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Chromium exhibits adverse effects at environmental relevant concentrations in chronic toxicity assay system of nematode *Caenorhabditis elegans*

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ABSTRACT

Here we investigated whether the assay system (10-d) in *Caenorhabditis elegans* can be used to evaluate chronic toxicity of chromium (Cr(VI)) at environmental relevant concentrations ranging from 5.2 μg L^{-1} to 260 μg L^{-1}. The results indicated that lethality, locomotion behavior as revealed by head thrash, body bend, and forward turn, metabolism as revealed by pumping rate and mean defecation cycle length, intestinal autofluorescence, and ROS production were severely altered in Cr chronically exposed nematodes at environmental relevant concentrations. The most surprising observations were that head thrash, body bend, intestinal autofluorescence, and ROS production in 13 μg L^{-1} Cr exposed nematodes were significantly influenced. The observed adverse effects of Cr on survival, locomotion behavior, and metabolism were largely due to forming severe intestinal autofluorescence and ROS production. Therefore, our findings demonstrate the usefulness of chronic toxicity assay system in *C. elegans* in evaluating the chronic toxicity of toxicants at environmental relevant concentrations.

1. Introduction

Chromium (Cr) and its compounds have been recognized as having potential severe adverse effects on health for more than 180 years. Because Cr is widely used in the chemical industry for different applications such as pigments, metal plating, or leather tanning and in chemical production, different species of Cr can be released into the environment (Unceta et al., 2010). Moreover, Cr in contaminated water and soil has hazardous effects on fish, wildlife, and invertebrates (Eisler, 1986; Lushchak et al., 2009).

Although water is important for survival and existence of life, wastewater from the urban and the industrial workplaces usually contain toxic metals including Cr (Salf et al., 2005). Besides the fact that industrial effluents are the most potential water pollutants, sewage water may also contain significant amount of heavy metals such as Cr (Latif et al., 2008). It was reported that 0.004–5.62 mg L^{-1} Cr existed in irrigation water samples from Korangi industrial area in Pakistan (Salf et al., 2005). Chromium contents in some of the tubewell water samples in Rawalpindi area were observed above the maximum permissible samples (Latif et al., 2008). Especially, it has been found that the naturally occurring Cr(VI) in ground and surface waters at values exceeding the World Health Organization limit for drinking water of 50 μg L^{-1} (Velma et al., 2009). Therefore, it is very important to evaluate the chronic toxicity of Cr at environmental relevant concentrations.

Caenorhabditis elegans, a free living nematode that lives mainly in the liquid phase of soils, is considered as an ideal model animal for the study of environmental evaluation and toxicity (Leung et al., 2008). *C. elegans* is now widely used in ecotoxicological studies utilizing various exposure media, including water, soil, and sediment (Mutwakil et al., 1997; Traunsperger et al., 1997; Peredney and Williams, 2000). Assays employing lethality, and sublethal endpoints including growth, reproduction, lifespan, locomotion behavior, perception, neuronal development, synaptic function, learning, memory, stress response, and oxidative damage have been developed and applied for environmental biomonitoring (Dhawan et al., 1999; Wang and Xing, 2008, 2009; Ye et al., 2008, 2010; Du and Wang, 2009; Guo et al., 2009; Xing et al., 2009; Tvermoes et al., 2010; Zhang et al., 2010; Jeong et al., 2011; Wu et al., 2011). Nevertheless, most of the studies performed in *C. elegans* are still focused on the evaluation and toxicology of acute exposure to toxicants.

In our previous study, specific developmental stage for adults from day 1 to day 10 was selected to evaluate the chronic toxicity of some heavy metals, because the population of dead nematodes and the lipofuscin deposition caused accumulation of intestinal autofluorescence increased sharply after day 10 in *C. elegans* (Shen et al., 2009). Such an assay system was further used to evaluate the chronic toxicity from Al_2O_3-nanoparticle exposure (Yu et al., 2011); however, it is still unclear whether such an assay system can be used to evaluate the chronic toxicity from toxicants at environmental relevant concentrations. In the present study, we selected the heavy metal of Cr(VI) to investigate its possible chronic toxicity after exposure at environmental relevant

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concentrations with the aid of lethality, locomotion, metabolism, intestinal autofluorescence, and reactive oxygen species (ROS) production as endpoints. Endpoints of lethality, intestinal autofluorescence, and ROS production have been already used in the chronic toxicity assay system in nematodes (Shen et al., 2009; Yu et al., 2011), and locomotion and metabolism are new introduced endpoints due to their closely associations with aging process. The main aim of this study was to examine the usefulness of this assay system in nematodes for evaluating the chronic toxicity from toxicants at environmental relevant concentrations.

2. Materials and methods

2.1. Reagents and strains

Five concentrations (5.2, 13, 26, 52, and 260 $\mu g\ L^{-1}$) of Cr(VI) solutions were used, and the selected environmental relevant concentrations were referred to previous reports (He et al., 2005; Salf et al., 2005). The used reagent was potassium dichromate (purity, 99.95%). Metal concentrations of exposed solutions were analyzed by atomic absorption spectrophotometry (AAS; Pye-Unicam model SP9, Cambridge, UK). The chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA).

Nematodes used were wild-type N2, originally obtained from the *Caenorhabditis* Genetics Center (CGC). They were maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50 at 20 °C (Brenner, 1974). Gravid nematodes were washed off the plates into centrifuge tubes, and were lysed with a bleaching mixture (0.45 M NaOH, 2% HOCl). Age synchronous populations were obtained by the collection as described (Donkin and Williams, 1995). The young adult were washed with modified K medium (50 mM NaCl, 30 mM KCl, 10 mM NaOAc, pH 5.5) (Williams and Dusenbery, 1990). Exposures were performed in 12-well sterile tissue culture plates as described (Mutwakil et al., 1997). Three hundred microliters of each diluted solution was added onto a NGM plate (6 cm diameter) containing 7.5 mL of NGM agar, and immediately spread to contain Cr at the indicated final concentrations. The bacteria (OP50) lawn was rinsed with 10 $mg\ mL^{-1}$ palmitic acid in ethanol to keep the nematodes from crawling off the surface of the agar plate because palmitic acid will precipitate on the plate to form a crunchy barrier that nematodes do not like to crawl through (Miler and Roth, 2009). The test NGM plates were freshly prepared prior to use. For the chronic exposure assay, 5′-fluoro-2′-deoxyuridine (FUdR), an inhibitor of DNA synthesis, was used to prevent production of offspring from reproducing without otherwise interfering with the organism's post-maturational development (Michell et al., 1979) by adding it into the test NGM plates at a final concentration of 25 μM. The exposures were performed from day 1 to day 10 on adult nematodes at 20 °C, and after exposure the toxicity of Cr was evaluated with endpoints of lethality, locomotion behavior, metabolism, intestinal autofluorescence and ROS production. From day 1 to day 10, the control or exposed nematodes were transferred to a new test NGM plate with food each day in order to ensure the food supply.

2.2. Lethality

For the lethality assay, a 1.0 mL aliquot of test solution for Cr was added to each of the wells of tissue culture plate, which was subsequently loaded with approximately 50 nematodes for each concentration. Following exposure, the wells were observed under a dissecting microscopy, where the inactive ones were scored. The nematodes were judged to be dead if they did not respond to stimulus using a small, metal wire. The lethality was evaluated by the endpoint of percentage of survival animals. Three replicates were performed.

2.3. Locomotion behaviors

Locomotion behaviors of nematodes were evaluated by head thrash, body bend, and basic movement (Wang and Xing, 2008). To assay the head thrash, nematodes were washed with modified K medium. Every nematode was transferred into a microtiter well containing 60 μL of modified K medium on the top of agar. After a 1-min recovery period, head thrashes were counted for 1-min. A thrash was defined as a change in the direction of bending at the mid body. To assay the body bend, nematodes were picked onto a second plate and scored for the number of body bends in an interval of 20 s. A body bend was counted as a change in the direction of the part of the nematodes corresponding to the posterior bulb of the pharynx along the *y* axis, assuming that the nematode was traveling along the *x* axis. To assay the basic movement, forward sinusoidal movement (forward turns) in a 20-s interval was measured (Murakami et al., 2005). Fifty nematodes were examined per treatment. All the nematodes were incubated under the same conditions, and the locomotion behaviors of control and treated nematodes were analyzed simultaneously to avoid the possible influences of light–dark cycle on the locomotion behaviors of examined nematodes. Three replicates were performed.

2.4. Metabolism

Pumping rate and mean defecation cycle length were selected to evaluate the metabolism state of nematodes (Iwasaki et al., 1995; Lazakovitch et al., 2008). To assay the pumping rate, nematodes were placed onto NGM places with food, and left undisturbed for 1 h before measuring. Pharyngeal pumping was counted for 1 min under DIC optics with a Ziess axioscope. To assay the mean defecation cycle length, individual nematode was observed for a fixed number of cycles, and a cycle period was defined as the interval between the initiations of two successive posterior body-wall muscle contraction steps. Thirty nematodes were examined per treatment. Three replicates were performed.

2.5. Endogenous intestinal autofluorescence

The photography of intestinal autofluorescence was performed as described (Shen et al., 2010). Intestinal autofluorescence caused by lysosomal deposits of lipofuscin, a valuable marker for cellular damage in cells, can accumulate over time in aging nematodes (Garigan et al., 2002). The images were collected for endogenous intestine fluorescence using a 525-nm bandpass filter and without automatic gain control in order to preserve the relative intensity of different animal's fluorescence. Adults cultured at 20 °C were photographed on the same day to avoid effects of light source variance on fluorescence intensity. Observations of the fluorescence were recorded and color images were taken for the documentation of results with Magnafire® software (Olympus, Irving, TX, USA). Lipofuscin levels were measured using ImageJ Software (NIH Image) by determining average pixel intensity in each animal's intestine. More than 50 animals were counted for the statistical analysis. Three replicates were performed.

2.6. ROS production

To quantify whether chronic Cr exposure increases ROS levels in *C. elegans*, the ROS production was assayed (Wu et al., 2011). The examined nematodes were transferred to 1 mL of M9 buffer containing 1 μM CM-H2DCFDA and pre-incubated for 3 h at 20 °C, and then mounted on 2% agar pads and examined with a laser