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Transgenerational effects of heavy metals on L3 larva of *Caenorhabditis elegans* with greater behavior and growth inhibitions in the progeny

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ABSTRACT

Heavy metals are ubiquitous environmental pollutants, and their toxic effects have been widely studied. However, their transgenerational effects between parent and progeny at environmental relevant concentrations need further investigations. Currently, L3 stage of *Caenorhabditis elegans* was exposed to aqueous metals (Cd, Cu, Pb and Zn) at environmentally realistic concentrations for 96 h. The whole exposure time covered the formation of sperm, ovum and eggs. Subsequently the behavior and growth indicators were measured. The parent nematodes were then bleached to gain synchronized eggs, which were cultured under non-toxic conditions to L3 stage when the same indicators were measured in the progeny. The parent suffered concentration-dependent inhibitions on behavior and growth. Based on the median effective concentration (EC_{50}) values, body bending frequency showed relatively higher sensitivity than other behavior indicators. The inhibitions on growth and behavior of progeny were more severe than those of the parent, based on their respective EC_{50} values. Interestingly, Cd was not the most toxic metal in either parent or progeny according to EC_{50} values, but its EC_{50} ratios between parent and progeny ($EC_{50,\ parent}/EC_{50,\ progeny}$) were the most significant, indicating its greatest transgenerational effects. The results demonstrated the higher sensitivity of L3 larva stage of *C. elegans* in the transgenerational effect studies than other life stages used before. Our findings suggested that parental exposure to heavy metals can multiply their harmful effects in following generations.

1. Introduction

Heavy metals are ubiquitous pollutants worldwide. Due to their persistence and accumulation in the environment, and their bio-magnification in organisms via food chain transfer, they are gaining more ecological and public concerns (Cain et al., 2011; Yi et al., 2011). Accordingly, their adverse effects have been widely studied. They can cause death, growth and behavior inhibitions, and oxidative stresses on numerous organisms (Arambašić et al., 1995; IARC, 1997; Dhawan et al., 2000; Anderson et al., 2001; Boyd et al., 2003; Pinho and Bianchini, 2010). However, most of the effects were observed within one generation of the test organism, while the transgenerational effects are increasingly recognized as one critical aspect of toxicity studies.

The phrase of transgenerational effects means that the parental experiences in critical development stages have influences on the phenotype, behavior and even health/disease of the progeny. Thus, the transgenerational effects are referred as "the developmental origins of health and disease" (Swanson et al., 2009; Zeng et al., 2011), and therefore can be used to indicate the long-term consequences. Heavy metals had been reported to provoke transgenerational effects. Exposure to copper (Cu) during the parental development stages of *Protophormia terraenovae* (fly) resulted in immune responses which were even apparent in the offspring that was not exposed to the heavy metal (Pölkki et al., 2012). Although the study considered the influence of early parental development, it only employed one exposure concentration, which was difficult to draw any concentration-dependent conclusions.

The transgenerational effects of cobalt (Co) (Wang et al., 2007b), ion (Fe) (Hu et al., 2008), nickel (Ni) (Wang and Wang, 2008), lead (Pb) (Wang and Yang, 2007) and zinc (Zn) (Wang et al., 2007a) have been demonstrated by the decreased locomotion and body sizes in the unexposed offspring of parentally exposed *Caenorhabditis elegans* (nematode). Although these studies considered multiple concentrations in the experiments, they employed L4 larva stage (except an un-identified life stage in the study on Ni), which is not persuasive enough to represent the early parental development according to the life cycle of *C. elegans*.

The nematode *C. elegans* is a good animal model for ecotoxicological studies because of its abundance in soil ecosystem, it is convenient handling in the laboratory, and its relative high sensitivity to different kinds of stresses or toxicants (Leung et al.,

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2008). This free living nematode is mostly single self-fertilizing hermaphrodite with an occasional rare male (Blaxter and Denver, 2012). It first produces sperm at L4 stage and then stores it to fertilize the eggs that develop later in development (Hill et al., 2006). Therefore, the aforementioned exposure at L4 stage may not include all of the sperm production. Instead, L3 stage of *C. elegans* will serve better to represent the early parental development, and should provoke greater transgenerational effects on the progeny.

Various indicators of *C. elegans* have been used to elucidate the toxicity of heavy metals. A common approach to assess the health status in *C. elegans* is to measure its locomotion behavior (de Bono and Maricq, 2005). As reviewed by Leung et al. (2008), a defect in the nematode locomotion reflects an impairment of the neuronal network. One recent study also demonstrated the correlation ($R^2=0.969$, $P<0.01$) between behavior (body bend) and reactive oxygen species (ROS) production (Wu et al., 2012b). Another general of nematode health parameter is the body length (body size or growth), which has been widely used in earlier reports (Anderson et al., 2001; Boyd et al., 2003; Wang et al., 2007b; Yu et al., 2012b).

In the present study, four typical heavy metals, cadmium (Cd), Cu, Pb and Zn, were chosen as tested chemicals. These four heavy metals usually paralleled in their occurrences and even overloads in water and sediments, and their environmental concentrations were usually at μmol/L and mmol/L (Zheng et al., 2008; Suresh et al., 2012; Wang et al., 2012; Yu et al., 2012a). Currently, L3 stage of *C. elegans* was exposed to aqueous metals (Cu, Pb, Cd and Zn) at environmentally realistic concentrations for 96 h, and then the behavior and growth inhibitions on the exposed parent (P_0) were measured. The unexposed progeny (F_1) was separated and the same indicators were measured. As expected, L3 stage of nematodes indeed showed higher sensitivity than L4 stage. The transgenerational effects of heavy metals at environmentally realistic concentrations indicated that parental exposure can multiply the harmful effects of heavy metal pollution in following generations.

2. Materials and methods

2.1. Tested chemicals

Stock solutions of $CuCl_2$, $Pb(NO_3)_2$, $Cd(NO_3)_2\cdot 4H_2O$, and $ZnCl_2$ were prepared with sterilized K-medium (Williams and Dusenbery, 1990). In an earlier report (Anderson et al., 2001), the median lethal concentration (LC_{50}) value of Cu^{2+} was 990 μmol/L, and the median effective concentration (EC_{50}) values on movement were 170 μmol/L, and 47 μmol/L for Cd^{2+} and Pb^{2+}, respectively. Meanwhile, 200 μmol/L of Zn^{2+} caused approximately 50 percent inhibition on body bends of *C. elegans* (Wang et al., 2007a). Based on the published data, the concentrations of each metal in the present study were selected on the attempt to avoid complete suppression on nematodes and to represent environmentally realistic concentrations at the same time. The concentrations were 0.022–220 μmol/L for Cd, 0.047–470 μmol/L for Cu, 0.0048–48.0 μmol/L for Pb, and 0.038–380 μmol/L for Zn. Each metal has five concentrations, which were diluted from corresponding stocking solutions with sterilized K-medium. The actual metal concentrations were determined by graphite furnace atomic absorption spectrometry (Perkin-Elmer, AA-600) (Santos et al., 2008; Yu et al., 2012b).

2.2. Preparation of nematode

C. elegans (wild-type N2) and *E. coli* OP50 (the nematode food), both stocked on a nematode growth medium (NGM) agar at 4 °C in the dark, were kind gifts from Institute of Development Biology and Molecular Medicine, Fudan University, Shanghai, China. The preparation of nematodes were performed according to earlier reports (Brenner, 1974). First, *E. coli* OP50 was inoculated from the stocked NGM agar into sterile lysogeny broth (LB) culture medium (10 g tryptone, 5 g yeast extract and 10 g NaCl in 1 L distilled water, autoclaved at 121 °C for 20 min and cooled to room temperature before used). The bacteria were incubated with a shaking speed of 180 rpm at 37 °C for 24–48 h, during which the NGM agar was prepared as follows: (1) 3 g NaC1, 2.5 peptone and 17 g agar in 975 mL distilled water; (2) after autoclaving at 121 °C for 20 min, 1 mL cholesterol in ethanol (5 mg/mL), 1 mL M $CaCl_2$, 1 mL M $MgSO_4$, and 25 mL M K_3PO_4 buffer (pH 6.0) are added in order, when the agar was 40–50 °C; (3) the NGM agar was poured into sterile Petri dishes (60 mm) to cool down to room temperature. *E. coli* OP50 suspensions (approximately 150–200 μL) were spread on the NGM agars, which were kept at 37 °C for 24 h to form bacterial lawn and was cooled down to 20 °C. Then, with the help of sterile pipette tip, one eighth of the stocked NGM agar, with nematodes on it, was cut off and transferred onto the freshly prepared NGM agars (8 in total) with the bacterial lawn. The 8 NGM agars with nematodes were kept in a 20 °C incubator for 4 d.

On the fourth day, *C. elegans* was washed off each of the 8 NGM agars with 1.5 mL sterile water, and transferred to a 15 mL sterile centrifuge tube (approximately 12 mL in total). After 20–30 min allowing the nematodes to settle, 6 mL supernatants were deserted, and the nematodes in the left 6 mL were inoculated onto 30 freshly prepared NGM agars with the bacterial lawn (approximately 200 μL on each). After incubation at 20 °C for another 4 d, nematodes on the 30 NGM agars were inoculated onto 120 NGM agars with the bacterial lawn in the same way. When nematodes on the 120 NGM agars were incubated for 4 d, they were inoculated onto 480 NGM agars with the bacterial lawn. Following incubation at 20 °C for 2 d, the nematodes were ready for age synchronization.

The gravid nematodes and newly produced eggs were washed off the 480 agars (2 mL sterile water for each agar) and collected into 48 sterile centrifuge tubes (15 mL in each). After 30 min allowing the nematodes to settle, 13 mL supernatants were deserted, and in each centrifuge tube were added 12 mL fresh Clorox solutions containing 0.5 M NaOH and 1 percent NaOCl (diluted from antiformin, 4–6 percent, Sinopharm Group Co. Ltd., China) (Emmons et al., 1979). After 10–15 min, the centrifuge tubes were centrifuged at 2500 rpm for 3 min at 20 °C. With supernatants deserted, 10 mL sterile water were added in each centrifuge tubes to wash the Clorox solution off the eggs, followed by a centrifugation at 2500 rpm for 3 min at 20 °C. The wash procedure was repeated twice. Then, the synchronized eggs in each tube were resuspended with 200 μL sterile K-medium and inoculated onto a NGM agar with the bacterial lawn (48 agars in total) for 36 h to obtain L3 nematodes (Van Gilst et al., 2005). The reason why L3 nematodes were chosen for toxicity tests was to cover the L4-adult molt, when the nematode sperm, ovum and eggs start to form (Hill et al., 2006), so as to provide a window for prenatal exposure. Before used, L3 nematodes were washed off the NGM agars and fasted in sterile K-medium for 2 h to digest the food in the guts (Reinke et al., 2010).

2.3. Toxicity experiment design

The toxicity experiments were performed according to previous study with some modifications (Wang et al., 2007b). The brief steps are as follows. The exposure to the nematodes was performed in the 96-well sterile culture plates with 8 rows and 12 columns. Two heavy metals, five concentrations for each, were arranged in 10 columns with 8 wells as parallel for each concentration. K-medium was arranged in the left two columns as the controls. Then, L3 nematodes were added in the wells. Each test well typically contained 100 μL metal solutions and 100 μL K-medium containing 100 nematodes. All exposures lasted 96 h and were carried out in the absence of food at 20 °C. After the exposure, nematodes from 3 wells for each of the five metal concentrations and 6 wells of the control were collected into respective 1.5 mL centrifuge tubes. After 30 min allowing the nematodes to settle, the nematodes in the bottom were transferred into new 1.5 mL centrifuge tubes, where they were washed with 1 mL sterile water. After 30 min of settlement, the nematodes in the bottom were used for the indicator measurements of the exposed generation marked as P_0.

On the other hand, nematodes from the 5 remaining wells for each of the five concentrations and 10 wells of the control were collected into respective 1.5 mL centrifuge tubes (1 tube for each metal concentration and 2 tubes for the control). After 30 min allowing the nematodes to settle, the nematodes in the bottom were transferred into new 1.5 mL centrifuge tubes (1 tube for each treatment), where they were washed with 1 mL sterile water. After 30 min of settlement, the nematodes in the bottom were transferred to toxicant-free NGM agars with the bacterial lawn. The nematodes were allowed to grow for 36 h to produce enough eggs for the aforementioned age-synchronization (Emmons et al., 1979), after which the age-synchronized eggs were allowed to grow on NGM agars with the bacterial lawn for another 36 h. Then, the nematodes were collected into respective 1.5 mL centrifuge tubes. After 30 min allowing the nematodes to settle, the nematodes in the bottom were transferred into new 1.5 mL centrifuge tubes, where they were washed with 1 mL sterile water. After 30 min of settlement, the nematodes in the bottom were used for subsequent indicator measurements of the progeny (F_1). The whole experiment was carried out independently in triplicate.

2.4. Growth and behavior indicator

Growth and behavior indicators were determined and calculated according to our previous study (Yu et al., 2011, 2012b). Briefly, the nematodes were transferred on NGM agar without the bacterial lawn. After 2 h allowing water evaporate, the nematodes were captured with the dissecting microscope. The images captured by the dissecting microscope were used. A sequence of polylines was drawn following