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Measurement of Genotoxic Endpoints in Earthworms Exposed to Radioactive Wastes from an Abandoned Uranium Mine

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Abstract—The main objective of this study was to assess the sensitivity of genotoxic endpoints, to be considered in earthworm's reproduction assays, performed under the evaluation of soils contaminated with radioactive wastes. Earthworms were exposed, under laboratorial conditions, to a contaminated soil from the Cunha Baixa uranium mine and to LUFA 2.2 soil (control soil). Earthworms (*Eisenia andrei* Bouché) were sampled from assay chambers after 0 h, 24 h, 48 h, 7 days, 14 days and 56 days of exposure. Some simple techniques like comet assay and flow cytometry were used to assess DNA damages and cytotoxicity in earthworm's coelomocytes. Higher DNA damage was recorded in earthworms exposed to the contaminated soil when compared to the control. Flow cytometry revealed that earthworm's coelomocytes were affected by the exposure to radioactive wastes, by causing depletion in cells responsible for the effector functions of the immune system of these organisms. These results suggest that DNA damages and cytotoxicity assessed by both techniques are sensitive endpoints for environmental risk assessment of radiological contaminated sites.

Keywords: *Eisenia andrei*, comet assay, uranium mine, coelomocytes, flow cytometry, cytotoxicity

INTRODUCTION

The legacies of past uranium mining and milling activities still are a cause of concern requiring assessment and the implementation of remedial actions. However, in the last few years, attention has been focused on impacts on public health and on the wider natural environment promoted by the full range of operational activities related to uranium mining and the rest of the nuclear fuel cycle (IAEA, 2005). In the case of the natural environment, these concerns include the risk of environmental degradation, contamination, reduced ecosystem viability and biodiversity, aesthetics, public amenities, access to land, and

quarantining of land for future beneficial land use (IAEA, 2005). Abandoned or inactive mines are of particular concern and require continuous control and monitorization (Santos Oliveira and Ávila, 2001; Carvalho *et al.*, 2009a, b). The determination of the risks of such hazards is usually carried out through chemical analysis of environmental samples, neglecting the assessment of biological effects (Antunes *et al.*, 2008a). Among soil organisms, earthworms, are considered to be of particular interest, as they are either directly or indirectly, important agents in modulating the transfer of inorganic and organic toxicants due to their presence in contaminated soils (Cooke *et al.*, 1992), and for many years they have been considered an interesting biological indicator of many metals in soils (Suthar *et al.*, 2008). Moreover, the impact of earthworms on soil properties and plant growth has been widely documented during the last two decades. From these studies abundant evidence has emerged that earthworms are powerful regulators of soil processes, participating in the maintenance of soil structure and regulation of soil organic matter dynamics (Lavelle *et al.*, 1997).

The bioaccumulation of radioactive substances and heavy metals is responsible for cellular and genetic damages that can seriously affect human health, as well as other important groups of aquatic and terrestrial organisms. Regarding uranium radiotoxic and chemotoxic properties, new endpoints to evaluate cellular and DNA damages seem to be highly relevant (Svendsen *et al.*, 2004; Barillet *et al.*, 2005), as uranium acts on the formation of oxidative DNA damages; this is due to the redox chemistry of transition metals (such as uranium), which activates oxygen species in the course of redox reactions, and increase the production of free radicals due to the emission of alpha particles (Barillet *et al.*, 2005). Thus, there is a need to understand the interactions between uranium and living organisms in order to provide useful tools for the prediction of possible genotoxic and citotoxic effects of environmental exposure.

The study of coelomocytes in earthworms is relevant, because these leukocytes, located around the intestinal tract, are particularly exposed to soil pollutants and are involved in the process of cell immunity (Dhainaut and Scaps, 2001; Manerikar *et al.*, 2008).

The present study was conducted to investigate the possibility of adding genotoxicity and cytotoxicity biomarkers to the list of endpoints presently used to monitor the soil environment. Among the molecular compounds of the cell, DNA is an important target of environmental stress in both aquatic and terrestrial organisms (Frenzilli *et al.*, 2001). In this experiment, animals were subjected to a laboratory exposure of 56 days to a contaminated soil from the Cunha Baixa uranium mine, previously described in Pereira *et al.* (2008) and a standard reference soil LUFA 2.2. During that period, organisms were sampled in order to assess biomarkers (described above).

MATERIALS AND METHODS

Soils tested

For this study, two soils were selected; the standard natural soil LUFA 2.2

(control soil) and a contaminated soil from the Cunha Baixa uranium mine. In this mine, the soil contamination results from the deposition of mine tailings and sludge from the effluent treatment pond and from runoffs from the aquatic system (Antunes *et al*., 2008b). The soil selected for the experiment soil is composed by sludge from the treatment pond which is highly contaminated with heavy metals and radionuclides (Pereira *et al*., 2008).

After discarding the superficial layer (plant debris and humus), the first 20 cm of soil were collected and sieved to discard the >2 mm fraction. LUFA 2.2 was purchased from LUFA Speyer. Prior to the test, pH of both soils was measured and the Water Hold Capacity (WHC) was adjusted to 40%.

Test organisms

The specimens of *Eisenia andrei* used in this study were obtained from laboratory-reared batch cultures under controlled environment (temperature 20 ± 2°C; photoperiod 16^{L}:8^{D}). Earthworms were selected according to international standard guidelines (ISO, 11268-2: 1998; OECD, 2004) regarding weight and maturation state, only adult earthworms with clitellum and individual mass between 250 and 600 mg were used. Before exposure, the organisms were acclimatized for 24 h in containers with LUFA 2.2 (control soil).

Laboratory exposure

Organisms were exposed to the soils in test chambers, with lids bearing one opening at the top (for ventilation) (Antunes *et al*., 2008a). Prior to the test, these chambers were filled with 500 g of each soil tested. A static design was employed, using 685 animals. This experiment was carried out for 56 days, during which we sacrificed animals in 6 exposure periods: 0 h (before exposure), 1 day, 2 days, 7 days, 14 days and 56 days of exposure. For each exposure period and soil tested, 5 pools of 3 animals were used for comet assay and flow cytometry analyses.

During the experiment, organisms were fed, once a week, with 5 g per test chamber of dried horse manure, previously dried at 105°C and finely ground.

The experiment was carried out under a controlled environment (temperature 20 ± 2°C; photoperiod 16^{L}:8^{D}).

Coelomocytes extrusion

Earthworm coelomocytes were obtained using the modified protocol of Reinecke *et al*. (2004). The animals were exposed in microtubes, to an irritating extrusion fluid to which they react strongly by secreting coelomic fluid containing the coelomocytes. After the cells extrusion, the worms were removed from the microtubes, washed with distilled water and placed back into the substrate. The extrusion fluid containing cells was centrifuged and the supernatant removed. The cell pellet was suspended and washed three times in PBS, using microcentrifugation, for 3 min at 380 g.

The same cell suspensions were used either for comet assay and flow cytometry analysis.

Comet assay

The comet assay was conducted under yellow light, to prevent UV-induced DNA damage, and performed as described by Nogueira *et al.* (2006), with a few minor modifications: normal microscope slides, not fully frosted slides, were used; the slides, were covered with the first agarose layer and left to dry to enable the adherence of the gel layer to the slides; only two layers of agarose were used (the first dried layer and the layer with the cells).

Visual scoring of cellular DNA on each slide was based on the categorization of 100 randomly-selected cells. The comet-like formations were visually graded into five classes, depending on DNA damage level and were adapted from García *et al.* (2004): undamaged—no tail visible (class 0) low damage—tails with low fluorescence and head still round and bright (class 1) medium damage—head and tail equally bright (class 2) high damage—small head, and a long and very bright tail (class 3) extreme damage—very long tail, while head is no longer round (class 4).

Flow cytometry

The flow-cytometric analyses were performed on a FACSCanto II (BD Biosciences, Erembodegem, Belgium). Cells were incubated with 2 μl of Draq V for 30 minutes, at room temperature in the dark. During analytical experiments, 50,000 threshold events per worm sample, were collected and analyzed on the basis of their size and complexity. The resulting files were analysed using Infinicyt 1.2 software (Cytognos).

Statistical analysis

Data will be compared using two-way ANOVAs (with exposure time and concentration as factors), followed by Dunnett and Tukey multi-comparison tests (when applicable), to discriminate significant differences between exposed and unexposed (control) animals. The significance level in all analyses will be 0.05.

RESULTS AND DISCUSSION

Here we present preliminary and qualitative data that was so far obtained. These data still need to be fully analyzed to allow deeper conclusions. However, a preliminary analysis of the results clearly shows the tendencies of the results that are expected to be obtained.

The exposure to the uranium mine soil seem to have caused significant DNA damages after 24 h, 7 days and 56 days of exposure compared with the control (data not shown). The increase in DNA damages was probably caused by the production and intracellular accumulation of ROS, induced by the exposure of earthworms to uranium and its daughter radionuclides, present in the uranium mine soil (Barillet *et al.*, 2005). Moreover, some researchers (Hamilton *et al.*, 1997; Miller *et al.*, 2002) support the existence of two possible molecular mechanisms that could result in uranium chemically induced strand breaks: (1) indirectly by free-radical generation (Fenton-type reaction) or (2) through direct

interaction (DNA hydrolysis). Therefore, the evaluation of metal sublethal toxicity should always include biomarkers of DNA damage because such damage may result in inappropriate gene expression and, subsequently, in more concerning genotoxic and mutagenic effects (Hartsock *et al*., 2007). Nevertheless, an apparent decrease in DNA damages was observed after 48 h and 14 days of exposure (data not shown). This may be due to the repair of the DNA damages by the cells, however, metal ions interfere with distinct steps of diverse DNA repair systems, such as base excision repair and nucleotide excision repair, which are important processes of maintaining DNA integrity (Hartwig and Schwerdtle, 2002). Because DNA damage recognition requires the preferential binding of one or more proteins to damaged DNA compared with undamaged DNA, potential interactions of carcinogenic metal compounds with these proteins may compromise the processes, thus diminishing DNA repair (Hartwig and Schwerdtle, 2002). Nevertheless, there are other possible explanations for this decrease in DNA damage like, for example, an elimination of the severely damaged cells by apoptosis, which is not likely to be detected by this version of comet assay (Hartmann *et al*., 2003; Collins, 2004; Hartmann *et al*., 2004: Collins *et al*., 2008) or a possible cell turnover.

Regarding flow cytometry analysis, we could observe that after 24 h of exposure, there seem to be a significant increase in number of cells (data not shown) that participate in various vital processes as metabolic and regulatory functions, ensuring proper functioning of the whole organism (Affar *et al*., 1998; Adamowicz, 2005), followed by a progressive decrease trough time (unpublished data). Despite this decrease in the organisms exposed to the contaminated soil, the frequency of these cells is apparently greater in the control organisms. The increase in the frequency of these cells may be a response of the organisms to the environmental stress, to which they were subjected. Since the exposure to metals can cause perturbations in growth, reproduction and cocoon production (Reinecke *et al*., 2001; Homa *et al*., 2003), the release of these cells in the celoma may be an attempt to cope with this stress and ensure the organism's survival. However, looking at cells with effector functions like phagocytosis, encapsulation, nodulation and humoral immune responses (Bilej *et al*., 2000; Adamowicz and Wojtaszek, 2001; Adamowicz, 2005), we recorded a lower frequency of these cells (data not shown) in the organisms exposed to the contaminated soil, comparing with control organisms, in all exposure periods. This may be due to the fact that, the exposure to heavy metals can compromise immune cell viability and effector functions (Burch *et al*., 1999; Galloway and Depledge, 2001; Homa *et al*., 2003), making them more vulnerable to external aggressions like bacterial attack.

Overall, and qualitatively, it seems that genotoxicity occurred in earthworms exposed to radioactive wastes from an abandoned uranium mine. Therefore, DNA damage may be a suitable biomarker for the evaluation of the genotoxicity of this kind of residues, which can be easily detected by comet assay, in these cells. Moreover, flow cytometry revealed that earthworm's coelomocytes seem to be affected by the exposure to radioactive wastes, by causing depletion in cells responsible for the effector functions of the immune system of these organisms.

A quantitative analysis of these results cannot be included at this moment as data are still under analysis.

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