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Neutral Red Retention by Earthworm Coelomocytes: A Biomarker of Cadmium Contamination in Soil

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The earthworm *Metaphire posthuma* were used as a model to assess the toxic potential of cadmium incorporated into the soil by environmental or human activities. The retention period of neutral red in the lysosomes of the coelomocytes was used as a biomarker. The viability of harvested coelomocytes by a non-invasive extrusion protocol was 93% with no alteration by the dye during experimentation. The control cells retained dye for 119 and 121 min in normal soil and KCl, respectively, whereas a linear decline in the retention time in the treated earthworm coelomocytes was observed. This illustrated that the presence of cadmium caused damage to the lysosomes of the coelomocytes.

INTRODUCTION

In recent years, there has been an increase in the application of biomarkers to identify and quantify the potential adverse effects of biologically available chemicals on the environment. The uses of biomarkers have already been reviewed (McCarthy and Shugart, 1990; Huggett *et al.*, 1992).

Environmental chemicals cause lysosomal damage in aquatic organisms also (Moore, 1985, 1990). For example, lysosomes in the digestive gland cells are adversely affected when the mussels are exposed to xenobiotics (Moore, 1988). Lowe *et al.* (1992) developed an assay employing neutral red in isolated liver cells of fish for the study of lysosomal injury. *In vitro* lysosomal membrane damage in marine mussels was assessed by using neutral red retention method (Lowe and Pipe, 1994). In fact, the lysosomes of healthy cells can only retain the supravital dye after initial uptake which is not possible with damaged cells.

Further modifications were implemented to enable the use of the retention of neutral red to validate a non-specific and non-destructive biomarker method for use with earthworms (Weeks and Svendsen, 1996). The method uses changes to the lysosomal membrane stability as a means to measure of subcellular pollution-induced stress. The stability of the lysosomal membrane of earthworm coelomocytes can be assessed rapidly and simply using the neutral red retention assay developed by Weeks and Svendsen (1996).

The neutral red retention assay has been found to be sufficiently sensitive for measuring the sub-lethal effects of copper on the integrity of the lysosomal membrane of earthworm coelomocytes (Svendsen and Weeks, 1997). This technique has already been applied *in situ* to different earthworm species at different field sites, e.g., *Lumbricus castaneus* at a

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plastic fire site in U.K. (Svendsen *et al.*, 1996) and *Amynthas gracilis* (Kinberg) at a gold mine tailings dump in Zimbabwe.

The present communication report with the assessment of the toxic potential of cadmium by measuring the neutral red retention time in coelomocytes of the earthworm *Metaphire posthuma*.

MATERIALS AND METHODS

Earthworms *M. posthuma* were collected from Gheru garden and maintained in earthen pots with their native soil containing 30% moisture and 2% cow dung manure at 20 ± 2℃ in a BOD. They were acclimated for 7 days before commencing the experiment. Healthy and clitellate earthworms (average weight, 3.13 g; average length, 14.73 cm) were sorted out and kept for 24 h in a glass trough with little water for moisture to evacuate their gut prior to experimentation.

Cadmium chloride (AR grade) was used as a test chemical and the various doses were calculated in terms of Cd. A. stock solution of test chemical was prepared in double distilled water and the various test concentrations (5,10,20,40 and 80 ppm) were prepared from it. They were sprayed onto the soil using an atomizer and thoroughly mixed in a blender to get the uniform distribution of the metal throughout the soil particles. Treatment of Cd was given only once during experimentation. Twenty earthworms each were placed in Cd-treated and control soils. The same concentrations of potassium chloride were also used as control along with normal soil to determine the effect of chloride ions in the test chemical. All the containers were kept at 20 ± 2℃ in a BOD incubator. They were inspected daily for behavioral and gross morphological alterations. Among the 20 earthworms, 10 were used after 7 days, and the remainder after 15 days of exposure.

Harvesting the Coelomocytes

Coelomocytes from experimental and control earthworms were obtained by a non-invasive extrusion protocol (Pyambe *et al.*, 1990). Briefly, an earthworm was placed for maximum of 3 min in a 100 × 15 mm petri dish containing 3.0 ml of extrusion medium which consisted of 5% ethanol and 95% saline supplemented with 2.5 mg/ml EDTA and adjusted to pH 7.3 with NaOH. Extruded cells were transferred immediately into test tubes containing 17 ml calcium-free earthworm Ringer, washed 3 times, spun at 150 × *g* for 5 min and resuspended in 1ml Ringer at 4℃. Coelomocytes from each earthworm were collected and processed separately for all assays.

Cell Viability

To ensure the viability of cells during the process of harvesting, 20 μl of coelomocyte suspension was placed on a microslide and stained with 1 μl of Eosin Y solution (2 mg Eosin Y per ml in earthworm Ringer). Healthy live cells stained green whereas dead cells stained red.

Retention of Neutral Red by the Coelomocytes

The procedure described by Weeks and Svendsen (1996) was adopted. For the stock solution, 20 mg of neutral red was dissolved in 1 ml of dimethyl sulfoxide (DMSO). Subse-

quently, 10 μl of stock solution was diluted with 2.5 ml of earthworm Ringer giving a working neutral red concentration of 80 μg/ml. To avoid crystalization of the non-polar neutral red in aqueous Ringer, the working solution was renewed every hour. A coelomic fluid sample (20 μl) was placed onto a microscope slide and the cells were allowed to settle and adhere to the slide surface for 30 s. Next 20 μl of neutral red working solution was applied and covered with a coverslip. The drying of the slides was avoided by putting them in a desiccator having water at the bottom. These slides were thoroughly observed under optical microscope for retention of the dye.

RESULTS

Cell Viability

The viability of harvested cells was 93% and it was not altered/affected by neutral red staining during experimentation.

Neutral Red Retention

Two main types of coelomocytes are found in the coelomic fluid: granulated and agranulated. The neutral red retention was more prominent in granulated cells than agranulated cells. So only granulated cells were taken into account as they were found to be a good biomarker. The results of normal soil control, chloride control and treatment are presented in Tables 1 and 2. The normal soil control and chloride control coelomocytes retained dye for 119 and 121 min respectively. Thus, the chloride ion had ill effects on lysosomal membrane. A decline in retention was observed with each increase in cadmium exposure level under exposed conditions for 7 days, dye retention was 98, 68, 60, 25 and 18 min at 5, 10, 20, 40 and 80 ppm concentrations respectively, whereas it was 90, 46, 43, 20 and 19

TABLE 1

Neutral Red Retention in Minutes (7 Days) ($\bar{x} \pm s$)

Control		Treatment				
NS	KCl	5 ppm	10 ppm	20 ppm	40 ppm	80 ppm
119 ± 1.29	121 ± 1.28	98 ± 1.9*	68 ± 0.95*	60 ± 2.3*	25 ± 1.03*	18 ± 0.6*

Note. NS, Normal soil control; KCl, Potassium chloride control.
* $P < 0.001$, Statistical analysis by Student 't' test.

TABLE 2

Neutral Red Retention in Minutes (15 Days) ($\bar{x} \pm s$)

Control		Treatment				
NS	KCl	5 ppm	10 ppm	20 ppm	40 ppm	80 ppm
119 ± 1.29	121 ± 1.23	98 ± 0.9*	46 ± 1.1*	43 ± 0.9*	20 ± 0.5*	19 ± 0.5*

Note. NS, Normal soil control; KCl, Potassium chloride control.
* $P < 0.001$, Statistical analysis by Student 't' test.

min after 15 days. In both sets of experiment, a steady decline was observed from 119 to 18 minutes in 7 days and to 19 minutes after 15 days of metal exposure. Exposure beyond 40 ppm for 15 days resulted in mortality of earthworms due to necrosis of clitellum (Gupta and Sundararaman, 1991) and therefore only 4 animals were available for experimentation.

DISCUSSION

Neutral red, which is a weak cationic dye that penetrates cell membranes by nonionic passive diffusion, is retained intracellularly in the lysosomes of coelomocytes (Nemes, Dietz and Luth, 1979) by a mechanism that is similar to iron trapping (Peterson, 1979). The ability of cells to retain dye is dependent on the lysosomal viability. Though the lysosomes are involved mainly in intracellular digestion, they perform many other physiological functions (Moore *et al.*, 1982). They are membrane bound cytoplasmic organelles containing a wide variety of hydrolytic enzymes capable of breaking down most types of biological molecules. These lysosomes are main target for the toxic effects of environmental pollutants at a subcellular level (Moore, 1990). Lysosomal damage in marine organisms has been well documented and correlates with environmental stress (Moore, 1980).

Neutral red staining is a well established technique for the evaluation of cytotoxicity caused by chemical compounds, using cells from mammals, humans and fish as targets (Babich and Borenfreund, 1990). The relative release of the dye depends on the lysosomal function. In the present experiment, it is clear that Cd induced lysosomal damage, and the dye retention time gradually decreased as the concentration of Cd was increased. Weeks and Svendsen, (1996) studied the effect of copper on coelomocytes from the earthworm *Lumbricus rubellus* and found that the retention time decreased significantly with increasing exposure to Cu in the laboratory as well as in mesocosm studies. The present study confirmed that the lysosomes are the target organelles in a cell, and that they can be used to assess the toxicity of environmental pollutants.

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