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Assessment of oxidative stress and metabolic changes in common carp (*Cyprinus carpio*) acutely exposed to different concentrations of the fungicide tebuconazole

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ABSTRACT

The aim of this research is to determine the lethal concentration (LC_{50-96h}) of fungicide tebuconazole for common carp (*Cyprinus carpio*) and to investigate the occurrence of oxidative stress and metabolic changes after acute exposure to different concentrations of tebuconazole. For this purpose, several parameters were assessed as whole-body levels of thiobarbituric acid-reactive substance (TBARS), protein carbonyl, catalase, superoxide dismutase, glutathione S-transferase, nonprotein thiols, ascorbic acid, glycogen, glucose, lactate, protein, amino acids and ammonia in tebuconazole-exposed fish. The calculated LC_{50-96h} was 2.37 mg L^{-1}. Fish exhibited significant increase in TBARS levels in all concentrations used while the enzymatic and nonenzymatic antioxidants were decreased. Among the metabolic parameters, glycogen and glucose increased at 1.19 mg L^{-1} and protein levels decreased at 1.78 and 2.37 mg L^{-1}. In conclusion, the fish health was adversely affected by exposure to tebuconazole, and those changes can compromise animal survival in the natural environment. The results indicate that the some of the parameters measured like a possible biomarkers of exposure to tebuconazole for this species of fish.

1. Introduction

In southern Brazil, aquaculture is still considered a complementary activity that is developed in places such as lakes and reservoirs very close to or within agricultural areas (Cericato et al., 2008). The activities of agricultural origin pose risks to groundwater quality and surface water, especially the use of pesticides, which along with many of their metabolites, can cause harm to human health and the environment (Van der Oost et al., 2003). The fungicide tebuconazole belongs to the group of azoles and is widely used in paddy fields. Tebuconazole (Folicur®) is classified as toxic to aquatic organisms and may cause long-term adverse effects in the aquatic environment (Bayer CropScience Limited, 2005).

Toxic effects of pesticides have been studied in several fish species (Monteiro et al., 2006; Ferreira et al., 2010; Toni et al., 2010). Exposure to pesticides can cause oxidative stress in many aquatic organisms, since these contaminants can stimulate the formation of reactive oxygen species (ROS) or alter the antioxidant defenses (Monserrat et al., 2007). These highly reactive substances cause damage to lipids, proteins, carbohydrates, and nucleic acids (Sevgiler et al., 2004). The antioxidant system comprises a group of antioxidant enzymes and low-molecular-mass antioxidants, such as ascorbic acid and other nonprotein thiols (Winston and Di Giulio, 1991). The antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR). Glutathione peroxidase (GPx) and glutathione-S-transferases (GST) are from the second phase, which has the functions of detoxifying the enzymes that catalyze the conjugation of GSH with a variety of electrophilic compounds.

The common carp (*Cyprinus carpio*) is one of the most important cultivated fish in the world and arguably one of the most important aquaculture species. In Rio Grande do Sul (southern South America), the carps (several species) represent the fish group most cultivated (Baldisserotto, 2009). For this reason, this fish was chosen for this investigation. The aim of this study was to determine the lethal concentration (LC_{50-96h}) of tebuconazole for *C. carpio* as well as to analyze the oxidative stress parameters (lipid peroxidation and protein carbonyl), the effects of fungicide on enzymatic and nonenzymatic antioxidants in fish, and metabolic parameters were evaluated.

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2. Materials and methods

The experiments were conducted in March and April 2010, at the facilities of the Universidade de Passo Fundo, Rio Grande do Sul, Brazil. We used 90-day-old, mixed-sex common carp fingerlings, weighing 2.1 ± 0.3 g (S.E.M, $n = 500$). The fingerlings were kept in a 6200-L plastic tank before transferring into experimental tanks under natural photoperiod and were fed twice a day (10:00 and 16:00 h) with commercial extruded food at 5% of body weight (42% crude protein, 3400 kcal kg^{-1} DE).

Water temperature (26 ± 1 °C) and dissolved oxygen concentrations (5.6–7.5 mg L^{-1}) were measured with a YSI model 550A oxygen meter (Yellow Spring Instruments, USA). The pH values (6.6–7.0) (Bernauer pH meter), total ammonia-*N* (<0.5 mg L^{-1}) (colorimetric test), total alkalinity (60 mg L^{-1} $CaCO_3$), and hardness (65 mg L^{-1} $CaCO_3$) were also measured (colorimetric tests).

2.1. Chemicals

Commercial formulation of the tebuconazole fungicide is [1-*p*-clorofenil-4,4-dimetil-3-(1H-1,2,4-triazol-1-ilmetil) pentane-3-ol]. The trade name used in the Brazilian Market is Folicur®200EC (BASF) at 200 g i.a. L^{-1}. 5,5′dithio-bis(2-nitrobenzoic acid) (DTNB), 1-chloro-2,4 dinitrobenzene (CDNB), bovine serum albumin, Triton X-100, hydrogen peroxide (H_2O_2), malondialdehyde (MDA), 2-thiobarbituric acid (TBA), and sodium dodecyl sulfate (SDS) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Experiment 1 – LC_{50-96h} determination

2.2.1. Toxicity-testing protocol

Four-day static acute toxicity tests were performed in the laboratory to determine the LC_{50} values of tebuconazole in *C. carpio*. Fungicide concentrations of 1.0, 1.5, 2, 2.5, and 3.0 mg L^{-1} (nominal values) were used. After being acclimated to laboratory conditions, fishes were randomly distributed in each test aquarium. For each concentration, a group of 10 randomly selected fishes was placed in the plastic aquaria (40 L of continuously aerated water per tank). This procedure was repeated three times for each concentration. The experiment was carried out in a static-test design without water changes and fungicide replacement. The control carp groups were kept in clean water (without fungicide) as in the experimental sets. During the acute toxicity test (96 h), animals were not fed. The number of dead fishes was recorded at 24, 48, 72, and 96 h. Dead fishes were removed from the aquaria.

2.3. Experiment 2 – tebuconazole-induced changes

2.3.1. Experimental design

The experiment was conducted testing three sublethal and lethal concentrations of the fungicide tebuconazole (Folicur200CE™). The experiment consisted of five treatments with 4 replicates (total 20 tanks), containing 95-L chlorine-free, well-aerated tap water and 10 fingerlings. Treatment 1 comprised the control (C) group, in which the fingerlings were kept in water without contamination. In treatments 2–5, the fingerlings were kept in water containing the tested concentrations of the agrichemical (0.59, 1.19, 1.78, and 2.37 mg L^{-1}), corresponding to 25%, 50%, 75%, and 100% of the lethal concentration for acute exposure (LC_{50-96h}) determined in experiment 1. In all treatments, the stocking density was 0.22 g $L^{-1}$and the experiment was carried out in a static-test design. Usually, in such experiments, the fishes are not fed; however, metabolic parameters might be affected by starvation, and the fishes in this study were fed thrice during the 96 h of exposure (24, 48, and 72 h after the beginning of exposure) at a rate of 0.75% of their biomass. Food residues and feces were not removed, to prevent stress owing to the introduction of cleaning equipment. The water quality was monitored daily and dead fish removed twice a day.

2.3.2. Sampling

The fishes were anesthetized by administering buffered (NaH_2CO_3) MS222 (300 mg L^{-1}) into the tank. After loss of orientation and complete immobilization, the fishes were captured and immediately killed by cold shock and stored in liquid nitrogen until required for analysis.

2.4. Determinations

2.4.1. Biochemical determinations

The fishes were individually homogenized with five volumes of 0.1 M phosphate buffer (pH 7.2), using a motor-drive homogenize. Homogenates were centrifuged at 2000*g* for 10 min, and the resulting supernatants were stored (−70 °C) until further analysis. All measurements were performed in duplicate.

2.4.2. Lipid peroxidation estimation

Lipid peroxidation was estimated by a TBARS (thiobarbituric acid-reactive substances) assay, performed by a malondialdehyde (MDA) reaction with 2-thiobarbituric acid (TBA), which was optically measured according to Buege and Aust (1978). Aliquots of supernatants (0.25 mL) were mixed with 10% trichloroacetic acid (TCA) (0.25 mL) and 0.67% thiobarbituric acid (0.5 mL) to adjust to a final volume of 1.0 mL. The reaction mixture was placed in a microcentrifuge tube and incubated for 15 min at 95 °C. After cooling, it was centrifuged at 5000*g* for 15 min, and optical density was measured by spectrophotometer at 532 nm. TBARS levels were expressed as nmol MDA mg $protein^{-1}$.

2.4.3. Protein carbonyl assay

Supernatants (0.4 mL) were homogenized in 10 volumes (w/v) of 10 mM Tris–HCl buffer pH 7.4 using a glass homogenizer. Protein carbonyl content was assayed by the method described by Yan et al. (1995) with some modifications. Soluble protein (1.0 mL) was reacted with 10 mM DNPH in 2N hydrochloric acid (0.2 mL). After incubation at room temperature for 1 h in dark, 0.5 mL of denaturing buffer (150 mM sodium phosphate buffer, pH 6.8, containing SDS 3.0%), 2.0 mL of heptane (99.5%), and 2.0 mL of ethanol (99.8%) were added sequentially, vortexed for 40 s, and centrifuged at 10 000*g* for 15 min. Then, the protein isolated from the interface was washed twice by resuspension in ethanol/ethyl acetate (1:1) and suspended in 1 mL of denaturing buffer, and the carbonyl content was measured spectrophotometrically at 370 nm. Assay was performed in duplicate, and two tubes blank incubated with 2N HCl (0.2 mL) without DNPH was included for each sample. The total carbonylation was calculated using a molar extinction coefficient of 22 000 M cm^{-1}. The protein carbonyl content was expressed as nmol carbonyl mg $protein^{-1}$.

2.4.4. Antioxidant enzymes

Catalase (EC1.11.1.6) activity was assayed by ultraviolet spectrophotometer (Nelson and Kiesow, 1972). Samples of were homogenized in a Potter–Elvehjem 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. 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 0.01 mL homogenate. Change of H_2O_2 absorbance in 60 s was measured at 240 nm. Catalase activity was calculated and expressed in μmol min^{-1} mg $protein^{-1}$. SOD activity was determined in whole-body as the inhibition rate of autocatalytic adenochrome generation at 480 nm in a reaction medium containing 1 mM epinephrine