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Frontier Article

Oxidative stress responses in blood and gills of *Carassius auratus* exposed to the mancozeb-containing carbamate fungicide Tattoo

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ABSTRACT

Intensive use of pesticides, particularly dithiocarbamates, in agriculture often leads to contamination of freshwater ecosystems. To our knowledge, the mechanisms of toxicity to fish by the carbamate fungicide Tattoo that contains mancozeb [ethylenebis(dithiocarbamate)] have not been studied. The present study aimed to evaluate the effects of Tattoo on goldfish gills and blood, tissues that would have close early contact with the pollutant. Exposure of goldfish *Carassius auratus* to 3, 5 or 10 mg L^{-1} of Tattoo for 96 h resulted in moderate lymphopenia (by 8 percent) with a concomitant increase in both stab (by 66–88 percent) and segmented (by 166 percent) neutrophils. An increase in the content of protein carbonyl groups in blood (by 137–184 percent) together with decreased levels of protein thiols (by 23 percent) and an enhancement of lipid peroxide concentrations (by 29 percent) in gills after exposure to 10 mg L^{-1} of Tattoo demonstrated the induction of mild oxidative stress in response to Tattoo exposure. At the same time, the activities of selected antioxidant enzymes were enhanced in gills: superoxide dismutase by 18–25 percent and catalase by 27 percent. A 34 percent increment in low molecular mass thiol concentrations (mainly represented by glutathione) also occurred in gills and could be related to increased activity (by 13–30 percent) of glucose-6-phosphate dehydrogenase. The results indicate that Tattoo exposure perturbs free radical processes, i.e. induces mild oxidative stress and enhances the activity of certain antioxidant and associated enzymes in goldfish gills. It is clear that goldfish respond to the presence of waterborne pesticide by adjusting antioxidant defenses through upregulation of activities of antioxidant and associated enzymes.

1. Introduction

Dithiocarbamates (DTC) are used as fungicides for treatment of crops, vegetables, seeds, and ornamental plants and they are also used as accelerators in the rubber industry, animal repellents, and biocides in many household products. Intensive use of pesticides, particularly dithiocarbamates, in agriculture often leads to contamination of freshwater ecosystems through atmospheric fall-out, agricultural effluents and runoffs (reviewed in Rath et al. (2011)). Carbamate pesticides are produced in a wide spectrum of commercial forms, including dithiocarbamates, which are potentially hazardous compounds for aquabiota, particularly fish. The carbamate fungicide Tattoo contains mancozeb [ethylenebis (dithiocarbamate)] as a main constituent. Mancozeb was registered several times by US EPA (1987, 2005) and extensively used as an active ingredient of fungicides applied worldwide like Dithane, Fore, Manzeb, Nemispot, Zimaneb and others. The wide use of mancozeb in agriculture is due to its reported low acute toxicity and scarce persistence in the environment (Maroni et al., 2000).

It is known that mancozeb, similar to most DTC molecules, can exhibit both prooxidant and antioxidant properties (reviewed by Rath et al. (2011)). Mancozeb releases its constituent ions, manganese and zinc, during metabolism (Calviello et al., 2006; Nobel et al., 1995). Whereas these ions as well as the disulfide component can contribute to its prooxidant effects (Calviello et al., 2006; Nobel et al., 1995), the thiols may contribute to its antioxidant effects (Liu et al., 1996).

To the best of our knowledge, the mechanisms of Tattoo/mancozeb toxicity to fish have not been studied. Falfushinska et al. (2008) showed that Tattoo had low toxicity to frogs and this was, at least partially, related with oxidative damage to lipids and

Abbreviations: AChE, acetylcholine esterase; CP, carbonyl protein groups; DTC, dithiocarbamates; GPx, glutathione peroxidase; GR, glutathione reductase; G6PDH, glucose-6-phosphate dehydrogenase; GST, glutathione-S-transferase ; H-SH, high molecular mass thiols; LOOH, lipid peroxides; L-SH, low molecular mass thiols; ROS, reactive oxygen species; SOD, superoxide dismutase.

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proteins. The findings of these authors are in an agreement with data on mancozeb influence on cell cultures, where relationships between mancozeb toxicity and stimulation of processes involving reactive oxygen species (ROS) were obtained (Calviello et al., 2006; Medjdoub et al., 2011). In fish, gills are the prime target for waterborne toxicants and possess a detoxification system, although not as robust as that of liver (Li et al., 2009; Li et al., 2010a; Oliveira et al., 2009; Pandey et al., 2008). The suitability of fish gills for environmental biomonitoring has been extensively discussed (Dautremepuits et al., 2009; Oliveira et al., 2009; Pereira et al., 2010). In performing their gas-exchange function, gills are permanently in contact with environmental water and blood, providing a path for xenobiotic entrance into the blood stream and hence further distribution to all organs. Being an indicator of physiological conditions in animals (Li et al., 2011), blood exhibits pathological changes before any external symptoms of toxicity (Blaxhall and Daisley, 1973). Many studies have demonstrated changes in blood variables as a result of the effects of certain environmental conditions or the presence of contaminants (Borges et al., 2007; Li et al., 2010b; Li et al., 2011; Sudova et al., 2009; Vasylkiv et al., 2010, 2011). Hematological parameters in concert with investigation of leukocyte profile and some biochemical indices in fish blood can be valuable tools for nonlethal diagnostics of fish intoxication (Li et al., 2011; Svoboda et al., 2001; Vasylkiv et al., 2010). These have become an important means of understanding the possible mechanisms of toxicological impacts (Borges et al., 2007; Patíl and Kulkarni, 1993; Sudova et al., 2009).

Although a great number of studies concerning the effects of various pesticides on living organisms have been carried out, many aspects of carbamate pesticide effects on fish, particularly fish gills and blood remain unclear. Therefore, in the present study we aimed to investigate the effects of the carbamate fungicide Tattoo on gills and blood of a highly stress resistant species, the goldfish *Carassius auratus*, with special attention to free radical processes.

2. Material and methods

2.1. Reagents

Phenylmethylsulfonyl fluoride (PMSF), 1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), oxidized glutathione (GSSG), glucose-6-phosphate (G6P), ethylenediamine-tetraacetic acid (EDTA), xylenol orange, cumene hydroperoxide, ferrous sulphate, 2,4-dinitrophenylhydrazine (DNPH), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), KH_2PO_4, Tris(hydroxymethylaminomethane) and N,N,N′,N′-tetramethylethylenediamine (TEMED) were purchased from Sigma-Aldrich Corporation (USA). Hydrogen peroxide (H_2O_2) was from Merck (Germany) and $NADP^+$ was obtained from Reanal (Hungary). Acetylthiocholine iodide and NADPH was purchased from Carl Roth (Germany). All other reagents were of analytical grade. The commercial fungicide Tattoo that contains 301.6 g L^{-1} of mancozeb [ethylenebis (dithiocarbamate)] and 248 g L^{-1} of propamocarb-HCl [propyl 3-(dimethylamino)propylcarbamate-hydrochloride] was obtained from Bayer Crop Science (Germany).

2.2. Animals and experimental conditions

Specimens of goldfish (*C. auratus* L.), weighing 100–120 g, were obtained from a local commercial supplier (private fish farm, Tysmenetsky district, Ivano-Frankivsk region) in November 2010. Fish were acclimated to laboratory conditions for 4 weeks in a 1000 L tank under natural photoperiod in aerated and dechlorinated tap water at 19.0–19.5 °C, pH 7.6–7.8, 9.0–9.5 mg L^{-1} O_2 and hardness (determined as Ca^{2+} concentration) 39–40 mg L^{-1}. Fish were fed with commercial cyprinid pellets (Koi Grower, The Netherlands), containing 44 percent protein, 11 percent fat and 150 mg kg^{-1} vitamin C, to apparent satiation. Fish were fed during the acclimation period (4 weeks), but were fasted for 4 days prior to and during experimentation.

Experiments were carried out in 120 L glass aquaria containing 100 L of water, in a static mode, in the absence versus presence of Tattoo. Treatments were conducted following a standard acute toxicity test procedure, 96 h exposure to sublethal concentrations of Tattoo/mancozeb, chosen on the basis of a known mancozeb LC_{50} for goldfish of 9 mg L^{-1} (US National Library of Medicine, HSDB, 1995). Groups of five fish were placed in aquaria with different nominal concentrations of Tattoo: 3, 5 or 10 mg L^{-1}, which corresponded to 0.9, 1.5 or 3 mg L^{-1} of mancozeb, respectively, and exposed to these conditions for 96 h. No mortality occurred during exposures. These concentrations may be environmentally relevant in agricultural runoffs, because concentrations of Tattoo/Mancozeb a thousand times higher are recommended for crop treatment. Fish in the control group were maintained in the same manner, but without the addition of Tattoo to the water. Experiments were conducted in two independent replicates, and the total number of repeats for each parameter was five to six. Aquarium water was not changed over the 96 h course in order to avoid stressing of the fish. During the experiment levels of dissolved oxygen, temperature and pH were monitored every 24 h. After fish exposure, blood was quickly taken from caudal vessels using 50 mM EDTA as an anticoagulant. Fish were then quickly sacrificed by transspinal transsection without anesthesia (to prevent anesthesia-induced changes in parameters of interest) and gill filaments were dissected from gill arches, rinsed in ice-cold 0.9 percent NaCl, frozen and stored in liquid nitrogen until use.

All experiments were conducted in accordance with the institutional animal ethics guidelines of Precarpathian National University and were approved by the Animal Experimental Committee of Precarpathian National University.

2.3. Evaluation of hematological, biochemical parameters and leukocyte content in blood

2.3.1. Estimation of total hemoglobin concentration and hematocrit value

Total hemoglobin concentration was determined in whole blood by a "multi-wavelength" spectrophotometric method (Zwart et al., 1981) with some modifications as described earlier (Bilyi et al., 2000; Vasylkiv et al., 2010). Briefly, blood aliquots (0.01 mL) were added to 3 mM sodium-potassium phosphate buffer (pH 6.36) in a total sample volume of 2 mL and incubated for 2–3 min to hemolyse erythrocytes. This was followed subsequent absorbance measurements at six wavelengths. Total hemoglobin concentration (g L^{-1}) was calculated from the measured absorption values using the known molar extinction coefficients at the six analytical wavelengths, as described by Bilyi et al. (2000).

Hematocrit was determined following the procedure of Ptashynski et al. (2002). Immediately after blood sampling, a small amount of whole blood was transferred to microcapillary tubes, which were then carefully sealed at both sides and centrifuged (2000 g, 20 min, 4 °C) using an OPN-8 centrifuge (USSR). Hematocrit values were calculated as the percentage of red blood cell pellet in the total blood column.

2.3.2. Examination of leukocyte content

For microscopic examination of leukocyte content, small drops of whole blood were directly smeared on slides ($n=2$ per fish) and