Bull. Environ. Contam. Toxicol. (1997) 59:44-49

Bulletin of Environmental Contamination and Toxicology

Combined Non-Invasive Cell Isolation and Neutral-Red Retention Assay for Measuring the Effects of Copper on the Lumbricid *Aporrectodea rosea* (Savigny)

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Received: 2 December 1996/Accepted: 11 March 1997

Within the field of cellular biomarkers, a lysosomal membrane stability is (due to its non-specificity) one of the most integrative parameters for monitoring the adverse impacts of environmental pollutants on cellular functions (Svendsen et al. 1996). One of the easiest techniques to determine lysosomal membrane integrity is the neutral-red retention (NRR) assay; which was originally derived from mammalian cell culture viability screening. This assay has been successfully applied in the laboratory and during field studies in aquatic systems on fish and molluscs (Lowe et al. 1992: Lowe and Pipe 1994; Svendsen and Weeks 1995) and in terrestrial systems on earthworms (Svendsen et al. 1996).

The NRR assay as a biotest on membrane damage is based on the uptake of the supravital lipophilic compound neutral-red by transmembrane diffusion, and its subsequent protonization within the acid lysosomal compartment (Lowe et al. 1992). The capacity of lysosomes to retain the dye depends upon the maintenance of an internal low pH and, therefore, on the efficiency of membrane-bound proton pumps (Seglen 1983). According to their membrane integrity, which is a marker for cellular stress, the lysosomes tend to leak after a distinct time period (retention time) and the dye subsequently leaks into the entire cytosol of the cell producing a pinkish colouration. The most prominent disadvantage of the NRR assay in the past was the invasive puncture of the test animals in order to extract the cells. The use of a syringe in several cases led to mortality exept for comparatively big test species (e.g. some bivalves). Hence, each individual could only be used once per study.

Recently, Goven et al. 1994 introduced a non-destructive method which per-

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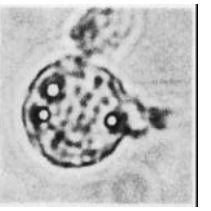

Figure 1. Light micrograph of a coelomocyte extruded from the lumbricid's body by mucus secretion.

mitted the extraction of healthy coelomocytes from earthworms *(Lumbricus terrestris)* for subsequent use in enzyme assays. In this paper we describe the combination of an analogous extraction technique with a modified NRR assay to integrate the advantages of non-destructiveness into a rapid low-cost bioassay.

MATERIALS AND METHODS

Individuals of the earthworm *Aporrectodea rosea* (Savigny) were collected by hand-digging from an uncontaminated grassland site close to the Monks Wood Nature Reserve, Cambridgeshire. UK. Collected worms were transferred to a large plastic container filled with a moistened sandy loam soil (96 % sand; 4 % clay; <1 % organic matter; pH 5.6) collected from Thetford Forest, Norfolk. UK, and the surface covered with dampened newspaper. Earthworms for use in experiments were starved for 3 days in Petri dishes (containing filter paper moistened with distilled water) under constant conditions of 12°C, 95 % relative humidity and constant artificial light (150 lux). Groups of similar sized adult earthworms were exposed to an increasing range of soil copper concentration viz. control (3 mg Cu/kg), 20, 40, 80, 160, and 320 mg Cu/kg soil on a dry-weight basis for a period of 10 days in clear plastic containers (15 x 8 x 6 cm) with ventilated lids. Copper was added by thoroughly mixing a solution of $CuCl_2$ (BDH Ltd. Poole, Dorset, UK) in distilled water with 600 g soil to produce the total amount of copper required for each soil exposure concentration (gravimetric water content of 15 %). Food in the form of 3 g of oven-dried horse manure (from a known source) was added to the surface of each container. The worms were left undisturbed throughout the duration of the experiment until harvested for analysis.

After 10 days of exposure to the different copper concentrations, coelomocytes were harvested from the earthworms using a modified non-invasive extrusion method based on Goven et al. (1994). The earthworms were transferred to a Petri dish and washed with distilled water. The worms were then placed on a wet Whatman filter paper (Whatman 541, diameter

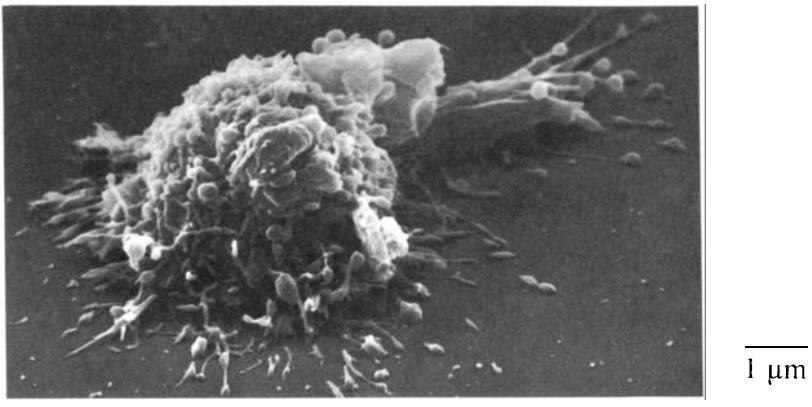

Figure 2. Scanning electron micrograph of a coelomocyte adhered to the slide surface.

11cm). The quarter of their posterior end was massaged to expel faeces from the lower gut to reduce contamination of the extrusion fluid. Subsequently, the worms were rinsed in a beaker containing a saline solution (0.85 g NaCl in 100 mL distilled water) and incubated for 3 min in a small vial (diameter 3.5 cm) containing 2 mL of extrusion medium which consisted of 5 % ethanol and 95 % of the above mentioned saline solution. supplemented with 2.5 mg/ml, EDTA and 10 mg/mL of guaiacol glycerol ether (BDH Ltd, Poole, Dorset. UK) adjusted to pH 7.3 with 1N NaOH. In this mild irritant solution the earthworms started to extrude mucus which contained many coelomocytes (Fig. 1). Subsequent to this, the earthworms were transferred into a rinsing saline solution to remove traces of the extrusion medium from the worm's surface. Ten mL *Aporrectodea*-balanced salt solution (ABSS) were added to the 2 mL cell suspension and lightly shaken. ABSS consisted of NaCl (71.5mM), KCl (4.8mM), $CaCl_2$ (3.8mM), $MgSO_4*7H_2O$ (1.lmM), KH_2PO_4(O.4mM). $Na_2HPO_4*7H_2O$ (0.3mM), and $NaHCO_3$(4.2 mM) in distilled water at pH 7.6. Twenty µL of the diluted cell suspension were placed in the center of circular markings (diameter 1.0 cm) on a light microscope slide and left undisturbed for 3 min. During this time, the coelomocytes (diameter 10-30 µm) adhered to the slide surface by developing several filamentous pseudopodia (Fig. 2). After the 3 min interval, the suspension droplet was removed and the adhered cells were rinsed 3 times with 40 µl ABSS. Finally, 17 µL ABSS and 20 µL of a neutral-red (NR) working solution (80 ug/mL) were added to the wet spots on the slide and lightly mixed by sucking the fluid up and down with a pipette. The NR working solution consisted of 10 µL of a freshly prepared neutral-red stock solution (20 mg of neutral-red (Sigma, Poole, Dorset, UK) dissolved in 1 mL of dimethyl sulphoxide (DMSO)) and 2.5 mL of earthworm physiological Ringer solution (6.0 g NaCl, 0.12 g KCl, 0.2 g

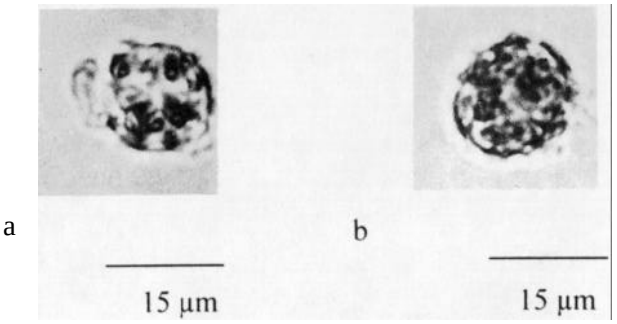

Figure 3. Coelomocytes of *Aporrectodea rosea* after the addition of neutral-red. Lysosomes immediately concentrate the dye and stain bright red (a). After deprotonisation, neutral-red diffused out of the lysosomes colouring the cytoplasm pink (b).

$CaC1_2$, 0.1 g $NaHCO_3$ in 1 L distilled water) (Lockwood 1971; Svendsen and Weeks 1995). For each individual worm, the NR working solution was freshly prepared. Each of usually 2 droplets on a slide was covered with a coverslip and examined alternately using a light microscope (Leitz, Wetzlar). The droplets were scanned horizontally and vertically at a constant magnification. High numbers of coelomocytes in the extrusion medium necessitated a dilution of the cell suspension in order to perform the neutral-red retention assay. From a methodological point of view, analysis of the cells in a single horizontal/vertical scan should not exceed 2-3 min, especially for higher copper concentrations which resulted in comparatively shorter retention times. For each full cross scan, the time required for scanning, the total number of observed coelomocytes, and the number of cells with fully red stained cytosols (Fig. 3) were recorded. Visualization was terminated after either 100 min of examination or in the interval in which the ratio of cells with fully stained cytosols exceeded 70% of the total cell number. To avoid disadvantages by extreme timespans required for scanning and the resulting additional stress in view of the fact that neutral-red is a photosensitising agent, high cell yields were diluted in order to guarantee a similar ceil density on each slide for every specimen. To obtain the NRR time at which precisely 70% of the extracted cells from one single earthworm had become stained, data recorded for every respective specimen was plotted vs. time (means of the scanning intervals) and subjected to regression analysis. Mean NRR time for each group of exposed worms was then calculated from the individual 70% NRR time data.

The viability rate of non-invasively isolated cells was checked for control earthworms by use of an Eosin Y test. After the rinsing steps, 17 µL ABSS and 1 µL Eosin Y solution (Sigma, Poole, Dorset, UK, 2 mg/mL) were added

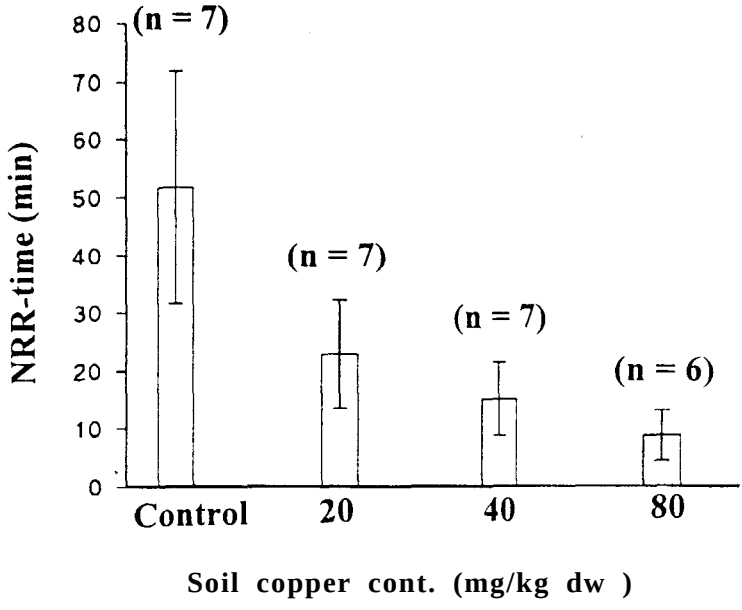

Figure 4. Neutral-red retention time (NRR-time) in minutes for coelomocytes extracted from *Aporrectodea rosea* exposed for 10 days to a graded series of copper concentrations (mg Cu / kg soil dw). The error bars represent the standard error of the mean (SEM) for each concentration. The number of separate individuals per concentration is given in parentheses.

to the slide. Live cells appeared green and dead cells were stained red.

RESULTS AND DISCUSSION

The extrusion medium used in the present study acted as a mild irritant, causing muscular contractions which result in the secretion of mucus usually containing large numbers of coelomocytes through pores in the body wall as shown for *Lumbricus terrestris* (Goven et al. 1994). The extraction of these cells in our modified procedure worked well in our experiments with *Aporrectodea rosea,* up to 600 isolated coelomocytes per droplet were counted.

After extrusion of the coelomocytes, all treated specimens showed minimal trauma and were able to bury again after a short recovery period. Coelomocytes collected by this extrusion process had a viability of >90 % assessed using the Eosin Y test as a measure of cell survivorship. Control worms even after 10 days in the experimental condition described had high cell viability counts; a key component in any biotest such as the one

described in the present study. The observed changes in NRR time for coelomocytes from earthworms exposed to the different soil copper levels showed that the NRR response was sufficiently sensitive even to low metal concentrations. Increasing copper concentrations resulted in a decrease in neutral-red retention time (Fig. 4). No results are presented for the worms at the highest copper concentrations (160 or 320 mg/kg soil dw) since these specimens failed to extrude a sufficient number of coelomocytes into the extrusion medium. This reduction in coelomic fluid extrusion capacity may be due to a sublethal toxic effect of copper and may represent a simple marker of stress itself. The combination of both techniques for the non-invasive coelomocyte extraction and neutral-red retention yielded the development of a non-destructive biomarker assay in earthworms. This rapid and low-cost method could help to save resources of animals for toxicity studies. Furthermore, the enormous advantage of this combined technique lies in the possibility to repeat or to start additional experiments with identical individuals.

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