ECOTOX BRASIL
SOCIEDADE BRASILEIRA DE ECOTOXICOLOGIA

Ecotoxicol. Environ. Contam., v. 15, n. 1, 2020, 9-13
doi: 10.5132/eec.2020.01.02

EEC

Genotoxic effect of the insecticide Chlorpyrifos on the erythrocytes of *Odontophrynus carvalhoi* tadpoles (Amphibia: Odontophrynidae)

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Received February 22, 2020; Accept April 01, 2020

Abstract

Chlorpyrifos is an organophosphate insecticide widely used and it acts as an inhibitor of cholinesterases, overstimulating the nerve endings. This study analyzed the genotoxic effect of the organophosphate insecticide Chlorpyrifos upon the induction of micronucleated erythrocytes in the blood of *Odontophrynus carvalhoi* Brazilian neotropical tadpoles submitted to four different nominal concentrations of this insecticide (10, 100, 200 e 400 μg L^{-1}) during 96 hours. The tadpoles were anesthetized, euthanized and their blood was collected through cardiac puncture and later on cyto smears were performed and stained with May Grunwald Giemsa. The frequencies of micronucleated erythrocytes were determined at different periods of exposure (24h, 48h e 96h) through the analysis of 1000 erythrocytes for each tadpole. The tadpoles that were submitted to the Chlorpyrifos showed high frequencies of genetic changes (micronuclei) when compared with the negative control. The differences in the frequencies of the micronuclei registered after 48 hours of exposure were statistically significant ($p<0.05$). Thus, it can be concluded that tadpoles of *O. carvalhoi* were susceptible to genetic alterations induced by the use of the commercial formulation of chlorpyrifos (Klorpan®), showing a clastogenic effect in the erythrocytes of this species. The use of this species as an animal model for genotoxic studies has shown promise.

Key-words: Amphibians; Chlorpyrifos; Erythrocytes; Micronucleus test; Tadpoles.

INTRODUCTION

The Brazil is the largest consumer of pesticides in the world, thus bringing about a huge input of contaminants in the environment (surface water, underground water, soil) generating chemical pollution, which threatens biodiversity, due to its magnitude and omnipresence (Schiesari & Grillitsch , 2010).

Organophosphates are widely used in agriculture and cattle pasture in the composition of insecticides, herbicides and plant growth regulators. The chlorpyrifos (O, O-dietil-O-3,5,6-trichloropyridin-2-il phosphorothioate; CPF) is an organophosphate insecticide commonly used in agriculture. It has toxic effects upon a wide variety of insects and is being used in a large scale in plantation fields. But it also has toxic effects on birds, fish and aquatic invertebrates (*Daphnia*) (Barron & Woodburn, 1995), and it is able to bioconcentrate in different groups of aquatic organisms (Arcaute *et al.,* 2012). According to Yin *et al.* (2009) some studies have shown connections between the decrease in amphibian populations and the presence of chlorpyrifos. Furthermore, there are reports that this substance can cause liver disorders, immunological abnormalities, neurochemical and neurobehavioral alterations, embryotoxicity, genotoxicity and teratogenicity in several organisms including amphibians (Barron & Woodburn, 1995; Widder & Bidwell, 2008; Bernabó *et al.,* 2011; Nobonita & Suchismita, 2013, Liendo *et al.,* 2015; Quiroga *et al.,* 2018, Salgado-Costa *et al.,* 2018; Rutkoski *et al.,* 2020).

In amphibians the high permeability of their integument gives these animals a high sensitivity to the exposure to aquatic

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pollutants. According to Bernabo *et al.* (2011) few studies discuss the effect of the chlorpyrifos upon amphibians. The toxicity of this substance was examined mainly in the species *Xenopus laevis*. Widder & Bidwell (2008) found species-specific differences in response to chlorpyrifos pesticide in terms of tadpole size, cholinesterase activity, and swim speed with *Hyla chrysoscelis* and *Gastrophryne olivacea* being most sensitive by *Rana sphenocephala* and *Acris crepitans* (native North American species of anurans). However, little is known as to brazilian neotropical species potentially exposed to this agrochemical.

Micronuclei are chromosome fragments or entire chromosomes that were not incorporated to the main nucleus in cells actively splitting of any tissue (Bosch *et al.,* 2011; Lajmanovich *et al.,* 2012). The micronucleus assay is a quick and sensitive test to detect structural and numerical chromosomal alterations (Heddle *et al.,* 1983). It is also a cytogenetic assay that is commonly used in several biological systems to monitor the environmental genotoxicity (Mersch *et al.,* 1997).

The aim of the present study was to assess, for the first time, the genotoxic potential of the organophosphate chlorpyrifos insecticide in tadpoles of *Odontophrynus carvalhoi* Savage & Cei, a brazilian neotropical species occurring throughout various states of Northeast Region of Brazil and also in the Minas Gerais state, Southeastern Region of Brazil, where there exists a intense agricultural activity.

MATERIAL AND METHODS

Chemical Aspects

Chlorpyrifos (Klorpan® 480 EC (emulsifiable concentrate), NuFarm®; consisted of 48,98% of purity, 480.00 g L^{-1} of O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate as active ingredient (a.i.); 495.77 g L^{-1} of xylol and 79.34 g L^{-1} other ingredients) (Nº CAS 2921-88-2).

Cyclophosfamide (CF) (Nº CAS 50-18-0) was used as a positive control in a concentration of 40ppm (mg L^{-1}). These tested solutions were prepared immediately before each experiment.

Tadpoles

The reproduction of *Odontophrynus carvalhoi* is associated with the rain period, occurring in a few days each year (Lisboa *et al.,* 2010). The tadpoles live in streams and puddles formed during the period of the highest rain falls (Juncá, Pers. Com.). Tadpoles of *Odontophrynus carvalhoi* were obtained from the hatching of two spawnings from two couples collected in amplexus in the municipality of Mucugê, State of Bahia, Brazil. Before the experiments, the tadpoles obtained from the spawnings were acclimatized in ten aquaria of 35 L each containing 30 L of well-ventilated water in a temperature of 20 to 25º C and a 12h-12h photo period. The newly hatched larvae were reared in a density of approximately two larvae per liter of water. The water was changed every two days and the larvae were fed *ad libitum* on a standard diet of the fish food NeonMEP200 complex®. Pre-metaphoric larvae in stages 27 to 31 sensu Gosner (1960) were used in the bioassays.

Experimental Design

Tests of sub-lethality were carried out during 96 hours according to the procedures adapted from literature (Fernandez, 1993; Campana *et al.,* 2003; Lajmanovich *et al.,* 2005; Marques *et al.,* 2009; Yin *et al.,* 2009; Li *et al.,* 2010; Bosch *et al.,* 2011), using solutions of chlorpyrifos with water without chlorine in the following nominal concentrations: 10, 100, 200 and 400 µg L^{-1} (4 treatments – C). Negative controls (CN) were kept during the period of the experiment containing only the tadpoles and the dechlorinated water. Positive controls (PC) were performed using CF in a concentration of 40 ppm (mg L^{-1}). All the test solutions were prepared immediately before each experiment and were renewed every two days. Tadpoles were fed daily throughout the experiment. The parameters that were evaluated in this work (micronucleated erythrocytes) were measured after 24, 48 and 96 hours for all the treatments. For each treatment (C10, C100, C200 and C400) and controls (CN and CP), 15 tadpoles were used, totaling 90 tadpoles per day of exposure and 270 tadpoles until the end of the experiment, which were kept individually in a polypropylene container of 800 ml capacity.

Test of Micronucleus

Each tadpole that had been previously anesthetized with Lidocaine hydrochloride gel (2%), euthanized and had blood samples collected through cardiac puncture. An aliquot of 10 µL of blood was collected with the help of micro pipettes and deposited in a micro tubule containing 500 µL of saline solution and 10 µL of ethylenediaminetetraacetic acid (EDTA). This compound was centrifuged in a CytoSpin type centrifuge. From this material cyto smears were prepared (two slides per tadpole). The slides were fixed with methanol during 10 minutes, dried at room temperature and finally stained with May Grunwald-Giemsa at 6 % in a *Sorensen's* buffer (pH 6.8) for 20 minutes.

For each tadpole, 1000 erythrocytes were analyzed in each replicated slides and the frequency of micronucleated cells was recorded using light microscopy, oil immersion and magnification of 1000X. Coded and randomized slides were scored blind by a single observer.The criteria adopted to determine the micronucleus was the same as the one proposed by Lajmanovich *et al.* (2005), Yin *et al.* (2009) and Bosch *et al.* (2011), namely, the micronucleus being that not refractive corpuscule, which presented shape, color, and intensity similar to the main nucleus, although it was not connected to it. The micronuclei had furthermore an area that was less or equal in size to one third of the size of the main nucleus.

Statistical Analyses

Mean values ± SD of MN/1000 cells were calculated. To test whether the differences between the control and experiment groups were significant, the non parametric Kruskal-Wallis test was used. The value of $p \leq 0.05$ was considered an indicative of significance. The Dunnet´s test was used to even further examine the main significant effects. All the tests were carried out with the software GraphPad Prism 5.00 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

The mature erythrocytes of *Odontophrynus carvalhoi* are oval-elongated of an approximate size of 20 x 14 µm. The nucleus is clearly evident with a well-defined shape and central position, which makes easier the identification of fragments in its cytoplasm. The micronuclei observed are spherical nuclear fragments separated from their parental nucleus (Figure 1). A single micronucleus was the predominant morphological abnormality in the erythrocyte cells that were analyzed.

Tadpoles that were exposed to chlorpyrifos show an increase in the micronucleate erythrocytes in all periods (24h, 48h and 96 hours of exposure) and at least one of the treatments (10,100, 200 and 400 µg L^{-1}) that were performed when compared to the negative control (Figure 2). After 24 hours of exposure, the frequencies of micronuclei were significantly different only between the negative control group and the treatment C100 (100 µg L^{-1}). However, in the 48h and 96 hour periods, all the tested treatments presented significant statistical differences when compared with the negative control group (Figure 2).

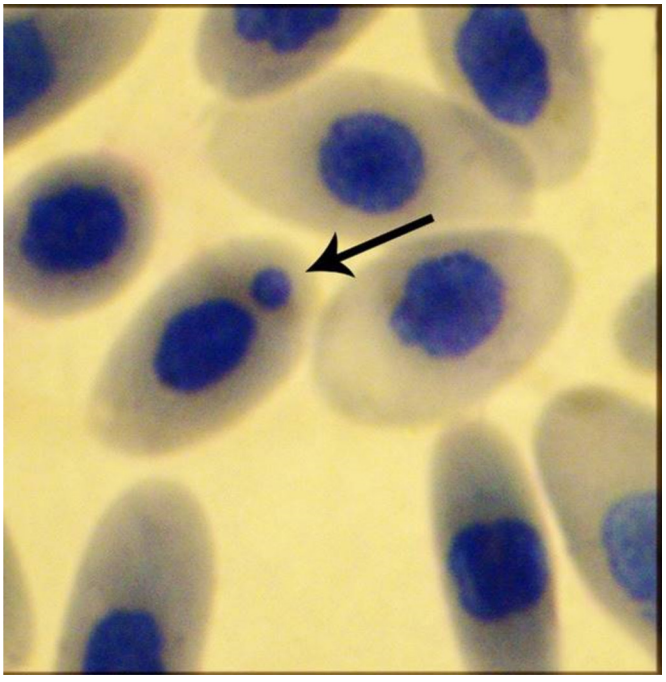

Figure 1: Erythrocytes in blood of *Odontophrynus carvalhoi* tadpoles exposed to the organophosphate insecticide Chlorpyrifos (10 µg L^{-1}, 96 hours). Blood smear stained with May-Grunwald-Giemsa method, 1000X. micronucleate erythrocytes (arrow).

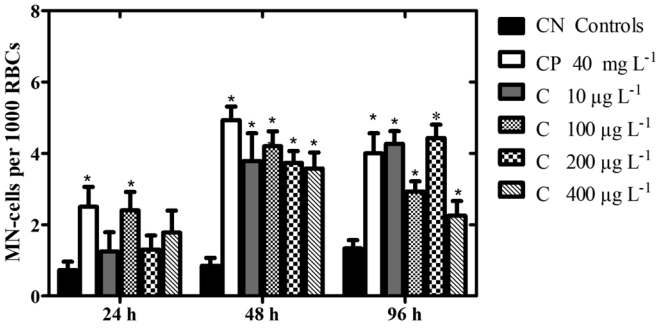

Figure 2: Mean and standard deviation (bars) of micronucleate (MN) erythrocyte (RBCs) (per 1000 cells) in *Odontophrynus carvalhoi* larvae treated with different nominal concentrations of test compounds. CN: Negative Control, CP: Positive Control, C: Chlorpyrifos; 24h: 24 hours; 48h: 48 hours and 96h: 96 hours of exposure. *p<0.05 in relation to the negative control (Kruskal-Wallis test).

There was an increase in the frequency of micronucleate erythrocyte cells when 24, 48 and 96h exposures are compared, thus showing that an exposure of 48 hours is a satisfactory time to carry out the analyses. There were deaths in all treatments. However, dead tadpoles were excluded from the analysis and we increased the number of tadpoles exposed in order to standardize the sample number of 15 tadpoles per treatment per day, totaling the 270 analyzed.

DISCUSSION

The present study shows that the chlorpyrifos presents a genotoxic effect, inducing the formation of micronuclei in tadpoles of *Odontophrynus carvalhoi* in all the concentrations tested in 48 and 96 hours. Yin *et al.* (2009) tested the effect of the chlorpyrifos upon Bufo *bufo gargarizans* tadpoles after 96 hours of exposure and found significant differences in most of the tested concentrations (0.16; 0.32 and 0.64 mg L^{-1}), except in 0.08 mg L^{-1}. This last concentration corresponds to 80 µg L^{-1}. However, methodological differences in experiments (concentration and commercial formulae tested) make direct comparisons among studies difficult. The present study showed that a lower concentration (10 µg L^{-1}) had a significant genotoxic effect, which reveals the greater sensitivity of the species *O. carvalhoi* to this pesticide. One can thus make evident the genotoxic effect of the chlorpyrifos in tadpoles of at least two species of frogs.

In this study, a significant increase in micronucleate erythrocytes in tadpoles of *O. carvalhoi* in 48 and 96 hours of exposure was found. Similar results were found for other chemical substances (Endosulfan, Cypermetrina, polluted water, Malation, Alficida®, petrochemical contaminants, Copper and insecticide Piretróide) while testing different species of amphibians (Campana *et al.*, 2003; Ferrari *et al*., 2005; Lajmanovich *et al*., 2005; Cabagna *et al.*; 2006; Huang *et al.*, 2007; Candioti *et al.*, 2010; Ossana *et al.*, 2010; Giri *et al.,* 2012).

Analyzing studies involving other amphibians species exposed to chlorpyrifos, using other endpoints, among them mortality, we found that the studies used concentrations

higher than that used in this paper, and the lower dose in these studies generally had no effect on the evaluated endpoints (see Arcaute *et al.,* 2011; Bernabó *et al*., 2011; Liendo *et al.,* 2015; Rutkoski *et al.,* 2020). This difference in sensitivity between species reinforces the idea of a possible species-specific response already mentioned in the introduction to this article. Thus, the species *O. carvalhoi* may be more sensitive to the toxic effects of chlorpyrifos in lower concentrations when compared to other species in the literature.

There was an increase in the frequency of micronuclei in the blood of the *O.carvalhoi* tadpoles throughout all periods of exposure to Chlorpyrifos. The frequencies of the micronuclei registered in the *O. carvalhoi* were lower to those registered for the *Bufo bufo gargarizans* tadpoles also exposed to the chlorpyrifos (Yin *et al.,* 2009). Although all concentrations tested showed a genotoxic effect on *O. carvalhoi* tadpoles, for better definition of the dose–response, it is necessary to use at least one more concentration (less than 10 μg L^{-1}). These differences merit further investigation.

The frequency reported in the control group with *O. carvalhoi* tadpoles (0.77± 0.47 micronuclei/1000 erythrocytes) can be used as a reference for comparison with other populations of this species present in the agro-ecosystem.

The analysis of micronuclei in erythrocytes of the peripheral blood of tadpoles is an effective and reliable tool to study the genomic instability induced by pesticides and other pollutants in aquatic environments all over the world (Relyea *et al.,* 2005; Peltzer *et al.,* 2008; Rocha *et al.,* 2011, Ossana & Salibián, 2013). Such pollutants may cause tadpoles to become vulnerable to opportunistic parasites and nuclear aberrations, erythrocytes or hemolysis (Peltzer *et al.,* 2008).

Our results show that the tadpoles of *Odontophrynus carvalhoi* were susceptible to genetic changes induced by the use of the commercial formula of chlorpyrifos (Klorpan®), interfering negatively in studies on development in environments where there is contamination by this substance. The intensive use of this organophosphate insecticide can cause genetic mutations in tadpoles that grow in environments, affecting or declining physical conditioning, species survival and loss of local diversity.

CONCLUSIONS

The results presented here constitute the first records of the impact of this insecticide on the species *Odontophrynus carvalhoi.* In all tested concentrations, genotoxic action was observed. The use of this species as an animal model for genotoxic studies has shown promise. These results may support further genotoxic studies for the species and for the understanding of actions for the development of sustainable agriculture and the preservation of amphibian communities, important agents of pest biocontrol.

ACKNOWLEDGEMENTS

We want to thank the Ministry of Environment of Brazil for the license to carry out the research (Licença ICMBio Nº 27305-1) and the Committee of Ethics on Animal Use of the University (CEUA-UEFS, protocolo Nº 02/2012)**.** Our thanks also to the reviewers for their comments and suggestions. To Marcelo F. Napoli, Mirco Solé, André L. Cruz and Denise C. Rossa-Feres for the suggestions. This research was funded by the National Council of Scientific and Technological Development - CNPq: Edital MCT/CNPq Nº 59/2009 - Program of Long Term Ecological Research PELD: Chapada Diamantina and Research Support Foundation of the Sao Paulo State – FAPESP: Project SISBIOTA: Anura Tadpoles of the Mata Atlântica, Amazônia, Pantanal, Cerrado and Zonas de Transição: Morphological Characterization, Spacial Distribution and Pattern of Diversity (FAPESP proc. 2010/52321-7 and CNPq proc. 563075/2010-4).

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