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Histological Alterations in the Body Wall of the Tropical Earthworm *Eudrilus eugeniae* Exposed to Hexavalent Chromium

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Abstract The tropical earthworm *Eudrilus eugeniae* was chronically exposed to hexavalent chromium (Cr) in its substrate over a concentration range from 0.24 to 893 mg kg^{-1}. Histological alterations in the body wall epithelium included cell fusion, reduction in thickness of the epithelial layer, a marked increase in pyknotic nuclei and epithelial sloughing. Similar changes were noted in the circular and longitudinal muscles with damage being indicated by the prominent inter-muscular cell spaces and disintegration. Many of these noted alterations intensified with increasing levels of exposure. It is significant that some of the changes recorded here were evident even at the lowest concentration of 0.24 mg kg^{-1}, an environmentally relevant concentration. Hence, the observed trends could be taken as an early warning to the imminent threats of heavy metal pollution to epigeic earthworm species.

Keywords Earthworms · Heavy metal · Histology · Pollution · Toxicity

Heavy metals are used in many industries around the world and their adverse effects have been known for a long time. Although many precautions have been taken to limit the exposure of heavy metals to humans, the adverse effects exerted on animals have been generally neglected. Chromium (Cr) is a heavy metal that exists in both trivalent and hexavalent forms; the former is needed in minute quantities for glucose and lipid metabolism in humans and animals (Rangasamy et al. 2013), but is known to be toxic at high doses, while the latter is highly toxic even at very low doses (Sivakumar and Subbhuraam 2005). Industries such as tanneries, paint, electroplating and dyeing, discharge untreated effluents which contain high levels of Cr. Therefore soil-dwelling and aquatic fauna inhabiting areas in the vicinity of contaminated discharges could be directly affected by Cr toxicity.

Earthworms are beneficial organisms playing multiple roles as detritivores, decomposers and aerators facilitating soil enrichment. In addition, epigeic earthworms are extensively used in producing vermicompost for agriculture. They are highly sensitive and therefore have been used as indicators of soil pollution (e.g. Lee 1992; Reinecke and Reinecke 2004) and as standard test organisms in ecotoxicological studies. Many such studies using earthworms have focused on monitoring the effects of contaminants on survival, reproduction and bioaccumulation (e.g. van Gestel et al. 1989; Robidoux et al. 1999; Hobbelen et al. 2006; Shi-ping et al. 2007; de Silva et al. 2010), but few have examined histological effects. In this study we report the effects of hexavalent chromium on the histology of the body wall of the tropical earthworm species *Eudrilus eugeniae*.

Materials and Methods

E. eugeniae specimens were collected from the culture facility at the Tea Research Institute, Sri Lanka, and acclimatized for 2 weeks in earthenware pots with banana stems as the substrate (as recommended by Jais and Hassan 2008; Kavitha et al. 2010). Adult worms with a well-developed clitellum and of a mean weight of 290 ± 9 mg were used for chronic exposure trials.

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To ensure that the tested concentrations were of direct relevance to field levels and to treat the substrate on which earthworms would be reared, we used Cr levels 0.002–0.20 mgL^{-1} as these are within previously recorded field levels in surface waters of Sri Lanka (CEA/Euroconsult 1993; Manage and Wijesinghe 2009). Additionally, to depict conditions that might occur at points of effluent discharge, we used two higher levels (2 and 20 mgL^{-1}) for treatment of the substrate. Analytical grade potassium dichromate (Sigma-Aldrich, USA, 99 % purity) was used to prepare the relevant test concentrations (Manerikar et al. 2008).

Banana stems collected from home gardens were cut into cylindrical blocks of 200 ± 9 g and 12 ± 2 cm diameter, washed thoroughly by soaking and rinsing in aged tap water and air dried, and subsequently soaked in the relevant test concentrations for 24 h. Stems used for control pots were soaked in aged tap water for a similar duration. To accurately ascertain the levels of Cr in banana stems after treatment with Cr solutions (as these are the levels to which the earthworms would be exposed), the banana stems were homogenized, dried at 105°C and digested with HNO_3 and H_2O_2 and analyzed (AA 6650 Shimadzu Atomic Absorption Spectrophotometer [Duisburg, Germany], graphite furnace for low concentrations – detection limit –0.04 μgL^{-1}; and flame AAS for high concentrations – detection limit –0.005 mgL^{-1}: 357.9 nm wave length; 10 mA current; 0.5 nm slit width). Analytical precision was monitored by running triplicates for each sample where a relative standard deviation (RSD) <10 % was recorded. Accuracy was ascertained by analyzing a Cr standard for AAS (Sigma Aldrich, Buchs, Switzerland), which gave a recovery rate of 99 %. Wet digestion is recommended as suitable for Cr by ASTM (2000) and this method gave a general recovery rate of 85 % for spiked banana stems.

Earthenware pots of 1 L capacity used for the trials were thoroughly washed and soaked in aged tap water for 1 week prior to use. Trials were conducted in triplicate for treatments and controls. Since this setup was also used to monitor effects of Cr on growth and mortality of the earthworms (not reported here), ten worms were carefully placed on the surface of the banana stems at the centre of each pot and covered with muslin cloth. Animals were kept under ambient conditions with natural light (approximately 12 h light: 12 h dark), temperature (28–31°C) and relative humidity (50 %–55 %). The moisture content was maintained throughout the trial by checking the moisture loss by weighing the pots at 3 days intervals and watering accordingly (de Silva et al. 2010). The substrates were replaced weekly with freshly treated stems.

At the end of 28 days, earthworms were sacrificed and used for histological examination of the body wall. Ethical clearance for toxicity trials was obtained from the Institute of Biology, Sri Lanka (Ref.No.ERCIOB103/02/13). Earthworms removed from the substrate were washed with distilled water and allowed to depurate for 24 h on moist filter paper, frozen (Sharma and Satyanarayan 2011; Morowati 2000), fixed in Zenkar's fixative overnight, dehydrated in a graded alcohol series and embedded in paraffin. Cross sections of 7 µm thickness were prepared using a Rotary microtome (Yamato, Tokyo, Japan) and stained with haematoxylin and eosin. Slides were examined under a light microscope (Nikon SE, Japan) at ×400 magnification and observations were noted. Measurements were made using an ocular micrometer. Images were taken under a phase contrast microscope (Nikon –1001v, Tokyo, Japan). Several parameters previously reported by Amaral et al. (2006) and Muangphra and Gooneratne (2011) as being affected in earthworms were examined, and some were quantified. The quantified parameters were thickness of the epithelium, the number of pyknotic (small and dark) and normal nuclei (large and light colored) in a given area of the outer epithelium, and the width of the space between the epithelium and the underlying circular muscle layer. A total of six earthworms were examined per treatment and the control, with five sections being taken from each. Readings were taken per section along radial lines (Fig. 1) (Muangphra and Gooneratne 2011) giving a total of 120 (6 × 5 × 4) observations per treatment and control. Pyknotic and normal nuclei in the epithelium were counted in microscopic fields located at similar positions i.e. at four points along the radial lines, to obtain 120 readings per treatment and control. Statistical comparisons were done

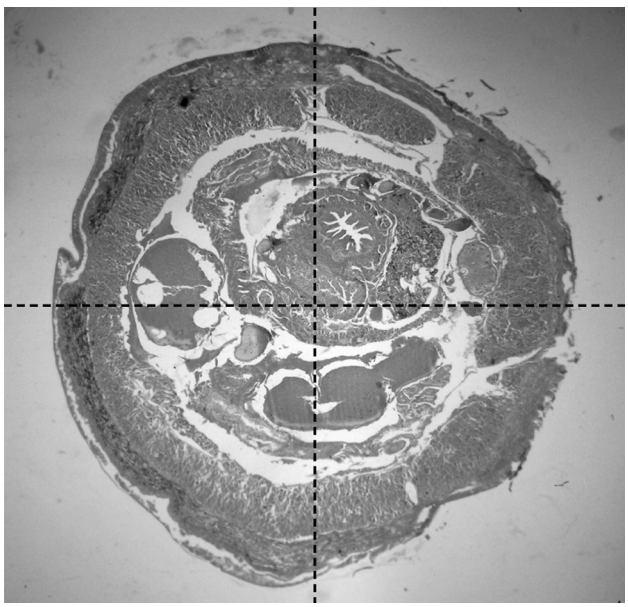

Fig. 1 Thickness of the epithelium was measured along the axes shown

between control and treatment values using one-way ANOVA and Tukey's tests, while the Pearson's correlation test was used to test for dose-dependency using Minitab 14.

Results and Discussion

The measured concentrations of Cr in banana stems (throughout the trial) were 0.24 ± 0.01, 1.69 ± 0.10, 13.5 ± 1.12, 146 ± 13.8 and 893 ± 55.8 mg kg^{-1}. Four of these levels were well within those recorded in polluted soils in Sri Lanka (e.g. 50–251 mg kg^{-1} – Domingo and Kyuma 1983; 103 mg kg^{-1} – Wijegoonawardena 1995; 1–250 mg kg^{-1} – Illeperuma 2000). No Cr was detected in the controls.

Typically the body wall of the earthworm consists mainly of the outer most cuticle secreted by the epithelium underlying it and the circular and longitudinal muscle layers. The epithelium is composed of a single layer including columnar cells, basal cells and mucous secreting cells (Edwards and Bohlen 1996). As observed, the body wall of the control group in the present study was in line with this typical structure. The epithelial layer contained neatly arranged intact cells with distinct margins and nuclei, and distinct basal and mucous cells (Fig. 2a). The muscle cells were also neatly compacted with no marked intercellular spaces. In contrast to the organized structure of the body wall of the control worms, several structural alterations were evident in Cr(VI) treated earthworms. Exposure to the metal caused serious disintegration of cell margins and fusion of cells in the epithelium (Fig. 2b) even at low doses of 0.24 mg kg^{-1} with severity of the damage intensifying with increasing levels of exposure. At 893 mg kg^{-1} the damage was intense with the entire epithelium being disintegrated; the epithelial cell layer was completely lost in certain regions.

Such histological alterations have been reported previously for earthworms exposed to other heavy metals and herbicides. For instance, the loss of cellular integrity and the fusion of and reduction in cell margins observed here have been documented by Lourenço et al. (2011) in *Eisenia andrei* exposed to soil contaminated with a collection of metals (not including Cr) and radionuclides. They further report that this damage ultimately leads to necrosis as the exposure prolongs. Gobi and Gunasekaran (2010) have reported cell fusion in the intestinal epithelium of *E. fetida* exposed to an herbicide.

The mean thickness of the epithelium of the exposed worms was significantly reduced compared to the control ($F_{5,714} = 111.83$, $p < 0.05$); mean thickness in the control earthworms was 61.6 ± 2.6 µm whilst the mean thickness in those exposed to 1.69 mg kg^{-1} was 28.0 ± 1.4 µm (Fig. 3). Muangphra and Gooneratne (2011) reported a significant decrease in the radial thickness of the body wall of *Pheretima peguana* exposed to neem extract (*Azadirachta indica*), which is a heavily used natural herbicide in the tropics. Amaral et al. (2006) provide evidence that the radial thickness of the intestinal epithelium is reduced in *Lumbricus terrestris* inhabiting volcanic soils containing mainly Zn and Cd. In contrast, an enlargement of the epithelial cell lining has been reported by Kılıç (2011) in *L. terrestris* in the Porsuk River Basin, Turkey, polluted with a combination of heavy metals which included Cd, Cr, Cu, Pd and Ni.

Further in the present study, a significant reduction was seen in the number of normal nuclei in the cells of the epithelium of the treated worms compared to the control ($F_{5,714} = 215.17$, $p < 0.05$) (Fig. 4), while a concurrent

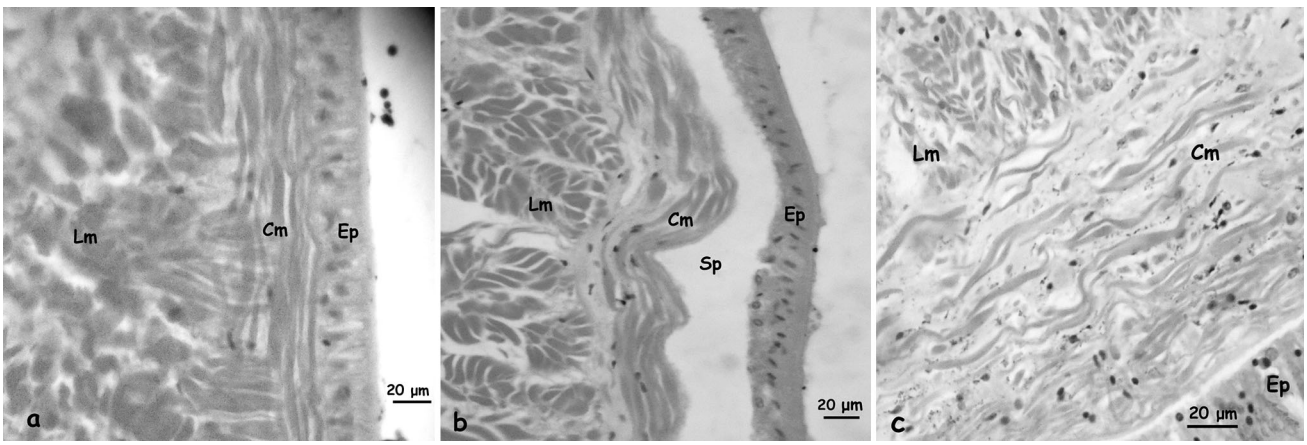

Fig. 2 **a** Body wall of a control earthworm with compact and distinct cells and normal nuclei in the epithelium (Ep) and intact circular (Cm) and longitudinal (Lm) muscle layers, and the body wall of Cr treated worms showing **b** disintegrated and fused epithelial cells (Ep) and the separation of the epithelium from the underlying circular muscle layer (Cm) as indicated by a space (Sp) at 146 mg kg^{-1} and **c** the thinning and dispersion of circular muscle cells (Cm) at 13.5 mg kg^{-1}

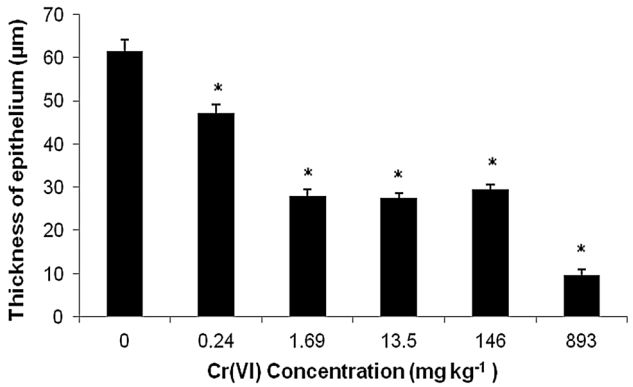

Fig. 3 Variation in the thickness (mean ± standard error) of the outer epithelium of earthworms exposed to different concentrations of Cr(VI). *Asterisk* indicates concentrations at which mean thickness was significantly different ($p < 0.05$) from that of the control

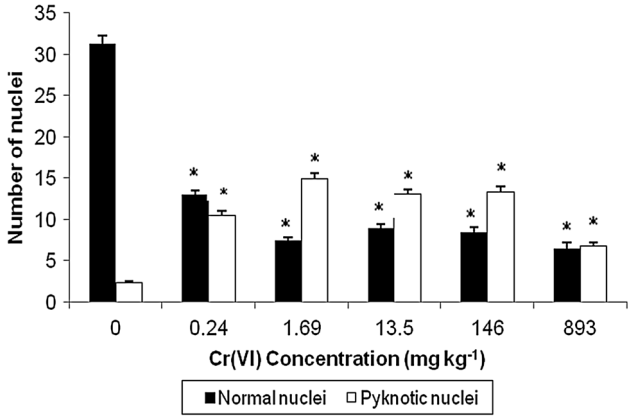

Fig. 4 Variation in the number of normal nuclei and pyknotic nuclei (mean ± standard error) in the epithelium of the earthworms exposed to different Cr(VI) levels. *Asterisk* indicates a significant difference ($p < 0.05$) from the respective control

significant increase was seen in the number of pyknotic nuclei ($F_{5,714} = 87.52$, $p < 0.05$) (Fig. 4) (normal nuclei, 31.3 ± 1.0 in control and 7.4 ± 0.4 in 1.69 mg kg^{-1}; pyknotic nuclei, 2.3 ± 0.2 in control and 10.5 ± 0.5 in 0.24 mg kg^{-1}). Nuclei in the epithelial tissues of earthworms exposed to 893 mg kg^{-1} were rarely visible because of the severe damage to the epithelium itself. The observation of pyknotic nuclei in epithelial cells has also been reported as a histological aberration by Morowati (2000) and Gobi and Gunasekaran (2010) in other earthworms exposed to different herbicides. The formation of pyknotic nuclei by the condensation of chromatin followed by nuclear fragmentation ultimately causes cell death. Thus, pyknotic nuclei in the outer epithelium of *E. euginiae* in the present study, which was observed in increasing incidence as levels of Cr exposure increased, may be taken as an early sign of cell necrosis. Although there was a significant difference in the values recorded for the above

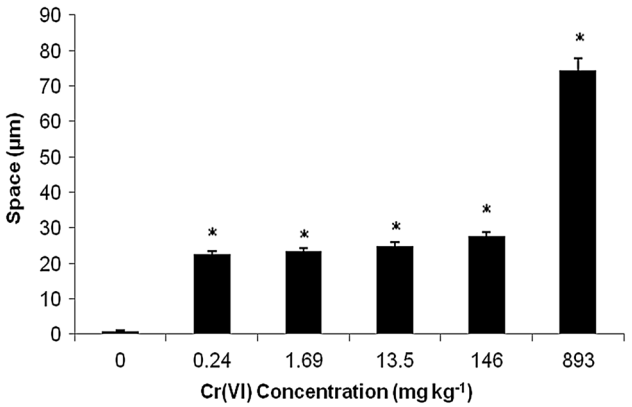

Fig. 5 Variation of interlayer space (mean ± standard error) in the body wall of earthworms exposed to different levels of Cr(VI). *Asterisk* indicates concentrations at which mean values were significantly different ($p < 0.05$) from that of the control

parameters between the controls and treatment, there was no significant dose-dependent trend (epithelial thickness $r^2 = 0.49$, $p > 0.05$; normal nuclei $r^2 = 0.13$, $p > 0.\ 05$; pyknotic nuclei $r^2 = 0.09$, $p > 0.05$).

Epithelial sloughing was apparent by the space created between the epithelium and the circular muscle layer (Fig. 2b). This interlayer space was more pronounced in treated earthworms than in the controls with a dose dependent trend being evident ($r^2 = 0.87$, $p < 0.05$) (Fig. 5) (thickness of space, 0.73 ± 0.3 µm in the control worms and 23.6 ± 0.6 µm in those exposed to 1.69 mg kg^{-1}). A similar histological alteration has been shown in the intestinal epithelium of the same test species *E. euginiae,* but exposed to Cu (Sharma and Satyanarayan 2011).

The circular and longitudinal muscle layers of the body wall of the Cr(VI) treated *E. eugeniae* were also damaged as indicated by the loss of structural integrity and increased intercellular spaces (Fig. 2c). The damage was more severe in those exposed to 893 mg kg^{-1}. Such damage to body wall muscles has also been documented elsewhere, for other earthworm species exposed to different toxicants. Lourenço et al. (2011) documented necrosis and loss of cell shape and organization of the muscle fibers in *E. andrei* exposed to metal and radionuclide contaminated soil, while gaps in muscles of the body wall in *P. peguana* exposed to neem extract has been reported by Muangphra and Gooneratne (2011). Many have speculated that the extensive deformations in the muscles and intestinal epithelium of earthworms exposed to heavy metals are due to metal bioaccumulation within these tissues (Amaral and Rodrigues 2005; Kiliç 2011).

The present study demonstrated that Cr(VI) induced structural alterations in the body wall of the studied tropical earthworm species. The body wall could be expected to be

a significant site of damage since dermal contact is one of the main routes of entry of toxicants into these animals (Lourenço et al. 2011). What is of significance is the fact that histological changes were induced at low and environmentally relevant concentrations of Cr in Sri Lanka. This paper for the first time showing dose dependency in Cr-induced histopathological damage in tropical earthworms, suggests that setting standards for the release of environmental contaminants is vital. The trend of increasing sensitivity of earthworms with elevated levels of Cr confirms their potential for use as an indicator species of soil toxicants.

Some are of the view that the ability of earthworms to accumulate heavy metals could be used as a beneficial trait for soil remediation (Iordache and Borza 2012). However, the histological damage observed in the present study, even at the lowest level of 0.24 mg kg^{-1}, suggests that such uses of earthworms may not be sustainable in the long term if soils are contaminated with certain metals.

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