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Genotoxicity evaluation of the insecticide imidacloprid on circulating blood cells of Montevideo tree frog *Hypsiboas pulchellus* tadpoles (Anura, Hylidae) by comet and micronucleus bioassays

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ABSTRACT

Acute toxicity and genotoxicity of imidacloprid (IMI) was evaluated on *Hypsiboas pulchellus* (Anura: Hylidae) tadpoles exposed under laboratory conditions. A lethal effect was used as the end point for lethality, whereas the frequency of micronuclei (MNs) and DNA single-strand breaks evaluated by the single cell gel electrophoresis assay were employed as end points for genotoxicity. Experiments were performed on tadpoles at stage 36 (range, 35–37) according to the classification proposed by Gosner. Mortality studies revealed an LC_{50} (96 h) value of 84.91 mg/L IMI (95% confidence limits, 77.20–93.04). While increased frequency of MNs was observed when 15 and 30 mg/L were assayed for 48 h, only 15 mg/L increased the frequency of MNs in tadpoles exposed for 96 h. Furthermore, other nuclear abnormalities, *i.e.*, binucleated cells and blebbed and notched nuclei, were induced in tadpoles exposed for both 48 h when treated with 15 mg/L and 96 h when treated with 15 and 30 mg/L. An increase in the genetic damage index was observed in tadpoles treated with 30 mg/L for 48 and 96 h. This study represents the first evidence of acute lethal and sublethal effects exerted by IMI on tadpoles of an amphibian species native to Argentina. Finally, our findings highlight the hazardous properties of this insecticide for nontarget living species exposed to this agrochemical.

1. Introduction

Amphibians represent important vertebrates in natural and agricultural ecosystems since they are included among the most important natural enemies of several agricultural pests worldwide. They possess certain characteristics rendering them a useful indicator species for measuring the effects of changes of the environment. Several reports agree in demonstrating that these vertebrates can be regarded as bioindicators of aquatic and agricultural ecosystems due not only to their sensitivity to habitat modification, but also to the presence of larvae stage. Amphibian larvae live in the aquatic environment and are sensitive to the pollutants (Brodeur et al., 2012; Pollet and Bendell-Young, 2000). At population level, decline of amphibian abundances have been observed, a phenomenon in most cases attributed to pollution of agricultural areas exerted by emerging pollutants, including agrochemicals (Mann et al., 2009; Relyea, 2009). However, other factors, *e.g.*, overexploitation, diseases, habitat loss and/or modification, introduced species, and climate change, also contribute to reduction of amphibian population (Mann et al., 2009). At the organism level, the growth, development, and susceptibility to disease are affected. Furthermore, at the molecular level, the induction of genetic injury into DNA after exposure to agrochemicals is perhaps the most relevant biological effect. A correlation between the use of agrochemicals and the decline of amphibian populations has been demonstrated (Beebee, 2005). The effects of pesticides, including insecticides and herbicides, are particularly detrimental to amphibian species. Several factors contribute to the high sensitivity of amphibians to pesticides: living in the aquatic environment and therefore exposure to various pollutants, the laying of unprotected eggs, and possessing highly permeable skin (Brühl et al., 2011; Sparling and Fellers, 2009).

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Hypsiboas pulchellus, the Montevideo tree frog, also called the common tree frog, is an arboreal anuran species in the family Hylidae. Its natural habitats are subtropical or tropical dry, seasonally wet or flooded lowland grassland, intermittent freshwater lakes, marshes and pastureland (Kwet et al., 2004). Is a very widespread and abundant species with an extensive distribution in Neotropical America, including Argentina, Brazil, Paraguay, and Uruguay, and species commonly found in the Pampasic region of Argentina (Cei, 1980). Previous studies have stressed that tadpoles of this species can be considered a suitable *in vivo* model for detecting lethal and sublethal effects induced by several emerging pollutants, including agrochemicals. Among them, the oxidizing agent potassium dichromate (Natale et al., 2006); the chemotherapeutic cyclophosphamide (Lajmanovich et al., 2005); the insecticides fenitrothion (Junges et al., 2010), cypermethrin (Agostini et al., 2010), and endosulfan (Lajmanovich et al., 2005); as well as the herbicide glufosinate ammonium (Peltzer et al., 2013) can be included.

Imidacloprid (IMI, $C_9H_{10}ClN_5O_2$), is a nicotine-derived systemic insecticide belonging to the neonicotinoid pesticide group. These insecticides act as insect neurotoxins and belong to a class of chemicals, the chloronicotinyl nitroguanidine chemical family, that affect the central nervous system of insects (Blacquière et al., 2012; Tomizawa and Casida, 2005). IMI works by interfering with the transmission of stimuli in the insect nervous system by blocking the nicotinergic neuronal pathway. This blockage leads to the accumulation of acetylcholine, resulting in the insect's paralysis and eventually death. It is effective on contact and *via* stomach action (http://extoxnet.orst.edu/pips/imidaclo.htm). Because IMI binds much more strongly to insect nicotinic neuron receptors than mammal neuron receptors, this insecticide is selectively more toxic to insects than mammals (NPIC, 2010; Tomizawa and Casida, 2005). IMI has been ranked as a Class II chemical (moderately hazardous) by the World Health Organization (WHO, 2002), whereas the U.S. Environmental Protection Agency (NPIC, 2010) has included the insecticide in Group E, compounds with no evidence of carcinogenicity, based on studies with rats and mice. Furthermore, it has not been included as a carcinogen by the International Agency for Research on Cancer (NPIC, 2010). Neonicotinoid insecticides, including IMI, are successfully applied to control pests in a variety of agricultural crops, affecting not only pest insects but also non-target organisms such as pollinators and aquatic invertebrates, *e.g.*, insect larvae living in water (Blacquière et al., 2012).

Studies of the deleterious effects induced by IMI have revealed that the insecticide should be considered as not acutely toxic for fish and amphibians, slightly toxic for zooplankton, moderately toxic for crustaceans, and highly toxic for annelids, but very highly toxic for insects (www.pesticideinfo.org). Among aquatic invertebrates, arthropods such as chironomids (Langer-Jaesrich et al., 2010; Stoughton et al., 2008) as well as ostracods and amphipods (Sánchez-Bayo and Goka, 2006; Stoughton et al., 2008) are extremely sensitive to imidacloprid exposure, with adverse effects observed on survival, growth, and reproductive success. Similarly, toxic effects have also been reported in aquatic vertebrates, namely, fish (Sánchez-Bayo and Goka, 2005) and amphibians, including two species widely distributed in Southeast Asia, *Pelophylax nigromaculatus* and *Rana limnocharis* (Feng et al., 2004).

There is an increasing interest in biomonitoring markers to provide a measurement as well as an estimation of biological exposure to genotoxic pollutants. To achieve this goal, several end points for testing both genotoxicity and cytotoxicity have been employed on aquatic organisms, including amphibians. However, analysis of the frequency of micronuclei (MNs) and the induction of DNA single-strand breaks by the single cell gel electrophoresis (SCGE) assay are the most frequently employed and recommended end points for detecting cytogenetic and DNA damage in circulating nucleated erythrocytes, respectively (Lajmanovich et al., 2005, 2013; Mouchet et al., 2007; Nikoloff et al., 2014; Vera-Candioti et al., 2010).

The aim of the present study is to characterize the acute toxicity of the insecticide imidacloprid on *H. pulchellus* tadpoles exposed under laboratory conditions using a static acute experimental method. This study was performed employing lethal and several sublethal short-term end points for genotoxicity, namely, the frequency of micronuclei (MNs) and the induction of DNA single-strand breaks.

2. Materials and methods

2.1. Chemicals

IMI [95.1%; CAS 138261-41-3; recommended application field ratio up to 700 g a.i. per hectare (CASAFE, 2011)] was kindly provided by Gleba, Argentina. Cyclophosphamide (CP; CAS 6055-19-2) and dimethyl sulfoxide (DMSO; CAS 67-68-5) were purchased from Sigma Chemical Co. (St. Louis, MO), whereas $K_2Cr_2O_7$ [$Cr_{(VI)}$] (CAS 7778-50-9) was obtained from Merck KGaA (Darmstadt, Germany). All other chemicals and solvents of analytical grade were purchased from Sigma Chemical Co.

2.2. Quality control

Determination of the concentration level of IMI in the stock and the test solutions was performed by QV Chem Laboratory (La Plata, Buenos Aires, Argentina) according to U.S. Geological Survey Report 01-4134. Imidacloprid levels were analyzed by high-performance liquid chromatography using an ultraviolet detector. Active ingredient samples from test solutions (30 and 100 mg/L) correspond to values obtained immediately after preparation (0 h) and 24 h thereafter. The detection limit for IMI was 0.5 mg/L.

2.3. Test organisms

Egg masses from *H. pulchellus* were collected from a temporary and unpolluted pond free from pluvial runoff from agricultural areas, in the vicinity of La Plata City (35°10′ S, 57° 51′ W; Buenos Aires Province, Argentina), at the late cleavage stage, stage 9 according to Gosner's classification (Gosner, 1960). Hatches were transported to the laboratory and then acclimatized to 16/8 h light/dark cycles in aquaria at 25 °C with dechlorinated tap water with artificial aeration. The physical and chemical parameters of the water were as follows (mean ± SE): temperature, 25.0 ± 1 °C; pH 7.5 ± 0.1; dissolved oxygen, 6.3 ± 0.3 mg/L; conductivity, 994 ± 8.5 μS/cm; hardness, 143 ± 23.5 mg/L $CaCO_3$. Boiled lettuce was supplied as a food source twice per week until the beginning of the experimental procedures.

2.4. Determination of LC_{50}

Experiments for toxicity assessment were performed on tadpoles at Gosner stage 36 (range, 35–37) (Gosner, 1960) following standardized methods proposed by the U.S. EPA (1975, 2002) and ASTM (2007) with minor modifications reported previously for native species (Nikoloff et al., 2014; Vera-Candioti et al., 2010). To determine IMI concentrations used in the acute toxicity tests, preliminary assays were performed. Experiments were performed in quadruplicate and run simultaneously for each experimental point employing five tadpoles maintained in a 500 ml glass container per replicate ($N = 20$), and exposed to six different concentrations of IMI (50, 75, 100, 150, 200, and 250 mg/L) for 96 h. Prior to use,