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Biochemical effects of clomazone herbicide on piava (*Leporinus obtusidens*)

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ABSTRACT

This study aims to verify the effects of the clomazone concentration used in rice fields on acetylcholinesterase (AChE), thiobarbituric acid reactive substances (TBARS), protein carbonyl and catalase activity in tissues of piava (*Leporinus obtusidens*). LC_{50}-96 h was 5.0 mg L^{-1} and the fish were exposed to 1/10 of LC_{50}-96 h: 0.5 mg L^{-1} of clomazone for 96 and 192 h. The same parameters were also assayed after a recovery period of 192 h in clean water. AChE activity was reduced only in the brain and heart of fish exposed for 96 h. AChE activity was decreased in the brain, muscle and heart tissues after 192 h of exposure. After 192 h of recovery period, AChE activity remained diminished in brain and muscle and showed a decrease in eye. However, after 192 h of recovery, AChE activity in heart was recovered. Fish showed increased TBARS levels in brain at all experimental periods. TBARS levels decreased in liver and muscle tissues after 192 h of exposure. The increase in muscle TBARS persisted in fish transferred to clean water. Protein carbonyl in the liver was increased in all periods studied including the recovery period. Catalase activity was reduced during all periods. The present study demonstrates the occurrence of disorders in AChE, TBARS, protein carbonyl and catalase activity in piava. The results also show changes in fish after exposure to an environmentally relevant concentration of clomazone. Most effects observed persisted after the recovery period. Thus, these parameters may be used to monitor clomazone toxicity in fish.

1. Introduction

In southern Brazil, the herbicide based clomazone (2-(2-chlorophenyl) methyl-4,4-dimethyl-3-isoxazolidinone) is extensively used in paddy rice fields to control agricultural pests (Jonsson et al., 1998). Although it is highly effective, it is toxic to non-target organisms, such as fish (Crestani et al., 2006). This herbicide causes groundwater contamination due to its water solubility (1100 mg L^{-1}) (Colby et al., 1989). Clomazone residues can last for up to 130 d in agricultural water and were detected in 90% of water samples collected from courses near rice cultivation regions (Zanella et al., 2002).

Many environmental impacts on fish caused by toxicants have demonstrated the effects of organic pesticides on several species. Pesticides cause changes in physiological and metabolic functions of the fish organism such as neurotransmission and functions of the immunity system (Gill et al., 1991; Aldegunde et al., 1999). One of these changes is the depletion of energy metabolism, because the intoxicated organisms spend more energy increasing the activity of several enzymatic systems to mitigate toxic effects (Dethloff et al., 1999). The most common indicator of neurological dysfunction is cholinesterase (ChE) activity, which is frequently used as an indirect measure of acetylcholinesterase (AChE; EC 3.1.1.7) activity. AChE is responsible for degrading the neurotransmitter acetylcholine for end cholinergic neural transmission. Organophosphate pesticides previously shown to inhibit brain AChE activity in fish include diazinon, malathion and folidol (Beauvais et al., 2000; Brewer et al., 2001; Aguiar et al., 2004). Several carbamate pesticides, including carbofuran, diuron, nicosulfuron (Bretaudt et al., 2000), and thiobencarb have also been shown to inhibit brain AChE activity (Sancho et al., 2000; Fernández-Vega et al., 2002). Similar results have been observed with other pesticides such as endosulfan (Dutta and Arends, 2003) and clomazone (Miron et al., 2005; Crestani et al., 2007). Pesticides may induce oxidative stress, leading to the generation of free radicals and causing lipid peroxidation (LPO) (Kehrer, 1993; Sevgiler et al., 2004). Fish are able to uptake and retain different xenobiotics in water via active or passive processes. Therefore, parameters measured in fish may be used to monitor the potential of contamination by pesticides to environmental and could be used to investigate biological effects such as enzymatic disturbances. The piava (*Leporinus obtusidens*) is a widely cultivated native freshwater fish from Southern Brazil with great commercial importance (Andrian et al., 1994; Glusczak et al., 2006). Thus, this fish was used in order to enhance the scarce information available in the literature on

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clomazone toxicity in native fish species. This study aims to assess some parameters of toxicity in piava and to find potential toxicity indicators for clomazone exposure.

2. Materials and methods

2.1. Chemicals

The herbicide clomazone (2-(2-chlorophenyl)methyl-4,4-dimethyl-3-isoxazolidinone) used in this study was obtained commercially from the FMC Corporation (Gamit; 36% purity, Philadelphia, EUA) and dissolved in water. Acetylthiocholine (ATC), 5,5′dithio-bis(2-nitrobenzoic acid) (DTNB), bovine serum albumin, Triton X-100, hydrogen peroxide (H_2O_2), malondialdehyde (MDA), 2-thiobarbituric acid (TBA), and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Fish

Piava fingerlings (*L. obtusidens*) of both sexes were obtained from the Santa Maria Federal University (UFSM) fish farm (RS, Brazil). Fish (weight, 8.0 ± 1.0 g; length, 6.0 ± 1.0 cm) were acclimated to laboratory conditions for 15 d, in tanks (250 L). They were kept in continuously aerated water in a static system and with a natural photoperiod (12 h light–12 h dark). Water parameters were measured every day and were as follow: temperature 23 ± 2.0 °C, pH 7.7 ± 0.2 units, dissolved oxygen 6.5 ± 1.0 mg L^{-1}, non-ionized ammonia 0.007 ± 0.01 mg L^{-1}, nitrite 0.03 ± 0.01 mg L^{-1}, alkalinity 66 ± 1.3 mg L^{-1} $CaCO_3$ and hardness 20 ± 1.4 mg L^{-1} $CaCO_3$. All water parameters were determined according to Boyd and Tucker (1992). During the experimental period, fish were fed ad libitum two times a day (8:30 and 17:30 h) with commercial fish pellets (42% crude protein, Supra, Brazil). Fecal remains and food residues were removed by suction every other day.

2.3. Experimental design

Previous experiments carried out in our laboratory established 7.32 mg L^{-1} (nominal concentration) as the LC_{50} 96 h for clomazone (Miron et al., 2005). The clomazone concentration usually recommended in rice fields is of 0.5 to 1.0 mg L^{-1} (Rodrigues and Almeida, 1998). The concentration chose (0.5 mg L^{-1}) was in accordance with the calculated concentration of clomazone used in rice fields. After the acclimation period, groups of 10 fish were transferred to glass boxes (45 L) with controlled aeration and temperature. Stock solutions were prepared by dissolving clomazone in water. This solution was added to the experimental boxes. Piavas 10 fish per box (triplicate) were exposed to 0.0 (control) and 0.5 mg L^{-1} clomazone for 96 h. In a second experiment, piavas (10 fish/box in triplicate) were exposed to 0.0 (control) and 0.5 mg L^{-1} clomazone for 192 h and, subsequently, 15 fish were removed and transferred to clean water. The herbicide concentration was monitored every 2 d by high-performance liquid chromatography (HPLC) (Zanella et al., 2002) to verify values in the experimental boxes. Clomazone concentration in the water after 48 h was approximately 90% of the initial concentration (data not shown). The water in the boxes was renewed every 48 h to maintain the concentration of clomazone constant during the period of exposure. Water quality parameters during the treatment period were the same as those for the acclimation period.

2.4. Sampling

After the experimental period, fish were killed by punching the spinal cord behind the opercula and were sampled. Brain, white muscle, eye, heart and liver samples were rapidly removed, washed in 150 mM saline solution, dried with filter paper, packed in Teflon tubes and kept at −4 °C for analyses. AChE activity, thiobarbituric acid reactive substances (TBARS), protein carbonyl and catalase activity were measured in this study.

2.5. Acetylcholinesterase assay

Tissue samples were weighed and homogenized in a Potter-Elvejhem glass/Teflon homogenizer with 150 mM NaCl. The homogenates were centrifuged for 15 min at 3000*g* at 5 °C and the supernatant was used as the enzyme source. Acetylcholinesterase (AChE; EC 3.1.1.7) activity was measured as described by Ellman et al. (1961) and modified by Miron et al. (2005). Aliquots of supernatant (50, 50, 100 and 200 μL) (brain, eye, muscle and heart, respectively) were incubated at 25 °C for 2 min with 0.1 M phosphate buffer pH 7.5, 1 mM and DTNB as chromogen. After 2 min, the reaction was initiated by the addition of acetylthiocholine (0.08 M) as substrate. The final volume was 2.0 mL. Absorbances were determined at 412 nm during 2 min. Enzyme activity was expressed as μmol of acetylthiocholine (AcSCh) hydrolyzed per minute per mg of protein.

2.6. TBARS levels

Peroxides produced can be quantified by a TBARS assay. This is performed by a malondialdehyde (MDA) reaction with 2-thiobarbituric acid (TBA), which is optically measured. Liver, muscle and brain homogenates (100–400 μL) were added to 8.1% sodium dodecyl sulfate (SDS), 2.5 M acetic acid (pH 3.4), 0.8% thiobarbituric acid and the final volume was adjusted to 2.0 mL. The reaction mixture was placed in a microcentrifuge tube and incubated for 90 min at 95 °C. After cooling, it was centrifuged at 5.000*g* for 10 min and optical density was determined at 532 nm. TBARS levels are expressed as nmol MDA per mg of protein according to Ohkawa et al. (1979).

2.7. Protein carbonyl assay

The liver tissue was homogenized in 10 volumes (w v^{-1}) of 10 mM Tris–HCl buffer pH 7.4 using a glass homogenizer. The protein carbonyl content was determined by the method described by Yan et al. (1995), with some modifications. Briefly, homogenates were diluted to 0.7–0.8 mg mL^{-1} of protein in each sample, and 1 mL aliquots were mixed with 0.2 mL of 2,4-dinitrophenylhidrazine (10 mM DNPH) or 0.2 mL HCl 2 M. After incubation at room temperature for 1 h in a dark room, 0.5 mL of denaturing buffer (150 mM sodium phosphate buffer, pH 6.8, containing SDS 3%), 2.0 mL of heptane (99.5%) and 2.0 mL of ethanol (99.8%) were added sequentially, mixed with vortex agitation for 40 s and centrifuged for 15 min. Next, the protein isolated from the interface was washed two times with 1 mL of ethyl acetate/ethanol 1:1 (v v^{-1}), and suspended in 1 mL of denaturing buffer. Each DNPH sample was read at 370 nm in a Femto Scan spectrophotometer against the corresponding sample (blank), and total carbonylation was calculated using a molar extinction coefficient of 22 000 M^{-1} cm^{-1}.

2.8. Catalase assay

Catalase (CAT; EC 1.11.1.6) activity was assayed by ultraviolet spectrophotometry (Nelson and Kiesow, 1972). Samples of liver were homogenized in a Potter-Elvejhem glass/Teflon homogenizer with 20 mM potassium phosphate buffer, pH 7.4 (with 0.1% Triton X-100 and 150 mM NaCl) (1:20 dilution), centrifuged at 10 000*g* for 10 min at 4 °C. Briefly, the assay mixture consisted of 2.0 mL potassium phosphate buffer (50 mM, pH 7.0), 0.05 mL H_2O_2 (0.3 M) and 0.05 mL homogenate. Changes in H_2O_2 absorbance in 60 s were