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Endosulfan-Induced Genotoxicity Detected in the Gilthead Seabream, *Sparus aurata* L., by Means of Flow Cytometry and Micronuclei Assays

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The relevance of the application of genotoxicity biomarkers in marine environmental monitoring has been increasingly recognized (Forbes 1998; Moustacchi 2000). The maintenance of DNA integrity is vital to the protection of genetic diversity in natural populations (Bickham et al. 2000). Additionally, the detection of structural/functional disturbances in the DNA enables the assessment of organism health and can assist in the prevention of the proliferation of DNA damage in the food chain and humans (Handy et al. 2001). Therefore, considering the risks associated with genetic damage, the need to develop, implement and standardize biomarkers of genotoxicity is of particular importance.

The study reported here is part of an investigation to examine and select biomarkers for monitoring genotoxicity in sentinel fish species of the Portuguese coast. Here we describe the leading steps to develop and test, under controlled laboratory conditions, the effectiveness and sensitivity of a set of chromosomal damage biomarkers using the gilthead seabream, *Sparus aurata,* as the model organism. *S. aurata* is a marine teleost widespread in Atlantic and Mediterranean coastal waters, and one of the most important commercial fish species in southern Europe.

Endosulfan, a chlorinated hydrocarbon pesticide, is among the most toxic pesticides for aquatic life, especially fish, and has been registered as a priority pollutant by the US Environmental Protection Agency (Cengiz and Ünlü 2002). It is used to control a wide range of insects in agricultural regions around the world (EPA 1980; Cengiz and Ünlü 2002). The exposure of aquatic organisms to low levels of endosulfan, has been reported to be extremely toxic to several fish and crustacean species, and capable of inducing various biochemical, physiological and histological alterations in vital cells (Bhavan and Geraldine 2000; Cengiz and Ünlü 2002). Endosulfan was found to modify the concentrations of several enzymes (acetylcholinesterase, phosphatases, glutathione S-transferase), impair growth and/or reproduction, and induce severe histopathological lesions (epithelial necrosis, lamellae hemorrhage and aneurisms, sloughing of the respiratory epithelium) in either fish or crustaceans species (Bhavan and Geraldine 2000; Cengiz and Ünlü 2002). However, there is a paucity of literature in relation to the genotoxic responses induced by endosulfan in aquatic organisms.

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Juvenile *S. aurata* were exposed to sub-lethal and environmentally realistic seawater concentrations of endosulfan (0, 0.1, 0.5 and 1.0 μgL^{-1}) for four days (96 hours). Genotoxicity was determined by analyses of erythrocytic micronuclei (EMN), erythrocytic nuclear abnormalities (ENA) and nuclear DNA content variation, in blood cells of *S. aurata*. In Portugal, this pesticide has been applied in rice cultures surrounding estuaries (Cerejeira et al. 1999) and therefore several fish, including juvenile *S. aurata* that use estuaries as nursing areas, are potential victims of endosulfan contamination.

MATERIALS AND METHODS

All chemicals used were supplied by Sigma-Aldridge. Citric acid (CAS Nº 6132-04-3, purity ≥ 98%), Dimethyl sulfoxide (CAS No 67-68-5, purity 99.9%), Igepal CA-630 (CAS Nº 9036-19-5, purity 99%), Propidium iodide (CAS Nº 25535-16-4, purity 95%), Potassium phosphate (CAS Nº 7778-8-770, purity 99%), Rnase A (CAS Nº 9001-99-4, purity > 90%) and Sucrose (CAS Nº 57-50-1, purity 99%).

Juvenile *S. aurata* weighing 0.7-1.5g were supplied by the "Ria Mãe" fish farm (Setúbal, Portugal). Fish were acclimated to controlled laboratory conditions in 60 L tanks with recirculating filtered seawater, for two weeks before the experiment began. Fish were exposed for 96 hours to five treatments: seawater control, solvent control (acetone) and three doses of endosulfan (0.1, 0.5 and 1.0 μgL^{-1} – nominal concentrations). These sub-lethal concentrations were selected according to preliminary acute toxicity tests. The experiment took place in beakers with 2 L of exposure volume containing 4 animals each; every treatment was carried out with 12 animals. The temperature and salinity of the assay were maintained at 20ºC and 33-34‰ and aeration was provided continuously. All of the test media in each aquarium was replaced at 24, 48 and 72 hours and the animals were not fed during the experiment. Survival was high and did not differ from the control (seawater control) in any of the endosulfan or solvent control treatments. At the end of 96 hours, blood was collected from all surviving individuals by cutting the caudal peduncle. Blood was stored in 1.5 mL microtubes with 800 µL of freezing buffer (250 mL H_2O/ 21.2 g sucrose, 2.9 g citric acid and 12.5 mL dimethyl sulfoxide; pH 7.6 - Collares-Pereira and Moreira da Costa 1999) for flow cytometry analysis and immediately frozen and stored at −80ºC until analysis. Blood smears were also prepared for the micronuclei assay (EMN and ENA frequencies).

Slides containing the blood smears from all individuals of each treatment were stained with Giemsa/phosphate buffer pH 6.8 (1:20) and mounted with Eukitt. For each slide 1000 mature erythrocytes were examined and the number of EMN and ENA were scored under 1000x magnification. The frequencies of EMN and ENA were expressed per 1000 cells (‰). The criteria for identification of EMN and ENA followed the procedures described by Carrasco et al. (1990) and Gravato and Santos (2002). Briefly, EMN were scored if nuclear structures were observed in the cytoplasm with the following characteristics: no refractory fragments of the same color as the main nucleus and with a round or ovoid shape about one-fifth of

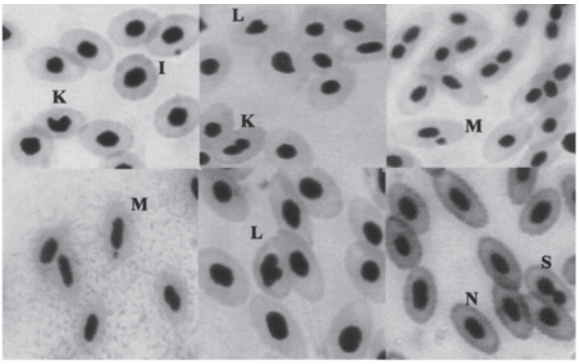

Figure 1. Erythrocytes of *Sparus aurata* with normal nuclear shape and nuclear alterations: N = normal shaped nucleus, M = micronucleus, L = lobed nucleus, K = kidney-shaped nucleus and S = segmented nucleus. The immature erythrocytes (I) were not scored. Giemsa stain, 1000 x.

A flow cytometry assay was used to measure the nuclear DNA content variation among the blood cells of each individual. The blood cell suspensions stored at −80 ℃ were thawed and a 100 μL aliquot was stained in the dark, with 200 μL of fluorescent dye (500 mL H_2O/500 mg Citric acid, 500 μL Igepal CA- 630, 25mg Propidium Iodide and 25mg RNAses A, pH 7.6). The quantification of nuclear fluorescence was carried out in a flow cytometer (Coulter Epics Profile II). Fluorescence was measured from 10,000 blood cells per individual and the nuclear DNA content variation was calculated by the coefficient of variation (CV) of the mean G1 stage cells.

Genotoxic effects of endosulfan were analyzed using one-way ANOVA separately for each dependent variable (CV, EMN and ENA). Post-hoc comparisons were carried out using the Least Significant Difference (LSD) test for multiple comparisons between pairs of means.

RESULTS AND DISCUSSION

The frequencies of EMN and ENA and the results of the flow cytometry assay as a function of endosulfan concentrations are presented in Figures 2 and 3, respectively. In all the assays no significant differences were detected between seawater and solvent controls. It was observed that EMN and ENA frequencies increased in a dose-dependent manner, and the two highest endosulfan concentrations (0.5 and 1 μgL^{-1}) were significantly higher relative to controls ($p < 0.05$ for EMN and $p < 0.01$ for ENA).

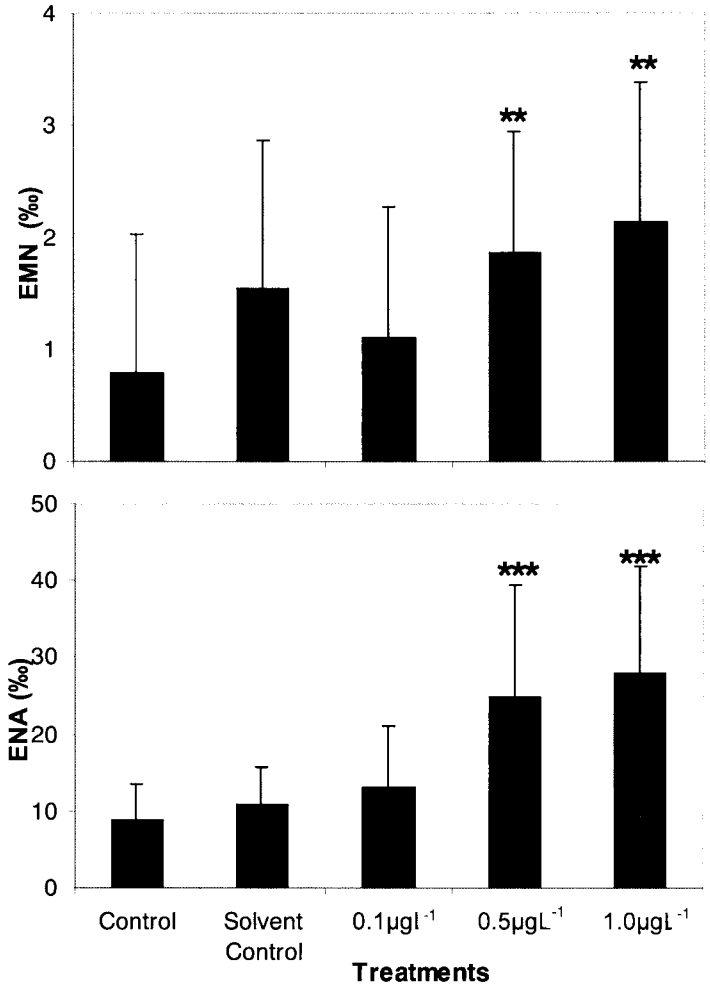

Figure 2. a) erythrocytic micronuclei (EMN) and b) erythrocytic nuclear abnormalities (ENA) frequencies in *Sparus aurata* as a function of endosulfan concentrations. Bars and error bars represent means and standard deviations. Asterisks indicate significant difference relative to control (seawater control): ** = $p<0.05$; *** = $p<0.01$.

The frequency of ENA followed a similar pattern of EMN and exhibited more sensitivity. Therefore, our results are in agreement with other studies that indicate the potential of micronuclei and nuclear abnormalities to screen for genotoxic effects of environmental contaminants. In the last decade, the detection of nuclear abnormalities, in addition to micronuclei, have been reported in aquatic organisms, such as bivalves and fish, after exposure to numerous genotoxicants.

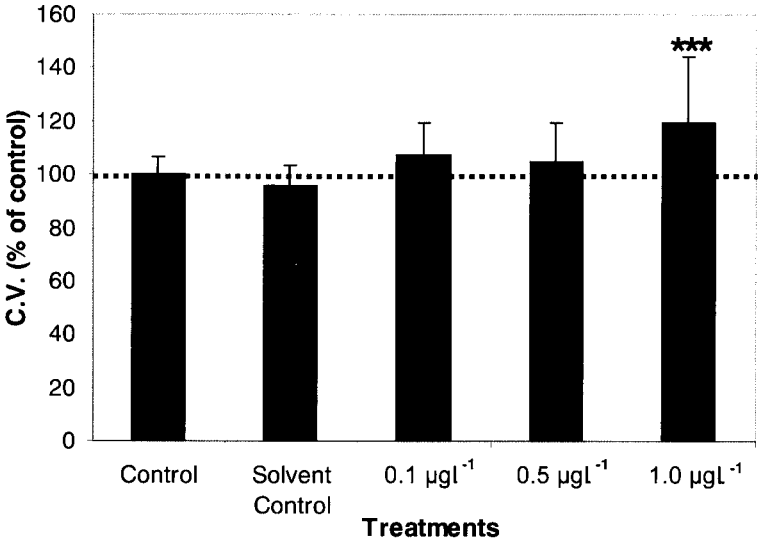

Figure 3. Nuclear DNA content variation (CV) in *Sparus aurata* blood cells as a function of endosulfan concentrations. All results are expressed as a percentage of control CVs. Bars and error bars represent means and standard deviations. Asterisks indicate a significant difference relative to the control (seawater control) samples: *** = $p<0.01$.

Several authors (Pacheco and Santos 1997; Ayllón and Garcia-Vasquez 2001; Gravato and Santos 2002) have described various alterations of nuclear morphology, similar to the nuclear abnormalities observed in this study, (lobed nuclei, kidney-shaped nuclei and segmented nuclei) and interpreted them as nuclear lesions analogous to micronuclei. However, more research is needed to understand the origin of these nuclear abnormalities and for standardizing the use of this biomarker, before including it in routine environmental studies.

For the flow cytometry assay, increased levels of nuclear DNA content variation (coefficient of variation, CV) were registered in response to endosulfan exposure in all treatments. However, only the CVs from the highest endosulfan concentration (1 μgL^{-1}) were significantly elevated over the seawater control (19 %, $p< 0.01$). The flow cytometry assay has been proposed to assess mutagenicity in organisms exposed to genotoxicants (Custer et al. 1994; Misra and Easton 1999; Custer et al. 2000). It has been recognized as a precise automated method, where genotoxic effects are related to an increase in cell-to-cell variation of DNA content resulting from unequal amounts of DNA being distributed to daughter cells when fragmented or acentric chromosomes are produced in the mother cell (Bickham 1990). In this study, flow cytometry successfully detected genotoxic effects of endosulfan in *S. aurata*, and therefore has potential to be more frequently used in environmental studies.

The results obtained in this study revealed the effectiveness of EMN, ENA and flow cytometry assays to diagnose genotoxic effect of endosulfan in *S. aurata*. Endosulfan concentrations as low as 1.0 μgL^{-1} or even 0.5 μgL^{-1} can be genotoxic to our model organism, producing significant chromosomal damage. On the whole, the findings of this study reveal the mutagenic potential of endosulfan and illustrate the genotoxic risks associated with endosulfan-contaminated runoff from agriculture areas.

The resources involved in the analysis of these biomarkers vary considerably and should be considered when selecting genotoxicity biomarkers for monitoring programs. Flow cytometry is a rapid procedure for assessing chromosomal damage, which requires expensive equipment and demands a high level of technical expertise. On the other hand, the micronuclei assay (EMN and ENA) is a simple and much less expensive technique. However, it requires the manual examination of many cells per slide, it is very time consuming, and should be performed by the same operator to prevent divergent interpretations.

According to He et al. (2000), evidence suggests that some chromosome abnormalities are a direct consequence or manifestation of damage at the DNA level. Therefore, as further research, it will be useful to combine measurements of DNA damage and chromosomal damage biomarkers in the same study to improve our understanding of the mechanisms underlying genotoxicity. For example, the comparison of the extent of DNA damage, such as DNA strand breakage, and the frequency of micronuclei or nuclear DNA content variation will allow one to estimate the amount of DNA strand breakage that may result in chromosomal damage.

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