In vivo Genotoxicity of the Pyrethroid Pesticide *β*-Cyfluthrin Using the Comet Assay in the Fish *Bryconamericus iheringii*

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Environmental pollution by pesticide residues is a major environmental concern due to the extensive use of these substances in agriculture. The insecticide *β*-cyfluthrin is a synthetic pyrethroid widely used in agricultural and other domestic activities. The aim of the present study was to assess the genotoxic effects of a sublethal exposure of the fish *Bryconamericus iheringii* (Characidae) to a commercial formulation of *β*-cyfluthrin using the comet assay. Fish were exposed to sublethal concentrations (4.2 and 5.6 μg/L) of *β*-cyfluthrin under static conditions during 24- and 48-h exposure periods. Fish in tap water were used as negative controls. Results obtained by the comet assay revealed genotoxic effects of the pyrethroid in the higher concentration and at the longer exposure period. The mean DNA damage index of fish exposed to 5.6 μg/L *β*-cyfluthrin for 48 h was significantly higher (145.9 ± 51.8) than in the control group (69.3 ± 39.5). These findings indicate that native fish species might be at risk for genotoxic damage in waters contaminated with *β*-cyfluthrin.

Key words: DNA Damage, Pesticides, *β*-Cyfluthrin

Introduction

Pesticides are extensively used all over the world, and environmental pollution by pesticide residues is a major environmental concern. In many situations, aquatic ecosystems form highly integrated parts of agricultural areas because they provide water and drainage facilities. Pesticide application techniques currently used for crop protection inevitably allow fractions of applied insecticides to enter aquatic ecosystems. Studies focusing on the detection of pesticides in aquatic environments have reported traces of these toxicants in various bodies of water, demonstrating that non-target species living in water catchments of agricultural areas are potentially at risk when they have similar toxicant receptors as the target organisms (Van Wijngaarden *et al.*, 2005).

Since DNA damage in aquatic animals may be associated with reduced growth, abnormal development, and decreased survival of embryos and adults (Lee and Steinert, 2003; Jha, 2008), genotoxic parameters are currently among the most valuable biomarkers for environmental risk assessment (Van der Oost *et al.*, 2003). In order to assess exposure to or effects of environmental pollutants on aquatic ecosystems, there is a suite of biomarkers which may be examined.

Fish have been used as convenient non-target aquatic bioindicator organisms for short-term aquatic genotoxicity assays (Dhawan *et al.*, 2009), since they can metabolize, concentrate, and store waterborne pollutants. Therefore, they can serve as a useful genetic model for the evaluation of pollution in aquatic ecosystems (Elliott *et al.*, 1988).

Among the tests used to investigate genotoxicity, the comet assay has found wide application as a simple and sensitive method for evaluating DNA damage in fish exposed to various xenobiotics in the aquatic environment (Dhawan *et al.*, 2009; Frenzilli *et al.*, 2009). According to Frenzilli *et al.* (2009), the comet assay is more sensitive than other biomarkers commonly used in genetic ecotoxicology.

Synthetic pyrethroids are a group of insecticides similar in structure to the pyrethrins. These compounds are generally recognized as potent

neurotoxicants, characterized by high insecticidal activity and low mammalian toxicity (Bradbury and Coats, 1989). *β*-Cyfluthrin, an important photostable synthetic fluorinated pyrethroid insecticide, is widely used in agriculture and as a household insecticide. It is one of the stereoisomers of the parent compound cyfluthrin (Vodeb and Petanovska-Ilievska, 2006).

Pyrethroids have been reported to be extremely toxic to fish (Sayeed *et al.*, 2003; Benli, 2005; Sepici-Dinçel *et al.*, 2009). Data on the genotoxic effects of synthetic pyrethroids are rather controversial, and different studies have reported different results depending on the test system or organism used in the experiments (Çavas and Ergene-Gözükara, 2003). Information on the genotoxic effects of pyrethroid insecticides on fish species is limited. However, some studies have demonstrated that synthetic pyrethroids are genotoxic to fish (Campana *et al.*, 1999; Çavas and Ergene-Gözükara, 2003; Simoniello *et al.*, 2009).

To date, no study concerning the genotoxic effects of *β*-cyfluthrin on fish has been reported in the literature. The present study aimed to assess the genotoxic effects of an acute and sublethal exposure to a commercial formulation of *β*-cyfluthrin using the comet assay in fish.

Material and Methods

Specimens of *Bryconamericus iheringii* (Characidae), measuring about 5 cm in length, were caught in the Feitoria River, in the city of Dois Irmãos, State of Rio Grande do Sul, Brazil. This species is appropriate for genotoxicity studies because it is a native, common, and not endangered fish species (Vari and Siebert, 1990). All animals were transported alive to the laboratory. The study was carried out according to the guidelines of the Feevale University's Committee for Ethics in Animal Research.

Healthy fish were placed in a 9-L aquarium, with well-oxygenated, dechlorinated tap water at room temperature, for a 48-h acclimatization period; they were then randomly divided into two groups of 20 specimens each and released into aquariums in which different concentrations of *β*-cyfluthrin were prepared. A commercial formulation of *β*-cyfluthrin (50 g/L, effective content 5%, trade name Turbo; Bayer CropScience LTDA, São Paulo, Brazil) was used in the experiments. Tests were conducted under static conditions using two sublethal concentrations of *β*-cyfluthrin:

4.2 and 5.6 μg/L. These concentrations were selected based on our previous investigations which demonstrated that 27.8 μg/L *β*-cyfluthrin is lethal to *B. iheringii* (unpublished data). In a third aquarium containing 10 individuals, a negative control test was performed using dechlorinated tap water. No food was supplied to the fish during the experiment. After 24 and 48 h of exposure, 10 individuals of each treatment group were killed, and blood samples were obtained for the comet assay. No fish died during the course of exposure.

The comet assay was performed on peripheral erythrocytes, according to Tice *et al.* (2000). Slides were precoated with normal-melting point agarose. A mixture of 5 μL of blood sample collected from caudal veins of fish with 95 μL of low-melting point agarose (0.7%) was added to the slide, which was immediately covered with a coverslip and then kept for 10 min in a refrigerator to solidify. After solidification of the gel, the coverslips were gently removed, and the slides were immersed into cold, freshly made lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10.2, to which 1% Triton X-100 and 10% DMSO had been added) and refrigerated at 4 °C for 1–24 h. After lysis, the slides were placed in a horizontal electrophoresis box side by side. The tank was filled with fresh electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) at 4 °C. The liquid covered the slides, which were then left in the solution for 20 min before the power was turned on. Electrophoresis was performed at 25 V and 300 mA (~ 0.95 V/cm) for 20 min. The steps above were carried out under red light to avoid induction of DNA damage. After electrophoresis, the slides were gently removed from the tank, and neutralizing buffer (0.4 M Tris, pH 7.5) was added to the slides dropwise three times, letting it sit for 5 min each time. The slides were rinsed three times with distilled water, air-dried for at least 24 h, and then fixed and silver-stained according to Nadin *et al.* (2001).

For evaluation of DNA damage, 100 cells per individual were analysed under an optical microscope at 400x magnification. All slides were coded and scored by a single observer. Cells were scored visually according to tail length into five categories, from undamaged (Type 0) to completely damaged (Type IV) (Anderson *et al.*, 1994). Based on the arbitrary values assigned to the different categories (from Type 0 ≙ 0 to Type IV ≙ 4), a genetic damage index was calculated for each fish

(Pitarque *et al.*, 1999). Therefore, the total score per individual ranged from 0 (all undamaged) to 400 (all maximally damaged).

Data were expressed as mean ± standard deviation. All analyses were carried out using the Statistical Package for the Social Sciences (SPSS) 15.0 for Windows. One-way analysis of variance (ANOVA) was used to determine the differences between treatment and control group means, considering a significance level of $p \leq 0.05$. The Tukey test was applied for post-hoc comparison.

Results and Discussion

The results obtained by the comet assay carried out on *B. iheringii* erythrocytes are shown in Table I. The Sample size was lower than 10 individuals due to loss of samples during the comet assay. Statistically significant differences between treatment and control groups were observed regarding the mean DNA damage index assessed by the comet assay ($p = 0.017$). Fish exposed to 4.2 *µ*g/L *β*-cyfluthrin during 24 and 48 h showed no difference from the control group, as well as fish treated with 5.6 *µ*g/L *β*-cyfluthrin during 24 h. However, the mean DNA damage index of fish exposed to 5.6 *µ*g/L *β*-cyfluthrin for 48 h was significantly higher (145.9 ± 51.8) than in the control group (69.3 ± 39.5).

Considering that information on the genotoxic effects of pyrethroid insecticides on fish species is limited, the aim of the present study was to assess the genotoxic effects of an acute and sublethal exposure to a commercial formulation of *β*-cyfluthrin using the comet assay. The main result

of the present study revealed that a 48-h exposure of *B. iheringii* specimens to 5.6 *µ*g/L *β*-cyfluthrin increased the DNA damage in fish erythrocytes. This result is in agreement with the other few studies which have evaluated the genotoxicity of pyrethroids in fish. Results obtained by Campana *et al.* (1999) demonstrated the genotoxic effects of lambda-cyhalothrin on erythrocytes of *Cheirodon interruptus interruptus*. Simoniello *et al.* (2009) reported that cypermethrin showed a significantly higher level of DNA damage in the fish species *Prochilodus lineatus* after *in vivo* exposure to different concentrations of cypermethrin.

Recent reports have demonstrated that pyrethroids stimulate the production of reactive oxygen species and produce oxidative damage to essential cell components. Sayeed *et al.* (2003) reported toxic effects on antioxidant/oxidant systems after exposure of green snakehead (*Channa punctatus*) to a sublethal dose of 0.75 *µ*g/L deltamethrin for 48 h. Sepici-Dinçel *et al.* (2009) concluded that exposure of carp (*Cyprinus carpio*) to a sublethal dose of cyfluthrin alters biochemical and physiological parameters and leads to histopathological changes in certain target tissues. Moreover, a potential oxidative stress-inducing effect of cyfluthrin, as lipid peroxidation, was suggested as a mechanism of action. Sadowska-Woda *et al.* (2010) demonstrated that *in vitro* administration of *β*-cyfluthrin resulted in induction of lipid peroxidation in human erythrocytes and significant changes in the antioxidant system, supporting that reactive oxygen species may be involved in the toxic effects of *β*-cyfluthrin. In this sense, one may speculate whether oxidative stress due to exposure to pyrethroids influences the cell physiology, leading to DNA damage observed in fish and other species. Further studies are warranted to address whether oxidative stress and DNA damage are correlated in fish exposed to pyrethroids.

Toxic effects of pyrethroids on non-target organisms have been reviewed and reported to be in the *µ*g/L toxicity range (Bradbury and Coats, 1989). In Brazil, the highest concentration of *β*-cyfluthrin recommended to be used in field crops is 12.5 mg/L. Although there are no available data on the occurrence of *β*-cyfluthrin in surface water, pesticide concentrations in water samples ranged from 0.005 *µ*g/L (Silva *et al.*, 2009) to 26.2 *µ*g/L (Marchesan *et al.*, 2010) in South Brazil. Thus, the genotoxic concentration detected in the present

Table I. DNA damage index estimated by the comet assay (mean ± standard deviation) in *B. iheringii* erythrocytes after different exposure times of the fish to two concentrations of *β*-cyfluthrin.

Treatment	n	DNA damage index
Control	9	69.3 ± 39.5[a]
4.2 *µ*g/L *β*-cyfluthrin		
24 h	7	101.7 ± 31.5[a,b]
48 h	8	118.7 ± 23.8[a,b]
5.6 *µ*g/L *β*-cyfluthrin		
24 h	10	102.1 ± 47.5[a,b]
48 h	9	145.9 ± 51.8[b]

Means followed by the same letters are not significantly different as determined by ANOVA followed by Tukey test.

study (5.6 µg/L) may be possibly found in the environment. Bradbury and Coats (1989) noted that fish are highly sensitive to pyrethrin and pyrethroid products and highlighted that contamination of lakes, streams, ponds, or any aquatic habitat should be avoided. Those authors are probably correct in their conclusion, considering that some studies indicate that fish erythrocytes may act as a surrogate tissue for detecting DNA strand breaks using the comet assay, which could be potentially used as a predictive tool for carcinogenic effects in fish (Jha, 2008).

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