



A new and sensitive method for measuring *in vivo* and *in vitro* cytotoxicity in earthworm coelomocytes by flow cytometry

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ABSTRACT

This study describes a new and sensitive method for measuring the *in vivo* and *in vitro* cytotoxicity of 2 earthworm species, *Eisenia andrei* and *Perionyx excavatus*, exposed to copper. Specifically, we measured the number of coelomocyte cells that were affected by copper following *in vivo* and *in vitro* exposure by flow cytometry, after calcein acetoxymethyl ester (calcein-AM) staining. We found that the coelomocyte viability of both earthworm species was noticeably reduced in the *in vivo* cytotoxicity test at concentrations of 100 mg/kg copper in dry soil. However, pathological symptoms, such as mucous secretion and bleeding, swelling, thinning, and fragmentation, and burrowing symptoms were not evident following exposure to copper levels of < 400 mg/kg dry soil. In conclusion, the present study demonstrates that calcein-AM is a more sensitive test of earthworm coelomocyte cytotoxicity compared to the traditional individual level toxicity test. Therefore, this test could be used to detect low levels of metal contamination in soils.

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1. Introduction

Earthworms are representative invertebrates of the soil ecosystem. The International Organization for Standardization (ISO, 2008, 2012b, a), the Organization for Economic Co-operation and Development (OECD, 2004), the American Society for Testing and Materials (ASTM, 2004), and the US Environmental Protection Agency (USEPA, 2012) have established standard methods for measuring the response of individual earthworm species by measuring mortality, behavior, pathological symptoms, body weight change, and reproductive activity. However, the sensitivity of this technique is low. Consequently, there have been attempts to develop more sensitive methods to evaluate earthworm responses at a cellular level.

Coelomocytes play an important role in the identification and elimination of xenobiotics (Kurek and Plytycz, 2003), immunological response (Hanna and Cooper, 1974; Hostetter and Cooper, 1972), and phagocytosis (Dales and Kalaç, 1992; Renzelli-Cain and Kaloustian, 1995; Stein et al., 1977) in earthworms. Existing studies of earthworm have investigated the effects of toxicants on the lysosomal stability of coelomocytes using neutral red retention assays, phagocytosis, DNA damage, and enzymatic responses (Scott-Fordsmand and Weeks, 2000).

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Various types of flow cytometric assays have been used to test the cytotoxicity of earthworm coelomocytes, as listed in Table 1. Flow cytometric assays use various fluorescent probes. Table 1 shows 9 fluorescent dyes that have been applied to earthworm coelomocytes in previous studies. JC-1 (5,5',6,6'-tetra-chloro-1,1',2,2'-tetraethylbenzimidazol-dazolcarbocyanine iodide) (Bearoff and Fuller-Espie, 2011; Cossarizza et al., 1995; Nacarelli and Fuller-Espie, 2011) and rhodamine 123 are related to mitochondrial membrane potential. Acridine orange (Cossarizza et al., 1995) measures mitochondrial mass. Propidium iodide (Bearoff and Fuller-Espie, 2011; Cossarizza et al., 1995; Fugère et al., 1996; Fuller-Espie et al., 2010; Massicotte et al., 2004; Sauvé et al., 2002) and 7-AAD (7-aminoactinomycin D) (Bearoff and Fuller-Espie, 2011; Fuller-Espie et al., 2010; Fuller-Espie et al., 2011; Nacarelli and Fuller-Espie, 2011) only stain dead cells, as they cannot permeate living cell membranes. Annexin V-FITC (fluorescein isothiocyanate) (Bearoff and Fuller-Espie, 2011; Fuller-Espie et al., 2010) binds to translocated phosphatidylserine in the membrane of apoptotic cells, and is generally used with propidium iodide to detect apoptosis. DCF (Dichlorofluorescein) (Komiya et al., 2003; Massicotte et al., 2004) and DHR123 (dihydrorhodamine 123) (Fuller-Espie et al., 2010; Fuller-Espie et al., 2011; Nacarelli and Fuller-Espie, 2011) react with reactive oxygen species (ROS), specifically H₂O₂.

Calcein acetoxymethyl ester (calcein-AM) has been used in several studies of earthworms. It stains live cells (Teplava et al., 2010), and is hydrolyzed by intracellular esterase to calcein,

which has a fluorescent property. Calcein-AM has been used to evaluate the esterase activities of several classes of organisms, including protozoa (Dias and Lima, 2002), waterflea (Teplova et al., 2010), fish (Iwanowicz et al., 2004; Sandbacka et al., 2000; Wilson et al., 1987), snails (Moran, 2000), plants (Cheng et al., 2001; Dassonneville et al., 2000), insects (Collins and Donoghue, 1999; Hunter and Birkhead, 2002), and a number of microorganisms (Kaneshiro et al., 1993). However, to the best of our knowledge, calcein-AM has not been previously used on earthworm species.

In this study, we used calcein-AM to stain earthworm coelomocytes, and report a new sensitive method for evaluating the cytotoxicity of earthworm coelomocytes by flow cytometry after calcein-AM labeling. Two earthworm species, *Eisenia andrei* and *Perionyx excavatus*, were exposed to copper in soil, and a bioassay was conducted to evaluate the responses of individual specimens. Copper was selected because it is a common soil pollutant, and has high ecotoxicity (Nor, 1987; Flemming and Trevors, 1989). Although organisms that survive copper exposure may represent more resistant individuals, the viability of coelomocytes in survivors may be used to quantify the toxicity levels in soil. Here, we directly extracted the coelomocytes from surviving earthworms, and labeled them with calcein-AM, to quantitatively measure the viability of coelomocytes by flow cytometry. Flow cytometry simultaneously measures multiple characteristics of single cells, including the relative size, granularity, and fluorescence intensity of cells, at a rapid rate. In addition, *in vitro* cytotoxicity was performed to compare the sensitivity of calcein-AM with other fluorescent probes. This study focused on confirming the utility of the flow cytometric assay with calcein-AM-labeled coelomocytes as a new and sensitive method to measure the viability of earthworm coelomocytes and, hence, infer cytotoxicity.

2. Materials and methods

2.1. Test species

The test species were *E. andrei* and *P. excavatus*. *E. andrei* is recommended as a test species by the OECD (2004), USEPA (2012), and ISO (2008, 2012b, a). The Asian earthworm, *P. excavatus*, is used extensively in toxicity tests (An and Lee, 2008; De Silva et al., 2010; Maboeta et al., 1999). Earthworms were maintained in standard OECD soil at $20 \pm 1^\circ\text{C}$ in darkness. Before the test, adult earthworms (weighing 300–600 mg) were maintained on a moist filter paper at $20 \pm 1^\circ\text{C}$ in darkness to remove all gut contents.

2.2. Test chemicals

Copper ($\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ > 99%) from Sigma Chemical Co., Inc. (St. Louis, MO, USA) was selected as a model chemical. Calcein-AM was purchased from Sigma Chemical Co., Inc. (St. Louis, MO, USA).

2.3. Individual-level toxicity test

Acute toxicity tests at an individual level were carried out using the modified OECD Testing Guideline (TG) No. 207 (An and Lee, 2008; OECD, 1984). 10 g of OECD standard soil was placed in a test unit (flat-bottom vial, ID 25 mm, height 50 mm, volume 20 mL). Each test solution was then spiked into the soil to represent 35% (v/w) of water content. A copper concentration of 0 (control), 100, 200, 400, 600, 800, and 1000 mg/kg dry soil was prepared. In the individual-level toxicity test, the control group (0 mg/kg copper concentration) was the earthworm group that was not exposed to copper. Previous studies have reported high copper concentrations of 2874 mg/kg (Fernández-Caliani et al., 2009) and 958 mg/kg (Cabrera et al., 1999) at contaminated sites. USEPA (2007) manages a ceiling concentration of 4300 mg Cu/kg of soils when applying sewage sludge on land. In this study, one earthworm was added to each test unit, and a Sili stopper was used to cover the test unit to prevent avoidance behavior or air stress. Earthworms were exposed for 7 days at $20 \pm 1^\circ\text{C}$ in darkness. The survival rate, abnormalities, such as mucous secretion, bleeding, swelling, thinning, and/or fragmentation, and burrowing rates were recorded by macroscopic examination.

2.4. In vivo and in vitro cytotoxicity tests

After exposure to each copper concentration in the individual level tests, the survivors were used to test cytotoxicity *in vivo* (Supplementary information Fig. S1). Coelomic fluids were directly extracted from survivors by inserting a hypodermic syringe into the clitellum (Svendsen et al., 1996). The fluids were then mixed with 0.1 mL LBSS (*Lumbricus* balanced salt solution), containing 1.5 mM NaCl, 4.8 mM KCl, 1.1 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 mM KH_2PO_4 , 0.3 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 4.3 mM NaHCO_3 , and 3.8 mM CaCO_3 (Sauvé et al., 2002). For the *in vitro* cytotoxicity test, coelomocytes were extracted from non-exposed earthworms. The extracted coelomocytes were directly exposed to copper for 1 h at $20 \pm 1^\circ\text{C}$ in darkness to assess the acute effects. The copper exposure concentrations were prepared at 0 (control), 1, 5, 10, and 50 mg/L in LBSS. The exposed coelomocytes were then rinsed twice with LBSS to remove copper from the medium. For the *in vivo* and *in vitro* experiments, the test concentrations and exposure duration were based on the sensitivity of the test type. Because the coelomocytes were placed in direct contact with copper for the *in vitro* test, the exposure concentrations were lower and exposure duration was shorter compared to those of the *in vivo* test. After the cytotoxicity test, the coelomocytes were stained by calcein-AM.

As shown in Table 1, all previous studies on earthworms, except 2 (Massicotte et al., 2004; Komiya et al., 2003), investigated cytotoxicity using fluorescent probes *via in vitro* tests. However, *in vivo* assays are required to compare the expression of cytotoxicity at low concentrations against individual-level effects. For this reason, we measured the *in vivo* cytotoxicity of copper after extracting coelomocytes from the survivors of individual toxicity tests.

2.5. Calcein-AM staining and flow cytometric analysis

A stock solution of calcein-AM (500 μM) was prepared by dissolving it in dimethyl sulfoxide (DMSO). Extracted coelomocytes were suspended in LBSS. The cell suspension (950 μL) was then mixed with 10 μL calcein-AM to give a final concentration of 5 μM calcein-AM. This solution was incubated for 1 h at $37 \pm 1^\circ\text{C}$ in darkness. Cell suspensions of *E. andrei* and *P. excavatus* after calcein-AM staining were centrifuged for 1 min at 5000 and 7000 rpm, respectively. Because the coelomocytes of the 2 species were of different sizes, the centrifuge was set to different spin conditions. The resultant pellets were resuspended in LBSS, and then flow cytometric analyses were conducted. We used a FACScalibur flow cytometer (BD Biosciences, USA) to analyze fluorescent calcein, by collecting data from 20,000 events in each sample with FL1 (500–560 nm band pass filter, green fluorescence) after excitation at 488 nm. The acquired results were analyzed using Cell Quest Pro software (BD Biosciences, USA), and coelomocyte viability was expressed as calcein intensity *via* histograms. In addition, the data of Forward-scattered light (FSC) and side-scattered light (SSC) from 20,000 events were also recorded to evaluate the effects of Cu on coelomocytes morphology after 1 h exposure in the *in vitro* test. FSC and SSC signals provide information about cell size (FSC) and cell granularity (SSC). In the *in vivo* and *in vitro* cytotoxicity tests, the control contained coelomocytes had not been exposed to copper. It is generally known that earthworm coelomocytes exert autofluorescence due to the riboflavin contents (Homa et al., 2010), which must be controlled for when conducting flow cytometric analysis (Fuller-Espie et al., 2010, 2011). Thus, we measured the calcein-AM-unstained coelomocytes treated with Cu in the *in vivo* test to evaluate their contribution to autofluorescence. The autofluorescence of coelomocytes at each concentration was measured by using coelomocytes without calcein-AM staining as negative controls. This procedure was followed to remove coelomocyte autofluorescence from the results.

2.6. Fluorescent microscopic measurement

Fluorescence images of coelomocytes before and after calcein-AM staining were taken using a fluorescent microscope (Olympus BX 51; Olympus Cooperation, Tokyo, Japan), which was equipped with a green filter (U-MWIB3) and a digital color camera (DP72, Olympus Corporation, Japan).

2.7. Statistical analysis

The effective concentrations (EC_{10} and EC_{50}) and no-observed-effect concentration (NOEC) for survival, abnormalities, burrowing rates, and skin irritation of earthworms exposed to copper in OECD standard soil were estimated. The EC_{10} values were calculated with Probit analysis, and the EC_{50} values were estimated using the computer program SPEARMAN (USEPA, 1999b) based on the Trimmed Spearman–Kärber method (Hamilton et al., 1977). The NOEC values were estimated using the Dunnett Program (Ver. 1.5) (USEPA, 1999a) based on Dunnett's procedure for multiple comparisons (Dunnett, 1955). The Dunnett Program calculates the minimum differences between the control and treatment means that were determined to be statistically significant. A 95% significance level ($p < 0.05$) was employed for all comparisons. Comparison of means was also conducted using ANOVA. To quantify the results of flow cytometry, replicates of the flow cytometric measurements were performed (triplicates in *in vitro*, 3–5 replicates in *in vivo*), and

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