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Effect of Sub-lethal Concentrations of Endosulfan on Phagocytic and Hematological Parameters in Nile Tilapia (*Oreochromis niloticus*)

M. I. Girón-Pérez · M. Montes-López · L. A. García-Ramírez · C. A. Romero-Bañuelos · M. L. Robledo-Marenco

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Abstract The effect of endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin-3-oxide), an organochlorine pesticide, was evaluated on phagocytic (phagocytic index and percentage of active cells) and hematological parameters in Nile tilapia. Experimental data showed that an acute exposure to endosulfan (4.0 and 7.0 μg/L) induces a significant decrease in the phagocytic index and the percentage of active cells in peripherical blood of Nile tilapia. However, hemoglobin concentration (Hb), hematocrit (Hto), red blood cell count (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were not significantly altered in fish exposed to endosulfan compared with control group.

Keywords Endosulfan · Nile tilapia · Hematology · Immunotoxicity

Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin-3-oxide) is an organochlorine insecticide of the cyclodiene subgroup, which is commonly used for a wide variety of food crops. However, the substance is highly toxic to non-targeted organisms (Ayub et al. 2003). Effluents of agricultural process may contain elevated levels of chemicals, such as pesticides, which will probably pollute the aquatic environments and induce alterations in fish (Hemmer et al. 2001). Consequently, contamination caused by pesticides, particularly endosulfan, may provoke mass mortality of fish and other aquatic species (Van Dyk and Greeff 1977).

Blood reflects the patho-physiological status of the body; therefore, blood parameters are important in diagnosing the structural and functional status of organisms exposed to toxic substances (Jenkins et al. 2003; Luskova 1996). Another physiological system frequently affected by pesticides is the immune system, particularly the innate mechanisms of the immune response, such as phagocytosis. This is a fundamental mechanism that eventually causes the destruction of pathogens (Neumann et al. 2001). Therefore, the alteration of phagocytic and other immune processes by exposure to pesticides could increase the susceptibility of the organism to infections.

Little information exists on the sub-lethal toxicity of commercial formulations of endosulfan on fish, and specifically, its biological effects on Nile tilapia (*Oreochromis niloticus*), a worldwide economically important freshwater fish (Fitzsimmons 2000). Some studies have shown the presence of endosulfan in aquatic ecosystems, where *O. niloticus* and other tilapia species can be found. Furthermore, accumulations of this pesticide have been shown in tissue samples of the mentioned species (Caldas et al. 1999; Zhou et al. 1999). The present study was carried out in order to determine the effect of sub-lethal concentrations of endosulfan (Thiodan 35 CE) on hematological parameters including: erythrocytes count, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular

M. I. Girón-Pérez (✉)
Laboratorio de Inmunotoxicología, Posgrado CBAP, Universidad Autónoma de Nayarit, Cd. de la Cultura Amado Nervo, Tepic, Nayarit 63000, Mexico
e-mail: ivangiron@nayar.uan.mx

M. Montes-López · L. A. García-Ramírez · C. A. Romero-Bañuelos · M. L. Robledo-Marenco
Laboratorio de Contaminación y Toxicología Ambiental, Universidad Autónoma de Nayarit, Cd. de la Cultura Amado Nervo, Tepic, Nayarit 63000, Mexico

hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and phagocytic function.

Material and Methods

Juvenile Nile tilapia (*O. niloticus*) were obtained from an aquaculture farm of SAGARPA (Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación) in Nayarit, Mexico. Selected male fish (2 months old and weighing approximately 80 g) were transferred in oxygenated containers to 40-L glass aquariums, which were maintained at a constant temperature (28 ± 2°C) with continuous aeration for a 10-day adaptation period in a bioassay laboratory before beginning the experiments. During this period, the fish were fed daily with dry commercial pellets (Purina^{MR}).

A commercial formulation (Thiodan 35 CE®, by Bayer), which is often used in Mexico, was used in the experiment. This stock solution of endosulfan was diluted with distilled water. Two sub-lethal concentrations (4.0 and 7.0 μg/L) were prepared by adding adequate volumes of stock solution to the aquariums. For each pesticide, ten fish were tested, and appropriate controls (fish in a similar condition, but without receiving pesticide) were run simultaneously. The bioassays were carried out in static conditions without solution replacements during a 96-h period. The average values for water quality were: temperature 28 ± 2°C, pH 8.0 ± 0.1, dissolved oxygen 7.0 ± 0.2 mg/L, and oxygen saturation 85.4 ± 2.4%.

After a 96-h exposure to endosulfan, the fish were rapidly anesthetized with methanesulfonate (MS-222, Sigma Chemical Co, USA). One milliliter of blood was obtained by cardiac puncture and placed in glass tubes containing EDTA (Sigma Chemical Co, USA), while the fish were sedated. In order to evaluate the red blood cell count (RBC), the blood was diluted to 1:200 with "Growers" liquid (Sigma Chemical Co, USA), mixed for 1 min and placed in a hemocytometer. The total RBC number was determined manually, using the standard formula:

$$\text{Erythrocytes/mm}^3 = (\text{counted cells})\,(\text{dilution factor})\,/\,(0.04\ \text{mm}^2)\,(\text{counted squares})\,(0.1\ \text{mm}).$$

The hemoglobin concentration was determined by using the cyanmethemoglobin method. Blood was diluted at 1:25 with "Drabkin's" solution (Sigma Chemical Co, USA). The mixture was kept at room temperature for three minutes, and the optical density was determined at 540 nm. Data were interpolated from a calibration curve previously obtained. In order to determine the hematocrit value, the blood was transferred to draw-in micro-hematocrit tubes and centrifuged for 5 min (Hermle Z 383 K) at 10,000 *g*. The MCV, MCH and MCHC were calculated according to the following formulae:

$$\text{MVC} = (\text{Hematocrit})(10)/\text{erythrocytes per } \mu\text{L}.$$

$$\text{MCH} = (\text{Hemoglobin})(10)/\text{erythrocytes per } \mu\text{L}.$$

$$\text{MCHC} = (\text{Hemoglobin})(100)/\text{hematocrit}.$$

Phagocytic functional assays were performed in vitro using the glass-adherence method: 200 μL of blood from the exposed groups (4.0 and 7.0 μg/L endosulfan, for 96 h) and non-exposed control groups were defibrinated with glass beans. The samples were placed on a glass coverslip and incubated in a moist chamber at 28°C for 20 min. They were then washed thoroughly with Hank's buffered salt solution (HBSS, Sigma Chemical Co, USA) and supplemented with 0.002% human albumin (HA, Sigma Chemical Co, USA) to eliminate the red blood cells. A mixture of 20% autologous serum and 80% of *Candida albicans* cells (equivalent to 1×10^6 yeast cells/mL) in HBSS-HA was added to the coverslip and incubated at 28°C for 40 min in a moist chamber. These samples were washed as described earlier and incubated again with HBSS-HA (28°C, 20 min) before given a final wash. The samples were stained using Wright's solution for 1 min. The percentage of Active Cells was determined by counting a total of 100 phagocytic (neutrophils and monocytes) and non-phagocytic cells. The results were expressed as the percentage of positive phagocytic cells. The phagocytic index was expressed as the average number of yeast cells engulfed per cell and calculated by dividing the total number of yeast cells engulfed by counted cells, in this case 100–200 phagocytic and nonphagocytic cells (Casas-Sólis et al. 2007; Muñiz et al. 2006; Watanuki et al. 1999; Ainsworth et al. 1991).

The phagocytic and hematological parameters of organisms intoxicated with commercial endosulfan (4.0 and 7.0 μg/L) were compared with the values of non-intoxicated fish. The data were analyzed by ANOVA and Tukey tests with 95% of confidence using Sigma Stat 2.03 software.

Results and Discussion

Blood samples from intoxicated and control fish were obtained for determining the hematological parameters. The results showed that endosulfan does not affect the hematological parameters (RBC, hemoglobin, hematocrit value, MCV, MCH, and MCHC) for neither of the two concentrations used in this study, as compared with the control group ($p > 0.05$) (Table 1).

In order to determine the phagocytic activity, two parameters were used: phagocytic index and percentage of

Table 1 Hematological values obtained from Nile tilapia (n = 10 per group) after exposure (96-h) to sub-lethal concentrations of endosulfan

Data were analyzed with ANOVA, and showed no significant difference ($p > 0.05$) between the three cases. Data given are mean ± SD

Hematological parameters	Endosulfan concentrations		
	Control	4.0 μg/L	7.0 μg/L
RCB ($\times 10^6$/μL)	1.6 ± 0.37	1.7 ± 0.31	1.5 ± 0.28
Hemoglobin (g/dL)	5.46 ± 1.34	5.46 ± 0.96	5.80 ± 1.07
Hematocrit (%)	22.51 ± 6.63	22.78 ± 4.47	24.17 ± 6.97
MCV (Fl)	140.09 ± 21.06	135.01 ± 16.69	156.36 ± 32.16
MCH (pg)	34.27 ± 4.63	32.44 ± 4.36	37.85 ± 5.03
MCHC (g/dL)	24.75 ± 3.67	24.04 ± 1.43	24.77 ± 3.72

active cells. The values of phagocytic index were 1.2 ± 0.15, 0.92 ± 0.26, and 2.8 ± 1.03 in fish exposed to 4.0 μg/L, 7.0 μg/L, and the control group respectively. However, the percentage of active cells in exposed fish (4.0 μg/L) was 61.5 ± 4.6, and in fish exposed to the maximum concentration (7.0 μg/L), it was 53.8 ± 13.0. In contrast, the control group presented a value of 79.1 ± 11.7. Results showed a significant decrease in levels of both parameters at all concentrations in comparison to the control group (Figs. 1 and 2).

This type of study, using a sub-lethal dosage, could be used to estimate water quality (Sunderam et al. 1994) due to the fact that physiological parameters could be measured in surviving organisms. The immune response and hematological values may be used as adequate biomarkers for evaluating the effect of pesticides at a sub-lethal concentration on aquatic organisms such as fish (Luskova 1996). As it was shown here, the immune response of tilapia is

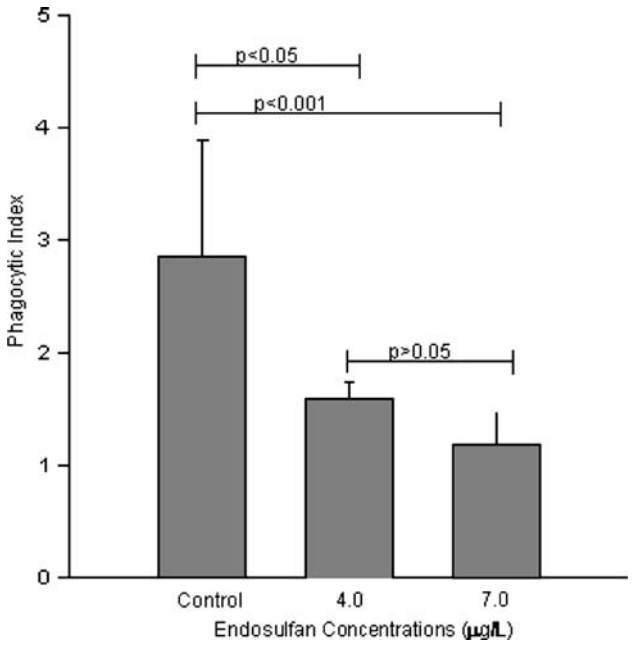

Fig. 1 Phagocytic index of blood cells from Nile tilapia exposed to endosulfan, during 96 h. Results are expressed as mean ± SD. One-way ANOVA and Tukey tests were applied

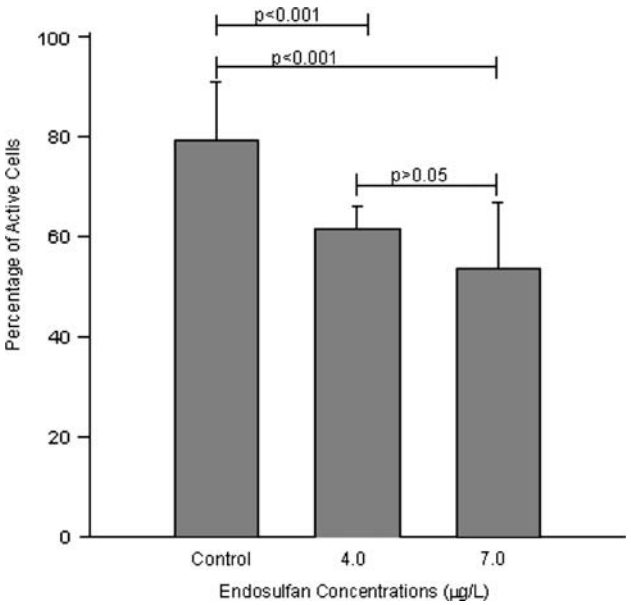

Fig. 2 Percentage of active cells from blood cells of Nile tilapia exposed to endosulfan, during 96 h. Results are expressed as mean ± SD. One-way ANOVA and Tukey tests were applied

sensitive to environmental parameters and relatively easy to measure.

Therefore, the present research was designed to evaluate the phagocytic function and hematological parameters in Nile tilapia after a sub-lethal exposure to a commercial formulation of endosulfan (Thiodan 35 CE®). The results showed that none of the hematological values determined here were significantly altered after 96 h at either concentration of endosulfan (4.0 and 7.0 μg/L). Conversely, Jenkins et al. (2003) carried out similar experiments with carp (*Cyprinus carpio)*, an economically important freshwater teleostean fish. Hematocrit, hemoglobin, and erythrocytes count values were significantly decreased by endosulfan. This difference could indicate that *C. carpio* is more sensitive to endosulfan than *O. niloticus*. The different results, however, could also be explained by the longer exposure time and higher concentration of endosulfan used by the authors or by a protective effect of

"inert" components of the commercial formulation of endosulfan used in our research.

The macrophages from tissues, neutrophils, and monocytes (in blood) are the principal phagocytic cells in fish. Their capability to carry out their function is associated with the state of activation of the innate immune response, which is highly influenced by xenobiotics, such as pesticides (Ayub et al. 2003). Our phagocytic function measurements showed that exposure to 4.0 and 7.0 μg/L of endosulfan significantly diminished the phagocytic activity and percentage of active cells. Similar studies were carried out in native Australian fishes and showed that endosulfan induces a significant decrease in granulocyte count, percentage of active granulocytes, and number of particles ingested by phagocytic cells. Nevertheless, the results of the in vitro effect of endosulfan on phagocytosis in head kidney cells from four Australian fish species were contradictory to this study. High endosulfan concentrations ($\geq$10 mg/L) caused a suppression of phagocytosis in rainbowfish. However, in silver fish, no effect was observed. Meanwhile, the same concentrations significantly increased the phagocytosis in golden perch and Murray cod (Harford et al. 2005). Although the immunotoxic properties of endosulfan vary between species, it is clear that endosulfan has an immunosuppressive effect in Nile tilapia. As shown in this paper, this could lead to a higher susceptibility to infection. This effect is not altered by the concentrations used in the present study. More concentrations might be assayed in order to obtain a more detailed look at toxic behavior of the pesticide in tilapia. However, we can speculate that a typical dose-response behavior is to be found. Meanwhile, our results allow us to propose that immunotoxicity of endosulfan on tilapia could be used to evaluate the effect of this pesticide on an aquatic environment.

More knowledge of tilapia immune response is evidently required for the development of further studies. Therefore, this parameter could be used to evaluate exposure to pesticides that may alter the health of tilapia, as shown previously using the organophosphate pesticide chlorpyrifos (Girón-Pérez et al. 2006). Taking this into account, fish cultured in areas close to agricultural activities could be affected by commercial formulations of pesticides, either by the active ingredients or the "inert" components (Cox and Surgan 2006). Hence, it is important to characterize physiological parameters that allow preventive actions in order to improve fish culture.

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