Bull Environ Contam Toxicol (2009) 83:155–158
DOI 10.1007/s00128-009-9771-z

Alkaline Comet Assay for Genotoxic Effect Detection in Neotropical Fish *Prochilodus lineatus* (Pisces, Curimatidae)

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Received: 23 July 2008 / Accepted: 5 May 2009 / Published online: 23 May 2009

Abstract Toxicants on fish may induce genetic alterations that can be used as genotoxic markers. We evaluated DNA damage using alkaline comet assay applied on erythrocytes after in vivo exposure of *Prochilodus lineatus* to different concentrations of Cypermethrin (0.300, 0.150, 0.075 and 0.000 μg/L) as a probable chemical mutagen. The results revealed a significantly higher level of DNA damage at all concentrations of Cypermethrin tested compared to control and background level ($p < 0.05$). We have standardized the technique for one of the most common native fish species that will be useful for biomonitoring genotoxicity in polluted waters of the region.

Keywords Comet assay · *Prochilodus lineatus* · Genotoxic effects · Cypermethrin

The significant increase of chemical releases into water resources has lead to serious deleterious effects for aquatic organisms, besides direct and indirect hazards to human health. Many of such chemicals can induce, apart from death of exposed organisms, other effects such as genetic disorders and physiologic alterations. Some substances, when present in low concentrations, may not cause acute detectable effects in organisms, but may, in the long run, reduce their life span (Nehls and Segner 2001). In last years, loss of biodiversity, population reduction and destruction of habitats have been registered as a result of industrial and farming activities that use hydrographic systems as receiver or vehicle of their wastes. Biocides are extensively used in the agricultural zone of the Paraná River basin in Argentina. Synthetic pyrethroid pesticides account for over 30% of the global pesticide use. Among them, Cypermethrin, single or in combination with other insecticides, is the most widely used to control many pests, including pests of cotton, fruit and vegetable crops. Nevertheless, there is very scarce information about the injurious actions of this compound in aquatic Neotropical organisms.

Ecotoxicology is an inter and multidisciplinary science combining the fields of ecology, chemistry and toxicology. In ecotoxicological studies, it is therefore important not only to link the fate and behaviour of the contaminants in the environment with the observed biological responses and potential ecological consequences, but also how different toxicological responses are expressed and linked to each other. In other words, it is important to dissect out the levels of toxicological responses before they could be linked to potential ecological consequences (Jha 2008). In recent years, there has been an increasing interest in the effects of toxicants on fish health due to the importance of fishing in rivers, ponds and estuaries exposed to wastes of the productive activity. Chemical contamination of water may affect molecular mechanisms of fish and induce genetic alterations that can be used as markers of DNA alterations in environmental pollution (Jha 2008). The estimation of early signs of danger using biomarkers in an ecosystem provides useful information

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for environmental biomonitoring and allows the development of control strategies and prevention measures (Markert et al. 2003).

The utilization of fishes as bio-indicators of pollutant effects is being more and more used, since such practice can help to detect possible environment problems. Results obtained in assays carried out with fishes can be useful for the evaluation of environmental presence of substances potentially teratogenic and carcinogenic in human beings (Matsumoto et al. 2006).

A variety of methods have been developed for detecting DNA damage. Genotoxic compounds may induce strand breaks directly or indirectly through the interaction with oxygen radicals or other reactive intermediates, or as a consequence of excision repair enzymes activity. If the damage produced is of high level, it can finally lead to cell apoptosis or necrosis. Generally, the alkaline (pH > 13) version of the comet assay (Singh et al. 1988) is recommended because it detects a broad spectrum of DNA lesions and has a very high sensitivity, measuring the migration of DNA from immobilized nuclear DNA. Under these conditions, DNA double and single strand breaks, and alkali-labile sites lead to increased DNA migration. Advantages of the comet assay for assessing DNA damage in aquatic animals include: (1) damage to the DNA in individual cells is measured; (2) only small number of cells are needed to carry out the assay (<10,000); (3) the assay can be performed on virtually any eukaryotic cell type; (4) it is a very sensitive method for detecting DNA damage. According to several authors (Vanzella et al. 2007; Ali et al. 2008; Cavalcante et al. 2008; Frenzilli et al. 2009), the comet assay has been successfully applied in erythrocytes of many fish species exposed to different genotoxic agents, allowing the evaluation of DNA alterations induced by xenobiotics. Among the advantages of this technique, it can also be mentioned that the size and number of chromosomes are not important and that mitotic activity is not required. The latter is especially important in fishes because the metabolic rate and metabolic index fluctuate considerably with temperature and thus mitotically active tissue is difficult to isolate.

The species *Prochilodus lineatus* is a widely distributed Neotropical fish, and represents 60% of the total ichthyomass of the Paraná River. Therefore, it could be considered a good species for the monitoring of aquatic environments (Parma et al. 2007).

In the present study, we evaluated genotoxic effects with the alkaline comet assay in *P. lineatus* exposed in vivo to sublethal concentrations of Cypermethrin. The information generated here will contribute to the utilization of this species for genotoxic biomonitoring in polluted water.

Materials and Methods

The specimens were obtained from a pristine environment next to Santa Fe city (Argentina) at 31° 39′ 36″S and 60° 35′ 26″W, they were transported to the bioassay laboratory in oxygenated recipients, and were acclimated for 24 h. A total of 60 juvenile specimens (mean weight: 41.7 ± 21.7 g, and length: 113.31 ± 19.49 mm), were used to carry out the experiment. Containers of 25 L were placed in an acclimatized laboratory with a constant temperature of 25°C, photoperiod of 12:12 h, and oxygen concentration between 5.7 and 6.8 mg/L.

For the experiment, 45 specimens (15 in each of 3 concentrations) were exposed to Cypermethrin "SHERPA O" (commercial formula: 25 g of Cypermethrin as active principle) as a probable chemical mutagen. They were exposed to the following sublethal concentrations during 96 h: 0.300, 0.150 and 0.075 μg/L of Cypermethrin. (Rodriguez-Cea et al. 2003; Parma et al. 2007). Another 15 specimens were used as intra-assay control without chemical.

Mortality, immobility and behavioural alterations in fish were registered every 24 h. Blood of all specimens was collected by the method of dissection of the caudal peduncle (Houston 1990) using heparinised micropipettes. Before running comet assay, cell viability was determined using the Trypan Blue exclusion method.

Fish erythrocytes were used to develop alkaline comet assay according to Singh et al. (1988), with some modifications necessary for it application in this cell population. Blood samples were diluted 1:200 v/v with RPMI 1640 and immediately used. In brief, 2 μL of each diluted blood sample were added to 100 μL of 0.5% (w/v) low melting point agarose (LMA) and deposited on microscope slides with normal melting point agarose (NMA), by duplicate, including negative and positive (H_2O_2 25 μM) controls. Finally, a third layer of 100 μL of LMA was pipetted out on the slide and allowed to gel at 4°C for 10 min. Afterwards, slides were immersed in freshly prepared, cold lysis solution (0.40 mL of Triton X-100, 5 mL of DMSO and 40 mL of stock lysis solution with 2.5 M NaCl, 100 mM EDTA, and 10 mM Tris, adjust to pH 10) and kept overnight at 4°C. After lysis, the slides were immersed in alkaline buffer (0.3 N NaOH, 1 mM EDTA, pH > 13) during 10 min for DNA unwinding, and electrophoresed in the same buffer. Electrophoresis conditions were: 10 min at 300 mA and 20 V (0.7 V/cm).

Then, slides were neutralised in 0.4 M Tris buffer (pH 7.5) with three changes of 5 min each, dehydratated in methanol for 5 min and left to dry at room temperature. The slides were stained with ethidium bromide (0.02 μg/ml).

Comet images were observed using a fluorescent microscope (Olympus CX-40) equipped with an excitation filter (Olympus U-RFLT 50). A total of 200 cells were counted in two replicated slides per sample to characterize DNA damage. The movements of DNA into the comet tail are facilitated by DNA strand breaks and relaxation of DNA supercoiling. The more damaged the DNA (i.e. the greater number of strand breaks) the longer comet tails and the greater DNA content in the tail. Comets were analyzed visually on a scale of 0–4 (categories depending on DNA damage level). The overall score, between 100 and 400 arbitrary units, is related to the DNA break frequency and a comet-like image indicates the presence of DNA breaks (Rodriguez Ferreiro et al. 2002). Comets with over 50% of DNA material in their tail and no detectable nuclei were classified as 'clouds' and not scored. Damage index comet assay (DICA) was calculated for each sample. Variations in background levels of DNA damage were analysed previously in erythrocytes of 40 specimens selected (length 119.53 ± 18.43 mm, weight 49.6 ± 20.3 g).

For statistical analysis, Kruskal–Wallis and Mann Whitney *U*-test were used to compare differences in DICA between control and exposed fishes to Cypermethrin, using SPSS 11.5 for Windows Version Software.

Results and Discussion

No fish mortality was observed during the test. Results of cell viability, obtained by the Trypan Blue dye exclusion method, were always in the range of 90%–95% for all samples as parallel tests. These results demonstrate that exposure to Cypermethrin and isolation process do not produce cell cytotoxicity.

DICA in *P. lineatus* erythrocytes showed significant differences ($p < 0.05$) between exposed groups and controls at all tested concentrations. Data is presented as mean ± SD of treatments. DICA at different concentrations of Cypermethrin were: 102.20 ± 3.10, 115.33 ± 15.94, 119.00 ± 10.74, and 202.87 ± 35.84 at 0.000 (control), 0.075, 0.150, and 0.300 µg/L, respectively (Fig. 1). Background DICA was 103.40 ± 9.09. The present results verified the genotoxicity of Cypermethrin at all concentrations tested.

In relation to the application of the comet assay in *P. lineatus* as a bio-indicator, our results corroborate the researches developed by another authors (de Campos Ventura et al. 2008), which showed that the comet assay in fish erythrocytes seems to be efficient to detect the genotoxicity of chemicals. In Fig. 2, damage is represented by an increase in DNA fragments that have migrated out of the cell nucleus, which form a distinctive comet tail. The tail length and DNA fragment contented in it are directly proportional to the amount of DNA damage.

Comet assay has proved to be a useful tool for measuring the relationship between DNA damage and exposure of aquatic organisms to genotoxic pollutants (de Andrade et al. 2004), being considered more sensitive than cytogenetic techniques to detect DNA damage. The evaluation of DNA damage in fish using the comet assay frequently involves the utilization of erythrocytes because of their ready availability and ease of collection.

Interaction of genotoxic agents with DNA can form alkaline labile adducts and other modifications, which can contribute to an increase level of DNA strand breaks via enzymatic removal of damaged nucleotides. The DNA fragmentation or DNA strand breaks are considered a kind of lesion potentially pre-mutagenic, with the production of breaks in DNA strands being related to mutagenic and carcinogenic properties of chemicals (de Campos Ventura et al. 2008). If not repaired, these DNA lesions can initiate a cascade of biological consequences at cellular, organic,

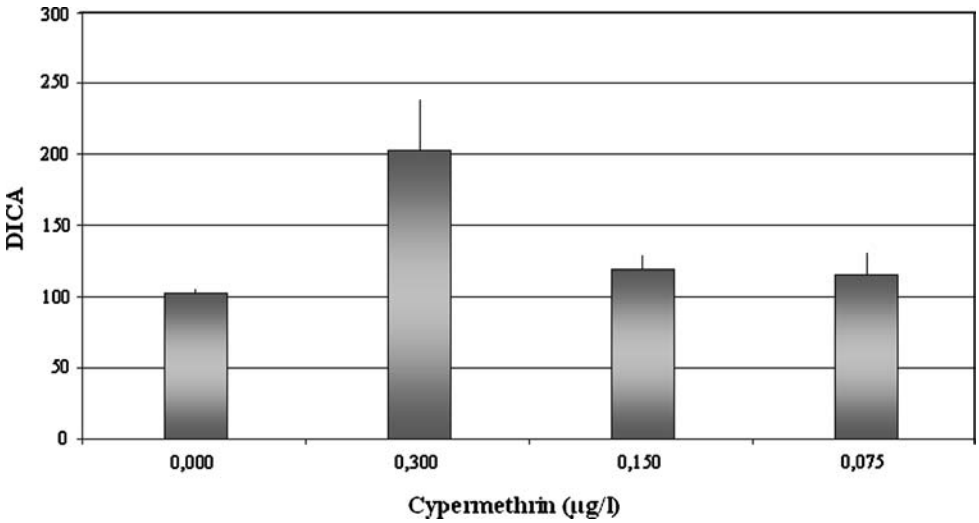

Fig. 1 Damage index comet assay (DICA) in fish exposed to different concentrations of Cypermethrin and control

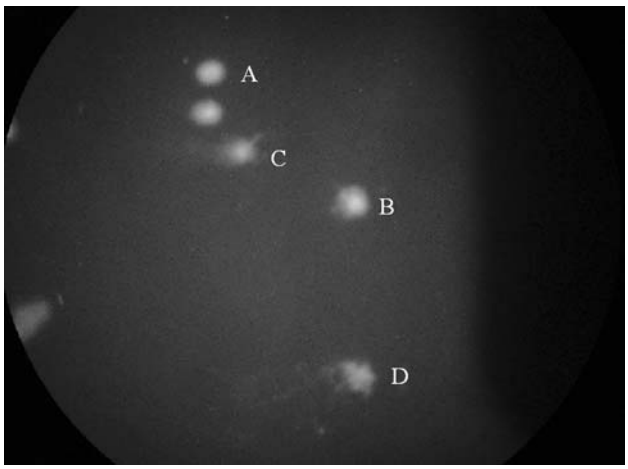

Fig. 2 Comet assay in erythrocytes of *Prochilodus lineatus*. **A** Class 0 (undamaged); **B** class 1 (slightly damaged); **C** class 2 (medium damaged); **D** class 3 (highly damaged)

individual, and finally at population and community levels. DNA damage in a variety of aquatic animals has been associated with reduced growth, abnormal development and reduced survival of embryos, larvae and adults.

Different studies (Nacci et al. 1996; Frenzilli et al. 2009) confirm that environmental contaminants can affect the genetic material of wildlife species and several mechanisms have been proposed to link these exposures with DNA strand breakage and repair. The significance of malignancy incidence in wild species, however, needs to be considered in the light of emerging scientific priorities, where humans are seen as part of the ecosystem. In this context, it has also emerged that increasing pollution could lead to higher incidence of cancer in the human population and concurrently, contribute to loss of biodiversity, the main aim of ecotoxicological studies (Jha 2008).

In the present study, we have standardized the comet assay for one of the most common native fish species of Neotropical region. In view of the results obtained in this work we conclude that the comet assay is an effective short-term test for in vivo monitoring of genotoxic agent effects in aquatic species and that *P. lineatus* is a promising sentinel organism for the evaluation of substances potentially mutagenic, teratogenic and carcinogenic in aquatic environments.

Acknowledgments The work was supported by research grant PI CAI + D N° 21/123 2006 from Universidad Nacional del Litoral, Argentina.

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