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Azinphos-methyl and chlorpyrifos, alone or in a binary mixture, produce oxidative stress and lipid peroxidation in the freshwater gastropod *Planorbarius corneus*

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ABSTRACT

Azinphos-methyl (AZM) and chlorpyrifos (CPF) are broad-spectrum organophosphate insecticides used for pest control on a number of food crops in many parts of the world that have been shown to inhibit cholinesterase activity in the non-target freshwater gastropod *Planorbarius corneus*. The present study was undertaken to determine: (a) whether AZM and CPF induce oxidative stress in *P. corneus*, and (b) whether a mixture of both organophosphates that causes a higher neurotoxicity than single pesticides also causes an enhanced oxidative stress. To this end, non-enzymatic and enzymatic parameters were measured in the soft tissues of snails acutely exposed to the insecticides in single-chemical (2.5 mg AZM L^{-1} and 7.5 μg CPF L^{-1}) and a binary-mixture (1.25 mg AZM L^{-1} plus 3.75 μg CPF L^{-1}) studies. At 24 h, all pesticide-exposed groups showed significantly decreased glutathione (GSH) and glutathione disulfide (GSSG) levels when compared to control animals. At 48 h, all exposed groups showed an alteration of the redox status (GSH/GSSG ratio) and a significant increase in malondialdehyde levels. The exposure for 48 h to AZM and CPF, alone or in the binary mixture, also resulted in a significant decrease of the antioxidant superoxide dismutase activity. The greatest decrease was observed with CPF exposure (59% of decrease relative to the control group). A significant increase in catalase and glutathione S-transferase activities was observed in CPF group and in CPF and AZM + CPF groups, respectively. The activities of glutathione reductase and glucose 6-phosphate dehydrogenase did not show significant changes with respect to controls in any treatment group. In conclusion, the data shown in the present study provide evidence that AZM, CPF and a mixture of both organophosphates are able to induce oxidative stress and oxidative damage in *P. corneus* tissues. However, no similarities between the degree of neurotoxicity and the degree of alterations of the measured oxidative stress parameters were found.

1. Introduction

Azinphos-methyl (*O*,*O*-dimethyl S-[4-oxo-1,2,3-benzotriazin-3(4H)-yl) methyl]triazin-3-ylmethyl]dithiophosphate, AZM) and chlorpyrifos (*O*,*O*-diethyl *O*-(3,5,6-trichloropyridin-2-yl) thiophosphate, CPF) are broad-spectrum organophosphate insecticides used for pest control on a number of food crops in many parts of the world (Loewy et al., 2011; USEPA, 2001, 2011). These insecticides can reach watercourses via runoff and spray drift and adversely affect aquatic invertebrate and vertebrate non-target species (USEPA, 2001; Gormley et al., 2005; Anguiano et al., 2012; Williams et al., 2014).

The primary acute mechanism of toxicity of AZM and CPF to vertebrate and invertebrate species is the inhibition of the enzyme

Abbreviations: AChE, acetylcholinesterase; AZM, azinphos-methyl; BHT, butylated hydroxytoluene; CAT, catalase; CDNB, 1-chloro-2,4-dinitrobenzene; CES, carboxylesterases; CPF, chlorpyrifos; CYP, cytochrome P450; GR, glutathione reductase; G6PDH, glucose 6-phosphate dehydrogenase; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione S-transferase; MDA, malondialdehyde; NEM, N-ethylmaleimide; OP, organophosphate; OPA, orthophthaldehyde; ROS, reactive oxygen species; SOD, superoxide dismutase; TBA, thiobarbituric acid; TEP, 1,1,3,3-tetraetoxypropane.

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acetylcholinesterase (AChE). This enzyme hydrolyses acetylcholine at cholinergic synapses and neuro-muscular junctions and the persistent inhibition of its activity causes neurotoxic effects. We have recently shown that AZM and CPF inhibit AChE activity in the non-target freshwater gastropod *Planorbarius corneus*. We have also shown that certain mixtures of AZM and CPF can act synergistically to inhibit AChE activity in this species (Cacciatore et al., 2013). These results are relevant since *P. corneus* is a hermaphroditic snail that usually inhabits small temporary ponds and streams near agricultural fields. It belongs to the Planorbidae family, the largest family of aquatic pulmonate gastropods which is distributed all over the world (Jopp, 2006).

Since AZM and CPF are organophosphorothioate compounds, their ability to inhibit AChE activity is directly related to their biotransformation to the corresponding potent oxon metabolite by the cytochrome P450 (CYP) enzymes. In addition, organophosphorothioates may be efficiently detoxified by reactions catalyzed by CYP (Gupta, 2006; Crane et al., 2012). The involvement of CYP in AZM and CPF metabolism can result in the production of reactive oxygen species (ROS) such as the highly reactive superoxide anion and hydrogen peroxide. Therefore, the exposure to AZM and/or CPF could lead to protein, DNA, or lipid damage due to an overproduction of ROS. Regarding this, several studies have already stressed the role of ROS production in the toxicity of organophosphate pesticides in several animal species (Oruç et al., 2004; Milatovic et al., 2006; Verma et al., 2007; Patetsini et al., 2013). However, information on the effects of organophosphates on oxidative stress parameters in freshwater gastropods is scarce. Even more scarce is the information on the effects of mixtures of organophosphates.

To control the production of ROS, aerobic cells have several non-enzymatic and enzymatic antioxidant defensive mechanisms. Oxidative stress develops when there is an imbalance between oxidants and anti-oxidants in favor of the formers. Among the non-enzymatic antioxidants, the abundant thiol glutathione (GSH) plays a central role in maintaining cellular redox status and protecting cells from oxidative injury (Dickinson and Forman, 2002). The two main antioxidant enzymes involved in the detoxification of ROS are superoxide dismutase (SOD) and catalase (CAT). SOD catalyzes the dismutation of the superoxide anion to hydrogen peroxide and water. CAT is a tetrameric enzyme located in peroxisomes that reacts efficiently with hydrogen peroxide to give water and oxygen as products.

Besides SOD and CAT, the GSH-dependent enzymes play an important role in various processes of detoxification and as antioxidants. Thus, the glutathione S-transferases (GSTs) are phase II detoxification enzymes that catalyze the conjugation of GSH with a variety of electrophilic compounds including the products of the cleavage of lipid peroxides (Cnubben et al., 2001; Stephensen et al., 2002). Glutathione disulfide (GSSG) is recycled to GSH by the enzyme glutathione reductase (GR) whose activity depends on the intracellular concentration of NADPH. The major source of NADPH is the pentose phosphate shunt. Therefore, the activity of the enzyme glucose 6-phosphate dehydrogenase (G6PDH) may limit the rate of NADPH production and, hence, the resistant of the cell to oxidative stress.

The present study was undertaken to determine: (a) whether the OPs AZM and CPF induce oxidative stress in *P. corneus*, and (b) whether a mixture of both OPs that causes a higher neurotoxicity than single pesticides also causes an enhanced oxidative stress. To this end, non-enzymatic and enzymatic parameters (GSH and GSSG levels, and the activities of SOD, CAT, GR, GST, and G6PDH) were measured in the soft tissues of snails acutely exposed to the insecticides in single-chemical and binary-mixture studies. Malondialdehyde (MDA) levels were also measured in the digestive glands of the snails. MDA is one of the final products of polyunsaturated fatty acids peroxidation and is known as a good marker of free radical-mediated damage and oxidative stress.

2. Materials and methods

2.1. Materials

1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), glutathione disulfide (GSSG), glucose 6-phosphate, NADPH, $NADP^+$, epinephrine, orthophthaldehyde (OPA), *N*-ethylmaleimide (NEM), butylated hydroxytoluene (BHT), thiobarbituric acid (TBA), 1,1,3,3-tetraetoxypropane (TEP), 2-mercaptoethanol, azinphos-methyl PESTANAL® (CAS N° 86-50-0, 97.2% pure), and chlorpyrifos PESTANAL® (CAS N° 2921-88-2, 99.9% pure) were purchased from Sigma–Aldrich of Argentina S.A. All other reagents and chemicals were of analytical grade.

2.2. Snails and general bioassay conditions

Adult *P. corneus* snails were purchased from Discus Morón S.R.L., Buenos Aires, Argentina. Afterwards the snails were reared in our laboratory in aerated glass aquaria (17–20 L), at a temperature of $22 \pm 2\,^{\circ}C$, and under a 14:10 (L:D) h artificial photoperiod regime. Animals were fed lettuce leaves *ad libitum*. For all the experiments, adult snails of similar size (10 ± 2 mm of shell length, 300 ± 36 mg of wet weight) were used.

Water quality characteristics were as follows: total hardness = 67 ± 3 mg $CaCO_3\,L^{-1}$; alkalinity = 29 ± 2 mg $CaCO_3\,L^{-1}$; pH 7.0 ± 0.2 and conductivity = $250 \pm 17\,\mu S\,cm^{-1}$.

The general experimental design consisted of 1 L glass vessels containing 600 mL of each treatment condition, i.e., different pesticide concentrations, solvent control and solvent-free control. During the treatments animals were not fed. All bioassays were performed at a temperature of $22 \pm 2\,^{\circ}C$, and under a photoperiod of 14:10 h light/dark without aeration. No mortality was observed either in control animals or in any of the treatments. Carbon-filtered dechlorinated tap water was used as the test medium.

The snails were exposed to 2.5 mg L^{-1} of AZM, to 7.5 μg L^{-1} of CPF, or to a binary mixture of AZM + CPF (1.25 mg L^{-1} of AZM + 3.75 μg L^{-1} of CPF). The concentrations were selected based on previous results from our laboratory concerning cholinesterase inhibition (Cacciatore et al., 2013). Single pesticide concentrations correspond to the levels that cause 60% cholinesterase inhibition at 48 h. The concentrations of AZM and CPF in the mixture correspond to a synergistic mixture in which cholinesterase inhibition is about 92% instead of the expected additive inhibition of 60% (Supplementary Fig. S1).

See Excel sheet 1 as supplementary file. Supplementry material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2015.07.009

Aqueous solutions containing the pesticides AZM and CPF were prepared by dissolving the pesticides in acetone, and diluting with an appropriate amount of dechlorinated tap water. Pesticide concentration was tested using HPLC with UV detector. Reversed-phase chromatography was performed on a Shimadzu Class-VP model with isocratic pump (LC-10AT VP), a variable wavelength programmable UV–visible detector (SPD-10A VP) and a Supelcosil LC-18 (250 mm × 4.6 mm, 5 μm particle size) column as described in Cacciatore et al. (2013). With this procedure, we were not able to test the concentration of CPF used for the exposure experiments due to the limit of quantitation of the method (0.053 mg L^{-1}). Therefore, the only concentrations tested corresponded to the stock CPF and AZM solutions and to the AZM dilution. The concentration values measured were always within the range 95–102% of the nominal values.