in exposed groups and non-exposed control group which indicates that the acute exposure to diazinon did not damage hepatic parenchymatous tissues. The activities of the GGT and the ALP were not significantly modified between study groups. This could sustain the idea that the diazinon is not hepatotoxic, at the time of exposure and with the concentrations used here; similar results have been also reported in carp (*C. carpio* L.) [25]. Moreover, the evaluation of the toxicity of the diazinon to tilapia and the effect of acute exposure
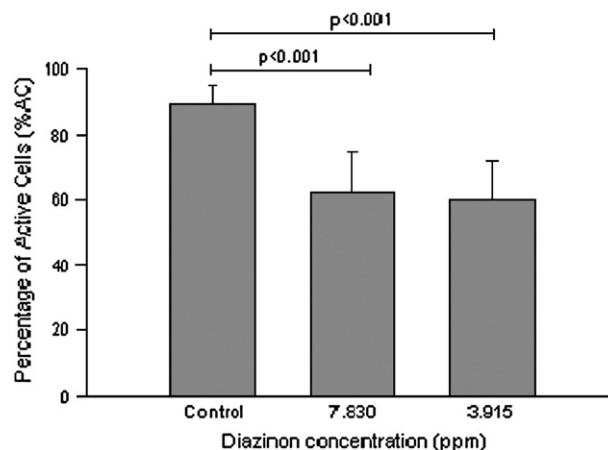

Fig. 3. Percentage of active cells. Determination of phagocytosis, expressed as percentage of active cells (%AC), in blood of in Nile tilapia exposed to LC_{50} and $\frac{1}{2}LC_{50}$ of diazinon during 96 h, and non-exposed fish (control). Results are expressed as mean $\pm$ SD, one-way ANOVA and Tukey test were applied.
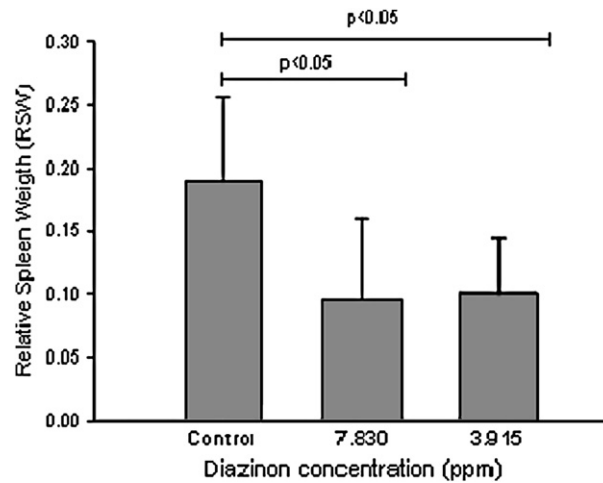
Fig. 4. Relative spleen weight. Determination of relative spleen weight (RSW) in Nile tilapia exposed to LC_{50} and $\frac{1}{2}LC_{50}$ of diazinon during 96 h, and non-exposed fish (control). Results are expressed as mean $\pm$ SD, and one-way ANOVA and Tukey test were applied.

to this insecticide on several innate and adaptive parameters (PI, %AC, RSW, total IgM concentration and SI) provided information on its immunotoxicity.

The principal cells involved in the phagocytic process are the macrophages in tissues and the neutrophils and monocytes in blood [26,27]. Exposure of fish to diazinon polluted water caused pathological changes in fish and activated the monocytes–macrophage [28]. The innate immune response of Nile tilapia to these OPs was evaluated through the determination of two phagocytic parameters: PI and %AC. Our observations indicated that acute exposure to diazinon significantly reduced the PI and the %AC in exposed groups compared with control group ($p < 0.05$), confirming other studies which showed that diazinon had a clear negative effect on the phagocytic capacity of fish [12,13]. Similarly, a lower number of monocytes in peripheral blood have been reported in mice exposed to diazinon at 25 mg, 2 mg and 0.2 mg/kg [29]. A decrease in neutrophil and monocyte counts as well an increase of the morphological abnormalities in spleen, thymus and lymph nodes from mice exposed to 300 mg/kg of diazinon for 45 days have also been reported [30].

The weight of the spleen can be used to assess the relative immune status of the fish. In the present study data showed that diazinon exposure significantly reduced ($p < 0.05$) the relative weight of this lymphoid organ, indicating an atrophy of this organ. Similar effects have been reported in mice chronically exposed to diazinon [29]. These authors also reported a statistically important decrease in cellular content of spleen and thymus weight.

The ability to respond to antigens by production of specific antibodies is directly related to the repertoire of B-cell clones. In fish the IgM is the most important antibody, with a tetrameric structure as opposed to the pentameric
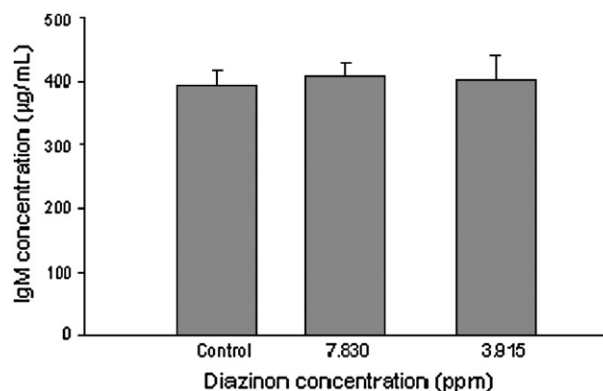

Fig. 5. IgM concentration. ELISA determination of total IgM concentration in plasma of Nile tilapia exposed to LC_{50} and $\frac{1}{2}LC_{50}$ of diazinon during 96 h, and non-exposed fish (control). The results were expressed as mean $\pm$ SD, and one-way ANOVA was applied.
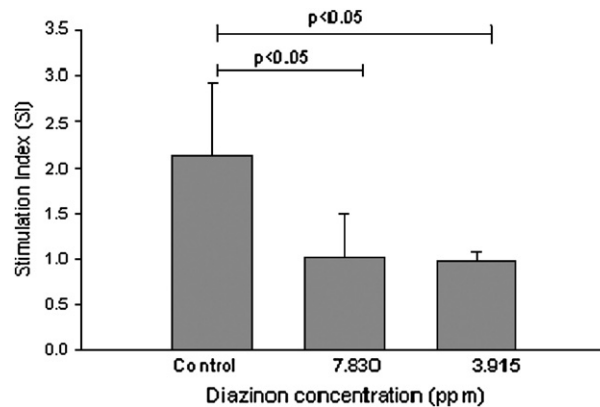
Fig. 6. Stimulation index. Determination of proliferation, expressed as stimulation index (SI), of spleen cells from Nile tilapia exposed to LC_{50} and $\frac{1}{2}LC_{50}$ of diazinon during 96 h, and non-exposed fish (control). The results were expressed as mean $\pm$ SD, and the ANOVA one-way and Tukey test was applied.

structure in mammals [31,32]. The total IgM concentration in plasma of exposed Nile tilapia was not statistically different compared with the control group. This could suggest that acute exposure to diazinon did not affect the humoral immune response of tilapia.

The in vitro proliferative response is evaluated through the stimulation index, and is one of the most reliable tests for assessment of the immunocompetence of lymphocytes after mitogenic stimulus [33]. Thus a reduction in the SI may be an indication of a lowering of the immunocompetence of the organism. Greatly reduced cellular proliferation was observed in splenocytes from exposed fish, stimulated with PMA and ionomycin, compared with cells from un-exposed fish ($p < 0.05$). Similar behavior was reported in studies made on Balb/C mice lymphocytes, which showed that diazinon had a negative effect on T lymphocytes [34]. Moreover, studies showed that the OPs chlorpyrifos presents a high toxicity in vitro on fish cell lines and in vivo on *O. niloticus* neutrophils [35,36]. It is also reported that diazinon and diazoxon (oxygen metabolite of diazinon) inhibited DNA synthesis in astroglial cells from rats and human astrocytoma cell lines [37].

The determination of the LC_{50} of diazinon to Nile tilapia indicated that this widely used OP is highly toxic for this fish. Using more than one technique we have demonstrated here that the acute exposure to diazinon caused severe negative effects on several parameters of the innate and adaptive immune responses of Nile tilapia, confirming its immunotoxicity to lymphocytes, phagocytic cells and spleen. However, it did not affect the biochemical parameters as determined through the enzyme activities in the liver. It is worth mentioning that no significant differences were observed between LC_{50} and $\frac{1}{2}LC_{50}$ groups for any of the parameters evaluated here. The low immune response of exposed fish will probably affect their capacity to tolerate biotic and abiotic stresses and make them more susceptible to infectious agents.

With regard to possible mechanism of immunotoxicity, the effects described here could be the result of different factors, e.g. the direct cellular toxicity for the diazinon or the indirect effect of some metabolite of this pesticide such as diazoxon [38]. Another factor could be the alteration of the neuro-immunological axis, due to variation of the neurotransmitter acetylcholine concentration as a natural result of intoxication with organophosphorus pesticides [39,40]. Thus further experimental work is required to investigate the immunotoxicological mechanisms of action of this OP in Nile tilapia.

Acknowledgments

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