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Cardio-respiratory function and oxidative stress biomarkers in Nile tilapia exposed to the organophosphate insecticide trichlorfon (NEGUVON®)

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ABSTRACT

The cardio-respiratory function, oxidative stress and fish antioxidants were analyzed in juvenile Nile tilapia exposed for 96 h to a sublethal trichlorfon (TRC-Neguvon®, Bayer) concentration of 0.5 mg L^{-1}. The exposure to TRC induced oxidative stress in the heart, as manifested by the glutathione S-transferase depletion and hydroperoxide elevation, and was the most sensitive organ when compared to the liver and gills, in which the antioxidant mechanisms against TRC exposure were sufficient to remove reactive oxygen species (ROS), preventing the increase of lipid peroxidation. TRC exposure also reduced O_2 uptake ($\dot{V}O_2$) and increased the critical oxygen tension (PcO_2), reducing the species capacity to survive prolonged hypoxic conditions. The heart rate and force contraction were significantly impaired, making the heart the most sensitive organ when exposed to the TRC.

1. Introduction

Organophosphate compounds (OPCs) are an important insecticide class, which are widely used in agriculture and domestic purposes to control insect pests (Rodrigues et al., 2001). In fish farming production systems, where fish are maintained at high densities, various pathologies, such as the epizooties caused by ectoparasites, are treated with OPCs. These chemotherapeutic agents actually constitute one of the main environmental problems derived from intensive fish culture (Lopes et al., 2006).

Trichlorfon [dimethyl, (2,2,2-trichloro-1-hydroxyethyl) phosphonate] is a selective OPC widely used to control a variety of arthropod pests, both as an agricultural insecticide and zoo vermicide (Lopes et al., 2006).

In Brazilian fish cultures, trichlorfon (TRC) is largely used to control *Lernaea* sp. and *Argulus* sp., two common ectoparasites causing epizooties (Lopes et al., 2006). According to Pavanelli et al. (2002), long-term baths at 0.5 mg L^{-1} TRC for 3 days are very effective against these ectoparasite infestations. Although the TRC dose to eradicate ectoparasites varies from 0.1 to 1.0 mg L^{-1} in ponds, farmers often apply excessive amounts of TRC in fish and agriculture farm management (Chang et al., 2006).

The primary effect of OPC on both invertebrate and vertebrate organisms, including humans, is the inhibition of the acetylcholinesterase (AChE). However, the effects of OPC are not restricted to the AChE inhibition. Chandrasekara and Pathiratne (2005) observed hematological alterations in common carp, *Cyprinus carpio*, exposed to 0.25 and 0.50 mg L^{-1} of TRC during 24 h. Veiga et al. (2002) observed that after 48-h exposure to sub-lethal TRC concentrations (0.2 μL L^{-1}), specimens of curimbatá, *Prochilodus lineatus*, presented kidney histopathological alterations such as glomerular expansion, changes in the tubular cells, including swelling and small and pyknotics cell nuclei advancing to a cariolisis and necrosis. Similar results were found by Rodrigues et al. (2001) in *P. lineatus* liver, after 24 and 48 h exposure to the same TRC concentration. Regarding the cardio-respiratory system of the fish, OPC effects include bradycardia and inhibited gill ventilation (Gehrke, 1988).

It has been reported that OPCs also induce oxidative stress, leading to reactive oxygen species (ROS) generation and alterations in antioxidants or free oxygen radicals scavenging enzyme systems in aquatic organisms (Hai et al., 1997; Yarsan et al., 1999; Peña-Llopis et al., 2003a; Mohammad et al., 2004; Monteiro et al., 2006).

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ROS, such as superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and highly reactive hydroxyl radical ($^{\bullet}OH$) can react with susceptible biological macromolecules and produce lipid peroxidation (LPO), DNA damage and protein oxidation, resulting in oxidative stress. Contaminant-stimulated ROS are associated with different pathologic processes involved in the etiology of many fish diseases and may also be a mechanism of toxicity in aquatic organisms exposed to pollutants (Monteiro et al., 2006).

Tropical ecosystems are currently threatened by human activities and environmental degradation. However, little research has been done to elucidate the impact of contaminants on tropical ecosystems and tropical fish species (Lacher and Goldstein, 1997; Almeida et al., 2005). Although OPCs containing TRC as the active substance have been extensively used in Brazil, there are few reports in the literature on TRC-induced cardio-respiratory effects, oxidative stress and its effect on fish antioxidants, which constitutes the main goal of the present study.

Nile tilapia (*Oreochromis niloticus*) is a widely used aquaculture species worldwide (Costa-Pierce, 2003) and has been termed the "aquatic chicken" for its extraordinary production capabilities (Coward and Little, 2001). Nile tilapia has been the most popular species in Brazil, and is cultivated in 22 Brazilian states with an annual production of between 30 and 40 thousand tons (Mainardes-Pinto et al., 1995; Lovshin and Cyrino, 1998). For these reasons, *O. niloticus* was chosen as a model to evaluate the sublethal effects of TRC on *in vivo* and *in vitro* cardio-respiratory function, as well as in the antioxidant system.

2. Materials and methods

This study was conducted in accordance with the COBEA (Brazilian College of Animal Experimentation) and duly approved by the Committee of Ethics in Animal Experimentation/Federal University of São Carlos, Brazil.

2.1. Chemicals

Commercial formulation of the organophosphorus pesticide trichlorfon (*O,O*-dimethyl *O*-2,2,2-trichloroethylene)-NEGUVON® (trichlorfon 97 g/100 g, Bayer) was used in the present work. All other chemicals and reagents were purchased from Sigma-Aldrich Chemical Co. and Merck.

2.2. Experimental design

Specimens of *O. niloticus* ($W_t = 150.6 \pm 0.6$ g; $L_t = 21.62 \pm 0.3$ cm) were obtained at the Águas Claras fish farm, Mococa, São Paulo State, Brazil. This weight range was selected to allow the *in vivo* experiments (respiratory variables and electrocardiography), which require implantation of polyethylene (PE) catheters to collect inspired and expired water and EGC electrodes. Fish were acclimated for 30 days prior to experimentation in 1000 L holding tanks equipped with a continuous supply of well-aerated and dechlorinated water at 25 ± 2 °C under natural photoperiod (~12h:12h). During this period, fish were fed *ad libitum* with commercial fish pellets (32% of protein). The physical and chemical parameters were kept nearly constant: pH 6.7–7.3, DO_2 6.0–7.3 mg L^{-1}, hardness 48–53 mg L^{-1} (as $CaCO_3$), alkalinity 40–43 mg L^{-1} (as $CaCO_3$), ammonium 0.1 mg L^{-1}; chloride 42 mg L^{-1}, and conductivity 110 μS cm^{-1}.

After acclimation, 60 fish were divided into two experimental opaque plastic boxes (250 L): the control group (C: $n = 30$) and the TRC group-fish treated with a sublethal concentration of 0.5 mg L^{-1} of active ingredient trichlorfon (Neguvon®, 97%) (TRC: $n = 30$). Alkahem et al. (1998) previously established a LC_{50} of 21.7 mg L^{-1} for *O. niloticus* exposed to TRC during 96 h. These procedures are in accordance with the OECD guidelines for testing chemicals, which recommend the maximum loading of 1.0 g fish L^{-1} for static and semi-static tests (Organization for Economic cooperation and Development (OECD), 1992). Thus, as fish had approximately 150 g, only one fish at time was exposed to TRC. To avoid prandial effects and prevent deposition of feces in the course of the assay, the fish were starved for 24 h prior to experimentation.

Opaque experimental tanks were used to avoid external disturbances and they were sealed with a dark cover to prevent sample volatilization. Dissolved oxygen, temperature and photoperiod were maintained as described for the acclimation period. The fish remained under a semi-static system for 96 h where the experimental TRC solutions were renewed every 24 h to maintain water quality and adjust the concentration of TRC. The C group was submitted to the same protocol but without adding TRC. During this period, sublethal effects such as the level of activity, swimming performance and color changes were monitored.

All the experiments described below were performed with both experimental groups.

2.3. Tissue samples

At the end of 96 h of exposure to TRC, fish from both experimental groups were killed by transecting the spinal cord and the mass, as well as the total length of fish were measured. After the biometry, the organs were carefully excised and washed in cold physiological saline (0.9% NaCl). The gills, liver and heart were excised and, subsequently, samples were taken and immediately frozen into liquid nitrogen. Frozen samples were stored at −80 °C until the biochemical determinations were carried out. The hepatosomatic index was calculated according to the following equation: [HSI = (liver weight/fish weight) × 100)].

2.4. Relative ventricular mass (RVM)

Control and exposed animals were killed and the body mass was measured (W_t-g). The heart was dissected and the ventricle carefully removed and weighed (W_v-g) to obtain the ventricular mass which was expressed as a percentage of body mass (relative ventricular mass, RVM-% of W_t).

2.5. Sample preparations

Samples of frozen tissue were quickly weighed and then homogenized at 18,000 rpm in a 0.1 M sodium phosphate buffer pH 7.0 at a ratio of 1:10 w/v. Homogenates were centrifuged at 12,000*g* for 30 min at 4 °C and the supernatants were used for biochemical analysis.

2.6. Antioxidant enzymes

All enzyme activities were measured spectrophotometrically at 25 °C.

The superoxide dismutase (SOD) activity was determined based on the ability of the enzyme to inhibit the reduction of nitro blue tetrazolium (NBT) (Crouch et al., 1981), which was generated by 37.5 mM hydroxylamine in an alkaline solution (Otero et al., 1983). The assay was performed in a 0.5 M sodium carbonate buffer (pH 10.2) with 2 mM EDTA. The reduction of NBT by superoxide anion to blue formazan was measured at 560 nm. The rate of NBT reduction in the absence of tissue was used as the reference rate. One unit of SOD was defined as the amount of protein needed to decrease the reference rate to 50% of maximum inhibition. The SOD activity was expressed in units per mg protein.

The catalase (CAT) activity was measured by decreasing the H_2O_2 concentration at 240 nm (Aebi, 1974). Decays in absorbance were recorded for 15 s in a 50 mM sodium phosphate buffer (pH 7.0) containing 15 mM H_2O_2 and the enzyme extract. CAT values were expressed as Bergmeyer units (B.U.) per mg protein. One unit of CAT (according to Bergmeyer) is the amount of enzyme, which liberates half the peroxide oxygen from the H_2O_2 solution of any concentration in 100 s at 25 °C.

The glutathione S-transferase (GST) activity was measured according to Habig et al. (1974) using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate. The assay mixture contained 1 mM CDNB in ethanol, 1 mM GSH, 100 mM potassium phosphate buffer (pH 7.0) and tissue homogenates. The formation of adduct S-2, 4-dinitrophenyl glutathione was monitored by the increase in absorbance at 340 nm against blank. The molar extinction coefficient used for CDNB was 9.6 mM^{-1} cm^{-1}. The activity was shown as the amount of enzyme catalyzing the formation of 1 nmol of the product formed per min mg $protein^{-1}$.

2.7. Lipid peroxidation (LPO)

The xylenol orange assay for lipid hydroperoxide (FOX—ferrous oxidation-xylenol orange) was performed as described by Jiang et al. (1992). Lipid hydroperoxide was determined with 100 μL of sample (previously deproteinized with 10% TCA) and 900 μL of reaction mixture containing 0.25 mM $FeSO_4$, 25 mM H_2SO_4, 0.1 mM xylenol orange and 4 mM butylated hydroxytoluene in 90% (v/v) methanol. The mixtures were incubated for 30 min at room temperature prior to measurements at 560 nm. The molar extinction coefficient of 4.3×10^4 M^{-1} cm^{-1} for cumene hydroperoxide (Jiang et al., 1991) was used. Lipid hydroperoxide levels were shown as nmol per milligram protein.

2.8. Reduced glutathione (GSH)

Reduced glutathione (GSH) levels were measured according to Beutler et al. (1963), using Elmann's reagent (DTNB). Supernatants of the acid extracts (1:1 v/v with 12% TCA) were added to 0.25 mM DTNB in a 0.1 sodium phosphate buffer,