Journal of Environmental Protection, 2015, 6, 1360-1367
Published Online December 2015 in SciRes. http://www.scirp.org/journal/jep
http://dx.doi.org/10.4236/jep.2015.612118

Scientific Research Publishing

Genotoxic Potential of the Insecticide Imidacloprid in a Non-Target Organism (*Oreochromis niloticus*-Pisces)*

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Received 12 November 2015; accepted 5 December 2015; published 9 December 2015

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Abstract

The indiscriminate use of pesticides has become a serious environmental concern. Insecticide imidacloprid (IMI) is the second most widely used pesticides worldwide. In 2010, 1.934 tons of IMI were sold in Brazil, mostly to be used in sugarcane crops. Several studies have detected its presence in the aquatic environment constituting a risk to non-target organisms. The aquatic animals are organisms used for environmental biomonitoring worldwide. They are considered excellent genetic models to detect environmental mutagens. Among animal species, the fish *Oreochromis niloticus*, commonly known as Nile tilapia, has been used to evaluate DNA damage. The present study therefore evaluated the effect of IMI on the genetic material of *Oreochromis niloticus* (Pisces) erythrocytes exposed to different concentration (250; 125 and 62.5 µg/L) of IMI used in growing sugarcane. The effect of the IMI was measured using the comet assay and the micronucleus (MN) test, assays that detected genotoxic damage. The results in the comet assay demonstrated that the concentrations tested induced primary damage to DNA. They also proved the occurrence of MN and nuclear abnormalities at the higher concentration used in the micronuclei and other nuclear abnormalities test. The insecticide IMI induced primary DNA damage at all concentrations and

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How to cite this paper: Ansoar-Rodríguez, Y., Christofoletti, C.A., Marcato, A.C., Correia, J.E., Bueno, O.C., Malaspina, O. and Fontanetti, C.S. (2015) Genotoxic Potential of the Insecticide Imidacloprid in a Non-Target Organism (*Oreochromis niloticus*-Pisces). *Journal of Environmental Protection*, **6**, 1360-1367. http://dx.doi.org/10.4236/jep.2015.612118

damage at the chromosomal level at the highest concentration tested. The results of this study show the potential risk of IMI in a non-target organism.

Keywords

Pesticide, Genotoxicity, DNA Damage, Comet Assay, Micronucleus Test

1. Introduction

The use of sugarcane as a biofuel has expanded in the last decade and Brazil becomes the largest producer in the world [1]. The increase in sugarcane crops is accompanied by the increase of pesticide consumption. The imidacloprid (IMI; 1-[(6-chloro-3-pyridinylmethyl]-N-nitro-2-imidazolidinimine) belongs to a class of chemical compounds called neonicotinoids that act on the central nervous system of insects and it is the second most used pesticide in the world [2]. In 2010, companies reported sales of 1.934 tons of IMI to the Brazilian Institute of Environment and Renewable Natural Resources, principally for use in sugarcane.

The IMI can persist in soil, with a half-life (28 - 1250 days) highly variable which varies greatly among soil type and other factors [3]. Also, depending on rainfall and soil conditions, 2.4% to nearly 80% of the mass of neonicotinoids (including IMI) could make their way into water bodies [4]. Due to its presence in various environments, inhabited by large numbers of organisms, toxicological studies are extremely important. Thus, the use of living organisms (bioindicator), capable of somehow indicating the presence of stresses generated by environmental pollutants [5] [6], is one way to monitor the negative effects in the environment. Due to the presence of significant levels that have been detected in water, it is very important to conduct studies on the effects of IMI on aquatic organisms as bioindicators. Fish are widely used because of capacity to accumulate contaminants and can show physiological, biochemical, histological or differentiated cell response [7]. These organisms may indicate variations in tolerance to environmental conditions created by the use of pesticides, including genetic change, which makes them excellent indicators with a high application for monitoring environmental genotoxicity [8]. Among the most commonly used species of fish is *O. niloticus*, known as Nile tilapia, considered optimal in laboratory studies for its multiple advantages such as a high rate of growth and reproduction, resistance and tolerance in laboratory conditions and adaptation in commercial alimentation [9].

There are many studies in target and non-target organisms, including mammals, birds, fish, insects, crustacean, molluscs and annelids, showing the toxicity of various neonicotinoids used in agriculture [3]. Furthermore, genotoxicology studies using biomarkers of DNA damage are very important. The tests most used to assess DNA damage in fish exposed to pesticides and other environmental contaminants are the comet and micronucleus tests, excellent tool in genotoxicology, both used in this study.

The alkaline single-cell gel electrophoresis assay, better known as the comet assay, detects strand breaks, alkali-labile sites, and delayed-repair sites as initial damage [9]. The micronucleus (MN) test associated with nuclear abnormalities (NAs) has been used for more than 30 years and also is one of the most promising trials in ecotoxicology for damage detection at the chromosomal level [10] [11]. These tests of genotoxicity are recognized due to their robustness, sensitivity and statistical power to evaluate DNA damage.

In this context, the present study provides more information about the effects of the used of this insecticide in non-target organisms, providing important data on ecotoxicological consequences.

2. Material and Methods

2.1. Test Compound-IMI

The compound used in this study was IMI (1-[(6-chloro-3-pyridinylmethyl]-N-nitro-2-imidazolidinimine; CAS N˚138261-41-3; molecular formula $C_9H_{10}ClN_5O_2$), Agricultural Experiment Station-SP, Lot EDE 0036241.

2.2. Treatment Solution

The Concentrations of IMI used in the present study for the exposure of *O. niloticus* were determined through

concentration used in growing sugarcane [12] and simulating natural insecticide dilution into water resources [3]. The insecticide IMI was applied in water at three concentrations C1 = 250 μg/L twice the concentration to simulate indiscriminate use, C2 = 125 μg/L based on the maximum concentration of the commercial product applied in sugarcane and C3 = 62.5 μg/L, half of the recommended concentration to simulate natural dilution.

2.3. Biological Material

The test organism used in the experiment was *O. niloticus* (Perciformes, Cichlidae) (n = 50), popularly known as Nile tilapia. Individuals with a mean size of 12 - 15 cm were analysed to avoid intra-specific differences related to size, age (two months) and average body weight (30 ± 2 g). The individuals analysed were reared on fish culture farms and kept in the Experimental Garden of the Institute of Biosciences, UNESP (São Paulo State University), Rio Claro, São Paulo, Brazil. They were brought to the laboratory and acclimated in aerated aquariums (size: length = 45 cm, height = 25 cm, width = 20 cm) for one week at a mean temperature of 23˚C, in tap water (pH = 8.3, Temp= 20˚C ± 2˚C) and 14 hours light/dark cycle.

2.4. Bioassay

Ten aquariums were used in the experiment, two for negative control (NC) (fresh water), two in which animals received an intraperitoneal (i.p.) injection the clastogenic agent cyclophosphamide (20 mg/mL) (in sterile water) (30 mL of cyclophosphamide/50 g of fish) for the positive control (PC) [13] and the remaining six were exposed to the three IMI concentrations (250; 125 and 62.5 μg/L). All treatments consisted of two replicates with five organisms, each one in a 40 L aquarium, which was aerated during the 96h exposure period [13] [14] and no food was supplied to the fish during the experiment. Approximately 0.3 cm^3 of whole blood was taken from each fish by heart puncture using heparinised syringes. The collected blood was used for the comet assay and the MN and NA test. The study was approved by "The Ethics Committee on Animal Use", UNESP, filed with the number 8937.

2.5. Comet Assay

The alkaline comet assay was performed as described by Collins (2004) with the modifications suggested by Caffetti *et al.* (2008) [15] [16]. The blood was obtained from the fish as described above and 3 μL aliquots were diluted in 1 mL of PBS (137 mM NaCl, 2.68 mM KCl, 8 mM HNa_2PO_4; H_2KPO_4 1.47 mM) to obtain the cell suspension. Microscope slide coded were coated with 1% (v/v) standard agarose and 10 μL of the diluted blood with 120 μl the 0.5% (v/v) low melting point agarose were applied to the slides at 37˚C. After, cover slips were placed on the slides for 10 min at 4˚C to ensure the formation of the microgel. The slides were placed in lysis buffer (1 mL of Triton X-100, 10 mL of DMSO, and 89 mL of solution plus, which included 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, ~8.0 g of NaOH and 10 mL of 1% (v/v) sodium lauryl sarcosinate), pH 10, in a refrigerator for 1 h. After lysis, the slides were incubated in 300 mM NaOH + 1 mM EDTA buffer (pH > 13) for 20 min to denature the DNA and then submitted to electrophoresis at 39 V and 300 mA for 20 min; field strength 0.8 V/cm. The slides were then neutralised with PBS for 15 min and fixed in absolute ethanol for 10 min. The slides were stained with Gel Red Nucleic Acid (Gel Stain Biotum 10,000× water) and analysed (blindly) under an Olympus BX60 fluorescence microscope equipped with filter excitation: k = 420 - 490 nm, emission barrier: k = 520 nm) and a 40× objective lens.

For each fish, 100 nucleoids were analysed per blood sample. The nucleoids were visually classified according to fragment migration as undamaged (class 0), slightly damaged (class 1), more damaged (class 2) and highly damaged (class 3) per Caffetti *et al.* (2008) [16]. This record is linearly related to the frequency of DNA strand breaks in the cell population in a study on a wide range of damage. The primary damage score was calculated in arbitrary units (AU) as the sum multiplying the number of observed nucleoids in each class by the class value (0, 1, 2 or 3). The results were reported as the averages in AU with their corresponding standard error of the mean (SEM).

2.6. MN and NAs Test

Approximately 0.3 cm^3 of blood from each fish was smeared on a slide coded (blood extensions). Three smears were performed for each exposed individual. The material was fixed in absolute ethanol for 10 min and dried for

24 h. After wards, the material was hydrolysed in 1 N HCl for 11 min in moist chambers at 60˚C. After this, the slides were washed in distilled water and placed in Schiff's reagent for 2 h. A total of 3000 erythrocytes form the fish were analysed (blindly) under an immersion objective (1000X). For the identification of MN, some criteria were adopted according to Huber and Streng (1983) [17], and the NAs were recorded according to the classification of Carrasco *et al.* (1990) and Bolognesi and Hayashi (2011) [10] [11]. The frequency and SEM values for MN and other NAs in the cells were quantified.

2.7. Statistical Analysis

All data were expressed as the mean ± SEM. The data do not follow a normal distribution (Shapiro-Wilk) and Kruskal-Wallis test show differences between groups. The results obtained in the comet assay and the MN and NAs test were compared with the NC and all groups by the non-parametric Mann-Whitney test. The program used was the Statistical Package for the Social Sciences for Windows, version 15.0, (SPSS Inc., Chicago, IL, EUA).

3. Results

3.1. Comet Assay

The results obtained using the comet assay in erythrocytes of *O. niloticus* exposed to different IMI concentrations and their respective NC and PC is presented in **Table 1**. The primary DNA damage was expressed in AU (means and SEM) and represents strand breaks, alkali-labile sites, and delayed-repair sites as initial damage. The erythrocytes of the NC group show a higher occurrence of classes 0 and 1 presented lower genotoxic damage than those that underwent PC and IMI treatments. Fish injected with cyclophosphamide presented erythrocytes with DNA damage index statistically significant in relation to NC. The erythrocytes observed revealed a higher rate of DNA damage (expressed in AU) in all groups exposed to IMI compared to the NC and did not find statistically significant differences between the AU values obtained in the different IMI concentrations.

3.2. MN and NAs Test

The means and SEM of MN and other NAs in erythrocytes of *O. niloticus* exposed to IMI and their respective NC and PC are shown in **Table 2**. The erythrocytes of the NC group show a low frequency of MN and other NAs. Higher chromosomal damage level was found on the PC, with a frequency of MN statistically significant in relation to NC. Also, statistical differences in MN frequency and some NAs such as blebbed nuclei (BL) and notched nuclei (NT), were observed between NC and the highest IMI concentration.

4. Discussion

The impact of IMI use under field conditions has not been well studied. The evaluation of the action of different concentrations of IMI and its consequences on the genetic material of fish is of the utmost importance. The

Table 1. Mean ± SEM expressed in arbitrary units (AU) of the primary DNA damage in the fish *Oreochromis niloticus* exposed to three concentrations of IMI and cyclophosphamide.

Groups	Classes				
	0	1	2	3	AU
NC (H_2O)	75.1 ± 7.9	19.7 ± 4.5	5 ± 2.8	3.4 ± 2.0	39.9 ± 12.4
PC (20 mg/mL)	20.7 ± 8.0	64.6 ± 6.4	11.7 ± 3.8	5.9 ± 2.0	105.7 ± 13.8*
IMI (250 µg/L)	19.3 ± 4.5	54.8 ± 6.7	13.7 ± 2.5	13.6 ± 3.9	123 ± 16.1*
IMI (125 µg/L)	28 ± 7.4	58.6 ± 5.3	9.3 ± 2.2	7.5 ± 1.9	99.7 ± 13.7*
IMI (62.5 µg/L)	36.7 ± 11.6	35.6 ± 5.8	17.4 ± 4.8	12.1 ± 4.1	106.7 ± 11.8*

NC: negative control; PC: positive control (cyclophosphamide); IMI: imidacloprid; AU: arbitrary units; *$p < 0.05$, values statistically significant, compared to negative control with the Mann Whitney test.

Table 2. Mean ± SEM of the frequencies of various erythrocyte abnormalities in 3000 erythrocytes from the fish *Oreochromis niloticus* exposed to three concentrations of imidacloprid (IMI) and cyclophosphamide.

Groups	Other nuclear abnormalities		
	MN	Blebbed Nuclei	Notched Nuclei
NC (H_2O)	1 ± 0.4	0.4 ± 0.3	0 ± 0
PC (20 mg/mL)	3.7 ± 0.8*	1.1 ± 0.3	0.5 ± 0.4
IMI (250 µg/L)	2.8 ± 0.8*	3.1 ± 1*	3.8 ± 0.8*
IMI (125 µg/L)	1.4 ± 0.7	2.4 ± 0.8	2.9 ± 1.6
IMI (62.5 µg/L)	0.8 ± 0.4	0.4 ± 0.3	0.7 ± 0.4

NC: negative control; PC: positive control (cyclophosphamide); IMI: imidacloprid; MN: frequency of micronuclei; *$p < 0.05$, values statistically significant, compared to negative control with the Mann Whitney test.

imidacloprid is one of the most widely used and sold neonicotinoids for the control of pests in different areas, however, it affects not only insect-pests but also non-target organisms. It can cause toxicity in ecosystem organisms, such as earthworms [18], amphipods, microalgae [19] and crustacean [20] among others. Also, the application of this insecticide has been associated with the death of bees in different regions of the country [21] [22]. Besides toxicity studies, it has been evaluated the genotoxicity in plants and animals. For example, DNA damage and increased frequency of MNs in frogs *Hypsiboas pulchellus tadpoles* (Anura, Hylidae) [23] and chromosomal aberrations and MNs in *Allium cepa* and *Tradescantia pallida* [12], showed genotoxic effects.

Different species of fish are used for evaluating pesticides and the choice of blood (erythrocyte) is made primarily because these cells are easily collected without sacrificing the animal and do not require additional separation methods [24] [25]. The use of different IMI concentrations caused a statistically significant increase ($p < 0.05$) in damage to the DNA molecule. This damage refers to strand breaks, alkali-labile sites, and delayed-repair sites, which indicates the genotoxic potential of the insecticide for this aquatic organism. The comet assay has been widely used in studies with pesticides; such a test was applied to the fish species *Channa punctatus* (Channidae) when exposed to atrazine. In this paper, the authors observed an increase in DNA damage in the erythrocytes of these fish [26]. The same results were observed in studies with the phorate pesticide, a genotoxic organophosphate, in fingerlings of *Labeo rohita* (Cyprinidae) [27], and Roundup® herbicide, evaluated in *Anguilla anguilla* (Anguillidae), also displayed genotoxicity [28]. The results obtained in this study corroborate previous studies with other pesticides and support the claim that the comet assay is a highly sensitive method for the detection of DNA damage induced by environmental pollutants.

With the increased use of pesticides against harmful plants and insects in the last decade, it has been observed that certain agricultural chemicals can cause changes that include the inhibition of cell division, the induction of chromosomal abnormalities and chromosomal damage [29]. Chromosomal aberrations induced by pesticides from different cultures are widely used as an indicator of genetic damage. The MN test associated with NAs is one of the most promising tests in ecotoxicological evaluations and the best one for the observation of damage at the chromosomal level [11]. This assay has been widely applied in measurement studies of genotoxicity of different chemicals in species of fish and the biological monitoring of contaminated areas [30] [31]. Based on the results obtained in this study, IMI caused a dose-dependent increase in the frequency of MN and other NAs as BL and NT, which was statistically significant ($p < 0.05$) in the highest concentration evaluated (250 µg/L) compared to the NC. The results obtained in this study corroborate other pesticide studies using different species of fish, such as the evaluation of Aficida® and Endosulfan insecticides in fish erythrocytes from *Cnesterodon decemmaculatus* (Poeciliidae) and *Carassius carassius* (Cyprinidae) by the MN test [32] [33]. These studies demonstrate the effectiveness of fish and the MN test as a model for the biomonitoring of aquatic ecosystems that may be affected by pesticides.

Different kinds of NAs are frequently observed in fish erythrocytes, although the mechanisms responsible have not been fully explained. The BL often is considered to be indicators of genotoxic damage and other NAs, such as NT nuclei, are mainly associated to cytotoxicity [11].

Several authors confirm that the comet assay is slightly more sensitive than the MN test in detecting early genetic damage. Furthermore, the comet assay identifies repairable DNA lesions; consequently, only a limited por-

tion of the induced primary DNA damage is assumed to lead to the serious damage represented by MN. The MN test detects unrepaired chromosome breaks while the comet assay detects strand breaks, alkali-liable sites, and delayed-repair sites that may or may not become repaired. A combination of MN test and comet assay enables comparison of the relative sensitivity of the two test systems [34]. A study in tadpole shows that IMI concentrations increased the frequency of primary DNA lesions estimated by comet assay. Additionally, the data revealed that the comet assay was more sensitive than the MN test in detecting early DNA damage when the same IMI concentrations were employed for tadpole exposure [35].

Pesticides can induce oxidative stress by generation of free radicals that interact with cellular membrane cause lipid peroxidation, alternations in membrane fluidity, DNA damage and finally carcinogenic effects [36]. For example, study indicates potential of IMI to develop oxidative stress and DNA damage in silkworms [37]. The significant increase in the lipid peroxidation can be possibly due to the reactive oxygen intermediates (ROS), which may lead to cell apoptosis [38]. Previous investigations have reported the induction lipid peroxidation by other pesticides such as endosulfan [39] and cypermethrin in fish [40].

The IMI has the potential to reach surface waters; it has been estimated to potentially reach such waters in concentrations up to 36 µg/L and has been detected in surface waters at concentrations up to 14 µg/L [41]. Higher concentrations can reach the water after application of this insecticide in crops of sugarcane and cause genotoxic damage to non-target organisms such as fish.

5. Conclusion

The results demonstrated that the concentrations tested in the comet assay induced primary damage to the DNA by increasing the frequency of strand breaks and alkali labile sites and increasing the frequency of MN at the highest concentration tested. Imidacloprid induced primary DNA damage at the concentrations tested and damage at the chromosomal level in the concentration of 250 µg/L. The results of this study help illustrate the potential ecological risk of IMI in aquatic environments and indirectly, to human health. The development of strategies for reduction in pesticide application and decreasing its impact on fish and other aquatic animals is necessary. This is a laboratory study previous to other field studies to be performed in areas where is applied this pesticide. While it is impossible to prevent the use of pesticides by humans, it would be beneficial to decrease the doses applied to farmland or to encourage the development of less toxic substances with similar effects that will enable future generations to live in healthier environments.

Acknowledgements

The authors thank the "Asociación Universitaria Iberoamericana de Postgrado" (AUIP) and the São Paulo Research Foundation (FAPESP, process 2012/50197-2) for financial support and American Journal Experts for providing language help.

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