Bull Environ Contam Toxicol (2009) 83:525–529
DOI 10.1007/s00128-009-9814-5

Effect of Cadmium on Haemocyte Viability of the Woodlouse *Porcellio laevis* (Isopoda, Crustacea)

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Received: 2 April 2008 / Accepted: 25 June 2009 / Published online: 8 July 2009

Abstract This study investigated the effects of cadmium on haemocyte viability of the woodlouse *Porcellio laevis*, using the trypan blue exclusion assay. The ultimate aim is to determine the usefulness of this cellular response as biomarker of cadmium exposure. Results showed that exposure to sublethal concentrations of cadmium, with concomitant cadmium accumulation in the body, significantly lowered the percentages of viable haemocytes in *P. laevis*. This response was already observed after the first week of exposure and could therefore possibly serve as an early warning of cadmium exposure. A field study is needed to validate these findings.

Keywords Woodlouse · Cadmium · Haemocyte viability · Trypan blue

Woodlice play a major role in the functioning of soil ecosystems by enhancing soil structure and by decomposing organic material (Kammenga et al. 2000) and are known to be macroconcentrators of metals such as zinc, copper and cadmium (Hopkin and Martin 1982; Martin and Coughtrey 1982). Laboratory and field studies in South Africa showed similar results for *Porcellio laevis* with cadmium (Odendaal and Reinecke 1999; 2004).

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Cellular biomarkers have often been proposed as tools to assess the ecotoxicological effects of environmental contaminants (Kammenga et al. 2000). Biomarkers in general have potentially useful features: their responsiveness and sensitivity may give an early alarm of toxicant impacts on organisms, well before ecological disturbances can be discerned. Also, they may give a more direct and accurate insight into the relationship between toxicant exposure (cause) and the biological response (effect) (Weeks 1998; Morgan et al. 1999).

In invertebrates, the function of cellular defense is mainly performed by circulating haemocytes, through phagocytosis (Galloway and Depledge 2001). Invertebrate haemocytes have been shown to sequester heavy metals such as cadmium, zinc and copper (Robinson and Ryan 1988, McIntosh and Robinson 1999). It is well known that cadmium damages cell membranes (e.g. Müller 1984; Steffensen et al. 1994; Tátrai et al. 2001), thereby changing the permeability of the membranes and eventually causing cell death. The trypan blue exclusion assay is a well-established technique used in medical research, to measure plasma membrane integrity and therefore cell viability (Müller 1984; Steffensen et al. 1994; Dacasto et al. 2001). This assay is based on the fact that cells with intact membranes will exclude the trypan blue dye, whereas cells with damaged membranes will take up the dye and the cytoplasm will be stained (Harbell et al. 1997). The trypan blue exclusion assay has however, not been investigated as a potential technique to use in ecotoxicological biomarker research.

The aim of the present study was to investigate the toxicity of cadmium to the terrestrial woodlouse *Porcellio laevis*, by studying the accumulation of cadmium over time and the corresponding changes in haemocytic viability, using the trypan blue exclusion assay. The ultimate aim is

to determine the usefulness of this assay as biomarker of stress resulting from cadmium exposure.

Materials and Methods

Experimental exposures in the laboratory were conducted using specimens of *P. laevis* collected from a compost heap at an unpolluted site. The woodlice used in the experimental exposures were those with a body length of between 7 and 9 mm. The sex of the animals was not taken into account in this investigation. Containers used in these tests were PVC cylinders of 110 mm in diameter and 130 mm in length. Plaster of Paris bottoms in each container provided a moistened environment in the containers. Decaying oak leaves (*Quercus robur*) collected from an uncontaminated site were used as substrate in the experiments. The leaves were shredded into small pieces and spread out on a flat surface to dry, at room temperature. To each of the containers used, 30 g dry mass leaves were added. Cadmium was sprayed onto the dried leaves, as $CdSO_4$ solutions. Concentrations used were 10 and 20 mg kg^{-1} (dry mass) $CdSO_4$. For each of the concentrations, three replicates with 12 woodlice each were prepared. A control in which the leaves were sprayed only with distilled water was also prepared. The exposure experiments were performed at 20–23°C, with a photoperiod of 12:12, for a period of 4 weeks.

Haemocyte viability of six woodlice from each treatment group was determined at the end of each exposure week. Twenty microliters (µl) of haemolymph was drawn from each woodlouse, into 30 µl of temperature-adjusted PBS, using a needle and syringe. The cell suspension was then added to an equal volume of trypan blue. After five minutes, the numbers of stained and unstained cells were counted under a light microscope, using a Neubauer haemocytometer chamber. No distinction was made between different types of haemocytes. The results were expressed as percentage viable cells. This was calculated by dividing the number of unstained cells by the total number of cells counted per sample.

Each week, after performing the cell counts, the woodlice were placed in small vials and stored in a freezer, for future metal analysis. For the latter procedure, woodlice were dried at 60°C for 48 h, weighed and digested in boiling 55% nitric acid, according to the procedure used by Odendaal and Reinecke (1999). The samples were filtered through 0.45 µm Millipore filter and made up to 10 mL with distilled water. To keep a check on possible contamination 'blanks' were also digested and analysed for cadmium. These 'blanks' did not contain any woodlouse samples. The analysis for cadmium was done with a Varian AA-1275 flame atomic absorption spectrophotometer. The detection limit of the FAAS for cadmium was 0.1 ppm. The cadmium concentrations were calculated by using the following formula: [(sample value−blank value) × 10]/mass. Cadmium concentrations were expressed as mg kg^{-1} dry mass.

The data from the experimental exposures were statistically analyzed by means of the One Way ANOVA. This was followed by multiple pairwise comparisons, using the Student-Newman-Kuels Method (Jandel Scientific Sigmastat 3.1).

Results and Discussion

Cadmium concentrations measured in specimens of *P. laevis* after exposure to different concentrations of cadmium sulphate are illustrated in Figs. 1, 2, 3. No cadmium was detected in control animals (Fig. 1). There were no statistically significant differences ($p > 0.05$) found in terms of cadmium accumulation over the 4 week exposure period, in woodlice exposed to 10 mg kg^{-1} cadmium sulphate (Fig. 2). In the 20 mg kg^{-1} exposure group, cadmium accumulation increased significantly ($p < 0.05$) over consecutive exposure weeks (Fig. 3).

Weekly comparisons between the 10 and 20 mg kg^{-1} exposure groups showed statistically significant differences ($p < 0.05$) after 1, 2 and 4 weeks of exposure. Cadmium accumulation after the first and second week was higher in the 10 mg kg^{-1} exposure group than in the 20 mg kg^{-1} group. However, after 4 weeks of exposure, cadmium accumulation was higher in the 20 mg kg^{-1} than in the 10 mg kg^{-1} exposure group (Figs. 2, 3).

The percentage viable haemocytes of *P. laevis*, after exposure to different concentrations of cadmium sulphate, are illustrated in Figs. 1, 2, 3. No statistically significant

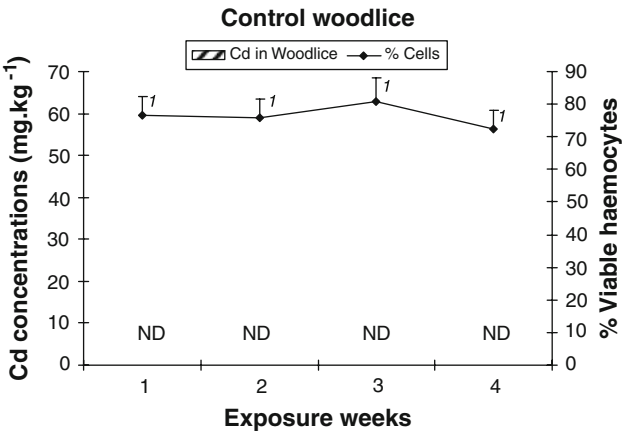

Fig. 1 Mean (±SD) percentage viable haemocytes of *Porcellio laevis*, from the control group, over a period of 4 weeks. ND = not detected. No cadmium could be detected in the control woodlice. Statistical significant differences are indicated by different letters in the case of cadmium in woodlice and different numbers in the case of percentage viable haemocytes. (n = 6)

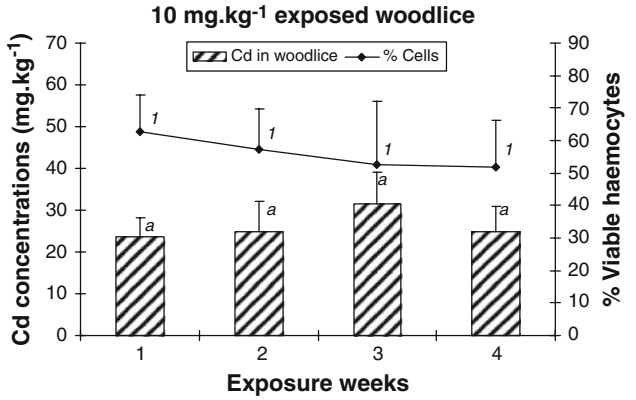

Fig. 2 Mean (±SD) body cadmium concentrations (mg kg^{-1} dry mass), as well as mean (±SD) percentage viable haemocytes, measured in *Porcellio laevis*, exposed to 10 mg kg^{-1} cadmium sulphate, over a period of 4 weeks. Statistical significant differences are indicated by different letters in the case of cadmium in woodlice and different numbers in the case of percentage viable haemocytes. (n = 6)

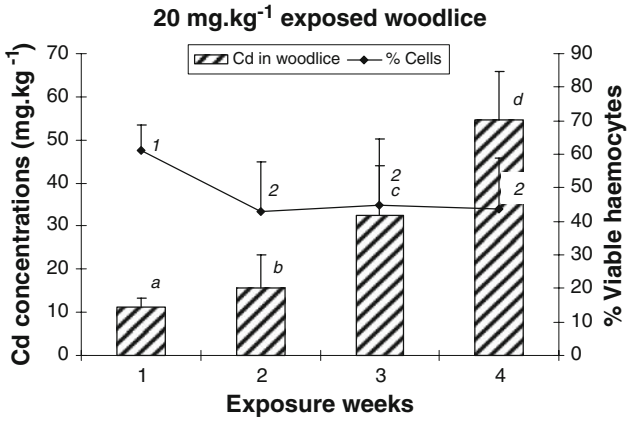

Fig. 3 Mean (±SD) body cadmium concentrations (mg kg^{-1} dry mass), as well as mean (±SD) percentage viable haemocytes, measured in *Porcellio laevis*, exposed to 20 mg kg^{-1} cadmium sulphate, over a period of 4 weeks. Statistical significant differences are indicated by different letters in the case of cadmium in woodlice and different numbers in the case of percentage viable haemocytes. (n = 6)

differences were found for control woodlice, when their percentage viable haemocytes were compared over the 4 weeks of exposure ($p > 0.05$) (Fig. 1). Similarly, no differences were found over the 4 week exposure period for woodlice exposed to 10 mg kg^{-1} cadmium sulphate ($p > 0.05$) (Fig. 2). In the 20 mg kg^{-1} cadmium sulphate exposure group, however, there was a significant ($p < 0.05$) decrease in percentage viable haemocytes from week 1 to week 2 but no differences from week 2 through to week 4 ($p > 0.05$) (Fig. 3).

Percentage viable haemocytes of the 10 and 20 mg kg^{-1} exposure groups were significantly ($p < 0.05$) lower over the entire exposure period, when compared to that of the control group (Figs. 1, 2, 3). Although the percentage viable cells of the 20 mg kg^{-1} exposed woodlice were consistently lower than that of the 10 mg kg^{-1} woodlice throughout the exposure period, these differences were not statistically significant ($p > 0.05$) (Figs 1, 2, 3).

Results showed that cadmium was accumulated in *P. laevis*, to concentrations greater than the exposure concentration. This has been illustrated before, for various woodlouse species (e.g. Hopkin 1990; Odendaal and Reinecke 1999; 2004). In the 20 mg kg^{-1} exposure group, a steady increase over consecutive exposure weeks was observed. A relationship between exposure concentration and internal dose was also seen in the fourth week, with the significantly higher body cadmium concentrations found in the highest exposure group. However, in the first half of the experiment, after the first and second weeks, the lower exposure group had accumulated higher body cadmium loads. This may be due to the ability of woodlice to detect cadmium concentrations and to show avoidance responses (Odendaal and Reinecke 1999). It could be that the woodlice in the present study, exposed to 20 mg kg^{-1} cadmium sulphate, detected the cadmium in their food to a greater degree than the woodlice of the 10 mg kg^{-1} exposure group, and therefore had a lower food intake. This would have resulted in a slower cadmium bioaccumulation rate.

The results of the present study also clearly showed that cadmium accumulation in *P. laevis* lowers the percentage of viable haemocytes: the percentages viable cells in the control group were consistently higher than in the cadmium-exposed groups. According to Sokolova et al. (2004), enhanced haemocyte death has serious implications for the animal, since it has the potential to cause immunosuppression and a reduced capacity to resist infections.

Since the trypan blue dye is only taken up by cells with impaired plasma membrane functioning, it is clear from the present results that the accumulated cadmium lowered plasma membrane integrity. However, other factors, apart from plasma membrane destabilization, may also have played a role in the lower percentages of viable haemocytes measured. Such factors include impaired lysosomal membrane integrity and mitochondrial damage. Lysosomes concentrate a wide range of contaminants such as metals, resulting in an increase in membrane permeability, loss of lysosomal contents into the cytosol and cellular damage (Moore 1990). This response, as measured with the neutral red retention time bioassay, has been illustrated for various invertebrates, exposed to a number of metals (e.g. Svendsen and Weeks 1995; Snyman et al. 2000; 2002; Matozzo et al. 2001; Hankard et al. 2004) and in particular for woodlice (digestive gland cells), exposed to cadmium (Nolde et al. 2006). Mitochondria are also known to be targets for cadmium accumulation (Dorta et al. 2003; Sokolova et al. 2004). In oysters, cadmium has been shown

to inhibit phosphorylation rate, decrease mitochondrial coupling and deplete intracellular ATP levels, which eventually lead to haemocyte apoptosis and necrosis (Sokolova et al. 2004).

In the 10 mg kg^{-1} cadmium sulphate exposed group, the percentage viable haemocytes followed the pattern of internal cadmium dose to some extent (Fig. 2). However, this was not statistically significant. In the 20 mg kg^{-1} exposure group, a significant decrease in percentage viable cells was already seen after the first and second weeks of exposure, after which the percentage of viable haemocytes stabilized for the remainder of the experiment, at ± 40% (Fig. 3). Since there were no significant differences in percentage viable cells between the two exposed groups, the response does not seem to be related to exposure concentration but to some extent to internal cadmium concentration. However, a wider exposure concentration range and a longer exposure time are needed, in order to make more accurate deductions. The fact that the percentage viable cells stabilized after the second week of exposure in the 20 mg kg^{-1}, may possibly be due to compensatory mechanisms such as increased proliferation and differentiation of immune cells, leading to elevated haemocyte turnover and associated energetic costs (Sokolova et al. 2004).

The fact that a statistically significant response was already seen after the first week of exposure in the exposed groups, relative to the control, indicates that this response may possibly serve as an early warning of cadmium exposure, which is advantageous if biomarkers are to be used in metal risk assessment (Kammenga et al. 2000). Steffensen et al. (1994) also measured a quick response to cadmium in human lymphocytes and monocytes after only 17–19 h of exposure.

It is concluded that haemocyte viability of *P. laevis*, as measured with the trypan blue exclusion assay, may possibly be a useful biomarker of cadmium exposure. The assay certainly is a very simple and cost-effective technique. However, much research is necessary before final conclusions can be drawn. A field study, especially, is needed to validate the laboratory findings. If this response proves to be useful in the field, it may be a valuable addition to a battery of biomarkers in toxicity assessments.

Acknowledgments Thanks are due to the Cape Peninsula University of Technology and the National Research Foundation for financial assistance.

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