IN VIVO AND *IN VITRO* CYTOTOXICITIES OF NICKEL ON EARTHWORM: A NEW METHOD FOR EARTHWORM COELOMOCYTES VIABILITY

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Introduction

Earthworms are representative and important invertebrates in terrestrial ecosystem. Although earthworms are widely used in toxicity test, there are limited studies of nickel to earthworm coelomocytes[1-16]. Previous studies evaluated the effects of nickel on earthworm survival[2,4,9,13,14,16], reproduction including cocoon production or juvenile production[4,5,9,10,16], accumulation[1,3,7,8,12,13-16], body weight change[1,7,16], 8-Hydroxydeoxyguanosine generation[8], DNA damages[10,11], riboflavin contents[12,14,15], burrowing[13,15], and freezing tolerance[9]. Only one study investigated the effects of nickel to lysosomal membrane stability[4].

In this study, flow cytometry with calcein acetoxymethyl ester (calcein-AM) staining were applied to investigate the *in vivo* and *in vitro* cytoxicity of nickel on earthworm coelomocytes. Because understanding of cytotoxic effects in low exposure concentrations is important, we used a new sensitive method for earthworm coelomocytes which play key roles in the cognition and elimination of foreign materials[17].

Calcein-AM has no fluorescent property, but if it is hydrolyzed by intracellular esterase, fluorescent calcein are produced. Therefore, evaluation of coelomocytes viability is available using this property. To the best of our knowledge, this is the first report of cytotoxic effects of nickel on earthworm coelomocytes using calcein-AM staining.

Materials and methods

We selected the earthworm *Eisenia andrei* and *Perionyx excavates* as test species. *Eisenia andrei* is recommended species by the test guidelines of OECD[18] and US EPA[19], and *Perionyx excavates* is an Asian earthworm[20]. Adult worm (weight of 300-600 mg) were exposed to nickel chloride ($NiCl_2$ > 98%, Sigma Chemical Co., Inc., St. Louis, MO, USA).

In the soil acute toxicity tests, modified OECD test guideline[20, 21] was applied. Ten grams of OECD standard soil (consisted of 69.5% sand, 20% kaolin clay, 10% sphagnum peat moss and 0.5% $CaCO_3$) were added to a glass test unit (flat-bottom vial, ID 25 mm, height 50- mm, volume 20 mL). Test concentration of 0, 100, 200, 400, 600, 800, 1000, 1500, 2000, and 3000 μg/g dry soil were prepared by spiking of nickel solution into soil as 35% (v/w) of moisture contents. Then, one earthworm was placed in a test unit and each unit was covered with a sili stopper to avoid air stress. During exposure duration of seven days, survival and abnormalities (mucous secretion and bleeding, swelling thinning, and fragmentation) were measured. *In vivo* cytotoxicities were investigated using survived earthworms after seven days.

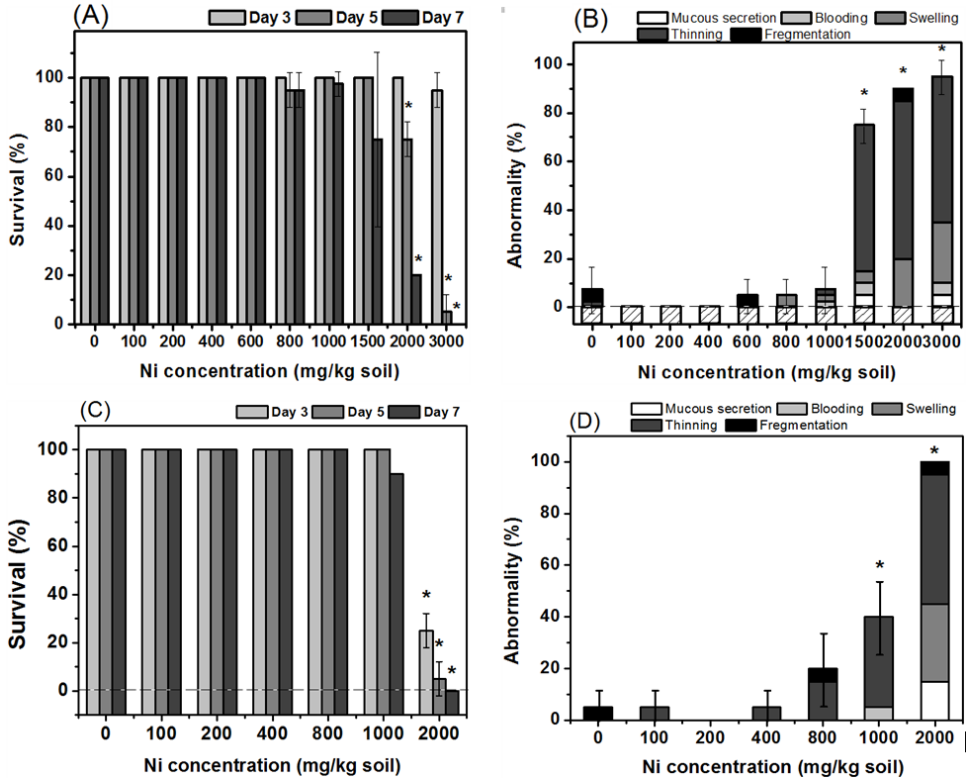
In vitro tests were conducted with healthy earthworm coelomocytes in LBSS (*Lumbricus* balanced salt solution) medium containing 1.5 mM NaCl, 4.8 mM KCl, 1.1 mM $MgSO_4 \cdot 7H_2O$, 0.4 mM KH_2PO_4, 0.3 mM $Na_2HPO_4 \cdot 7H_2O$, 4.3 mM $NaHCO_3$ and 3.8mM $CaCO_3$ [22]. Coelmocytes were collected with hypodermic syringe in the clitellum[23]. Test concentration of *in vitro* test were prepared as 0, 1, 5, 10, and 50 mg/L. Coelomocytes were exposed for one hour at 20±1℃ in darkness.

Coelomocytes viabilities were assessed with flow cytometry after calcein-AM staining (Sigma Chemical Co., Inc., St. Louis, MO, USA). To observe the inhibition of intracellular esterase activities by nickel, extracted coelomocytes were incubated with calcein-AM for one hour at 37±1℃ in darkness. Then coelomocytes were washed and analyzed with FACScalibur (BD Biosceinces, USA). We collected the data of 20,000 events in each coelomocytes samples with FL1, and analyzed the acquired results with Cell Quest Pro software (BD Biosceinces, USA).

Results and discussion

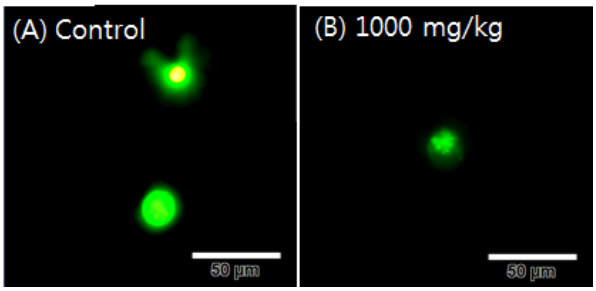
During exposure duration, negligible mortal effects as well as abnormal effects were observed up to concentration of 1000 mg /kg soil. However, survival rates of *E. andrei* were reduced by 75%, 20%, and 0% at concentration of 1500, 2000, and 3000 mg/kg soil (Fig. 1(A)). Also *P. excavatus* survival rate were observed as 90% and 0% at concentration of 1000 mg/kg soil and 2000mg/kg soil (Fig. 1(C)), respectively. In addition, abnormalities including mucous secretion, blooding, swelling, thinning, and fragmentation were not decreased upto 1000 mg/kg soil. While 75%, 90%, and 95% of abnormalities for *E. andrei* were observed at the concentration of 1500, 2000, and 3000 mg/kg soil, respectively (Fig. 1(B)). Abnormalities of *P. excavatus* were reduced by 20%, 40%, and 10% at the concentration of 800, 1000, and 2000 mg/kg soil (Fig. 1(D)), respectively.

Figure 1. Effects of nickel on earthworms exposed in the OECD standard soil for seven days. (A) is survival rate of *E. andrei*, (B) is abnormality rate of *E. andrei*, (C) is survival rate of *P. Excavatus*, and (D) is survival rate of *P. excavatus*. Error bar represents standard deviation of the mean and asterisk means significantly different with control mean.



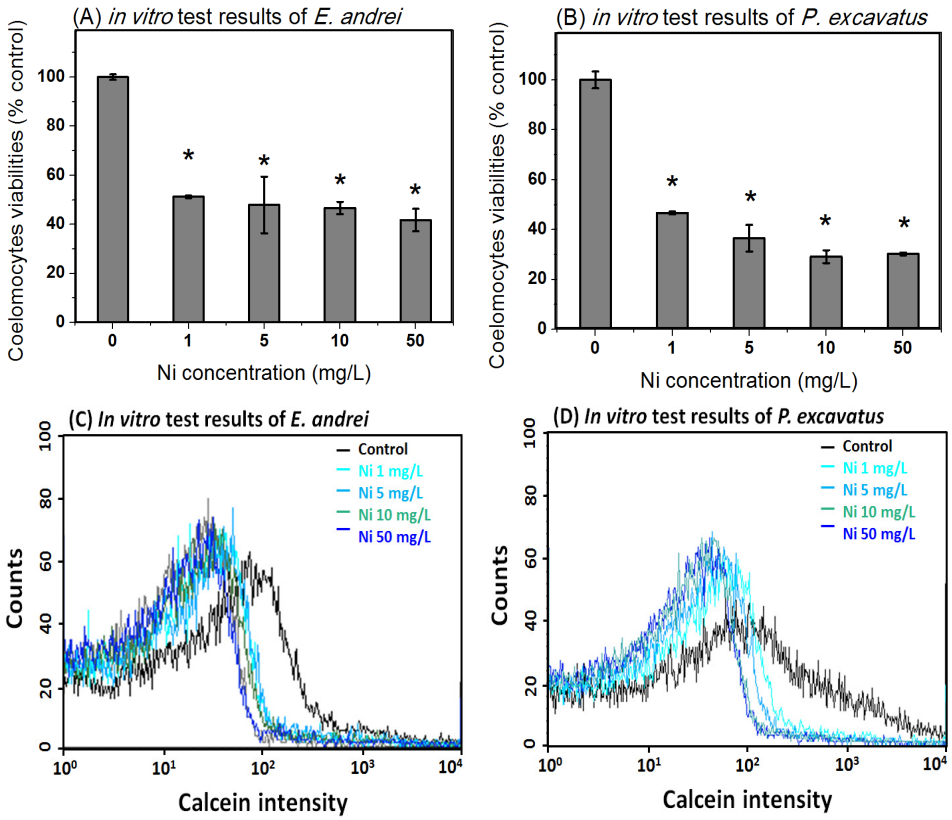
Even though two test earthworm were negligiblely affected by nickel up to 1000 mg/kg soil, *in vivo* cytotoxicity induced by nickel were observed at lower than 1000 mg/kg soil. Fig. 2 shows that *P. excavatus* coelomocytes exposed to nickel had relatively low fluorescent intensities (calcein intensity) compared to control.

Figure 2. Fluorescence images of coelomocytes stained with calcein-AM. (A) is control. (B) is *P. excavatus* coelomocytes exposed to nickel in OECD standard soil for seven days (*in vivo* cytotoxicity). Relatively low fluorescent intensity were observed at nickel exposure group.



In the *in vitro* cytotoxicity test, we also observed the decrease of coelomocytes viabilties compared to control from the lowest exposure concentration. As shown in fig. 3(A), coelomocytes viabilities of *E. andrei* were inhibited by 51%, 48%, 47%, and 42% and coelmocytes viabilities of *P. excavatus* were decreased by 47%, 37%, 29%, and 30% % at the concentration of 1, 5, 10, and 50 mg/L, respectively.

Figure 3. *In vitro* cytotoxicities induced by nickel were observed with significantly inhibition from the lowest exposure concentration (1 mg/L). (A) and (B) show the changes of coelomocytes viabilities after nickel *in vitro* exposure compared to control. Error bar represents standard deviation of the mean and sterisk means significantly different with control mean. (C) and (D) are histograms of flow cytometric measurements. Coelomocytes viabilities were expressed as calcein intensity in histograms.



To evaluate the toxicity of nickel to earthworm, we conducted soil acute assay, *in vivo* and *in vitro* cytotoxicity tests. Overall, *P. excavates* were sensitive than *E. andrei* to nickel in the bioassay and cytotoxicity test but survival rate and abnormality rate of both earthworms were negligibly affected up to 1000 mg/kg soil. According to Reineke et al. (2004)[6], nickel breaks the DNA strand and cause DNA-protein cross-links by ROS[24, 25], as well as inhibit DNA repair system[26]. Also lysosomal membrane stabilities of earthworm were affected by nickel concentrations in soil[4].

In this study, we observed that nickel induced cytotoxicity by inhibiting intracellular esterase activities in earthworm coelomocytes via *in vivo* and *in vitro* tests with flow cytometry after calcein-AM staining. We suggest that application of calcein-AM staining to earthworm coelomocytes is sensitive method. This study would provide fundamental data of nickel earthworm toxicity.

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