Accumulation and biological effects of metals in wild rats in mining areas of Zambia

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Abstract The lead–zinc (Pb–Zn) mine in Kabwe City and the copper–cobalt (Cu–Co) mine in the Copperbelt Province are major mining areas in Zambia. To examine the effects of metal pollution on wildlife, wild black rats (*Rattus rattus* and *Rattus tanezumi*) were captured in Kabwe and Chingola (in the Copperbelt Province), and in Lusaka (a noncontaminated site). Wild black rats in Kabwe accumulated significantly higher concentrations of Pb and Cd in various organs than rats from Lusaka. In Chingola, significantly higher concentrations of Cu, Co, Pb, and Cd were accumulated in wild black rats than in rats from Lusaka. These results were in accordance with metal accumulation patterns in soil. From toxicological aspects, concentrations of Pb and Cd in rats were generally low. However, metallothionein-1 (MT-1) and metallothionein-2 (MT-2) mRNA expression levels in wild black rats from Kabwe were significantly higher than those in rats from Lusaka. A generalized linear model (GLM) showed that concentrations of Zn and Cu had positive effects on the MT-1 and MT-2 mRNA expression. These results suggest that wild black rats in Zambian mining sites were exposed to metals that accumulated in their organs, causing biological responses such as MT mRNA induction. GLM indicated that heme oxygenase-1 (HO-1) mRNA expression could be a marker for Cr exposure.

Keywords Metal · Metallothionein · Hemeoxygenase · Biological responses · Generalized linear model · Wild rat

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Introduction

Environmental pollution due to rapid economic progress has had serious adverse effects on fish, food animals, wild animals, and humans in African countries (Nriagu

1992; Syakalima et al. 2001; Jumba et al. 2007; Yabe et al. 2010). The Republic of Zambia is rich in mineral resources such as copper (Cu), cobalt (Co), zinc (Zn), and lead (Pb). Zambia accounted for 3.7 % and 9.2 % of global annual Cu and Co production, respectively, in 2008 (USGS 2010:http://minerals.usgs.gov/minerals/pubs/country/2008/myb3-sum-2008-africa.pdf.). The major mining areas in Zambia are the city of Kabwe and the Copperbelt Province. Recently, environmental pollution due to mining and smelting activity has become a major problem in these two areas.

Kabwe City, the provincial capital of Zambia's Central Province, has a long history of extensive Pb and Zn mining. In the Kabwe mine, which operated from 1902 to 1994, cadmium (Cd), silver (Ag), Cu, and vanadium (V) were produced as by-products in addition to the main products of Pb and Zn (Kamona and Friedrich 2007). Recent studies recorded high concentrations of Pb, Zn, Cd, Cu, and arsenic (As) in Kabwe soil (Tembo et al. 2006; Nakayama et al. 2011). High blood Pb levels in children (60–120 μg/dL) were also reported (Copperbelt Environment Project 2006). Given these, Kabwe has been ranked among the top ten most polluted places in the world (Blacksmith Institute 2007).

In the Copperbelt Province, Cu–Co mines and smelter plants are extensive. Discharge of mining waste into the Kafue River, which flows through the center of Zambia, causes significant contamination. High concentrations of Cu and Co have been reported in water, sediment, and fish from the Kafue River (Pettersson and Ingri 2001; Choongo et al. 2005) as well as in soil (Ikenaka et al. 2010) in the Copperbelt Province. High Cu concentrations have also been reported in sediment and fish in Lake Itezhi-tezhi, which is located in the Kafue National Park, approximately 450 km downstream of the Kafue River from the Copperbelt Province (Nakayama et al. 2010).

In the present study, metal levels in soil samples from Chingola, one of the biggest mining cities in Copperbelt Province, were determined and compared with our previous study in Kabwe and Lusaka (as control area) (Nakayama et al. 2011). Furthermore, metal concentrations in liver, kidney, brain, and spleen of the wild rats from Kabwe, Chingola, and Lusaka were determined to assess the metal accumulation levels. These organs are known to accumulate metals efficiently (Ma 1989; Pereira et al. 2006; Sanchez-Chardi et al. 2007). To evaluate biological responses

to metal exposure at the subcellular level, mRNA expression levels of metallothionein-1 (MT-1), metal-lothionein-2 (MT-2) and heme oxygenase-1 (HO-1) in the livers and kidneys were analyzed. Metallothio-neins are proteins of low molecular weight and are induced by several metals (Amiard et al. 2006; Rogi-val et al. 2007; Swiergosz-Kowalewska et al. 2007). Therefore, MTs have been used as biomarkers for metal pollution in both aquatic and terrestrial animals (Amiard et al. 2006; Rogival et al. 2007; Swiergosz-Kowalewska et al. 2007). HO-1 is a cellular stress protein and its gene expression has been used as a marker for oxidative stress (Li et al. 2003). Metals such as As, Cd, Co, chromium (Cr), nickel (Ni), and Pb are known to generate reactive oxygen species (ROS); thus, HO-1 is also considered to be a biomark-er for metal pollution (Leonard et al. 2004).

Since ecotoxicological studies in African countries are scarce, the aims of the current study were to (1) identify the relationship between metal accumulation in wild rats and soil contamination in mining areas, (2) estimate the effects of metal pollution on wild rats in terms of metal accumulation and biological responses, and (3) offer a basis for similar studies of metal pollution in other areas in Africa.

Materials and methods

Study sites and sampling

This study was conducted at three areas, Kabwe, Chingola, and Lusaka (Electronic Supplementary Material Fig. S1). Kabwe is 130 km north of Lusaka, the capital city of Zambia. There is a Pb and Zn mine in Kabwe that was in operation until 1994. We collected soil samples and wild rats in Kabwe in May 2009 (Nakayama et al. 2011). Chingola is located in the Copperbelt Province, approximately 400 km north of Lusaka. Chingola is a thriving Cu and Co mining site, with large Cu–Co mines and other support industries. Sampling in Chingola was conducted in September 2008. Soil samples ($n=40$) were collected in Chingola. Approximately 50 g of soil from a depth of 0–5 cm was collected for each sample and kept in a plastic bottle. At least three composite soil samples were collected from each sampling point. Each sample was passed through a 2-mm sieve before extraction. Wild rats (*Rattus* spp.) were collected in residential areas, commercial areas,

and farms in Kabwe ($n=20$) and Chingola ($n=13$). In Lusaka, 18 rats were captured at the University of Zambia (UNZA) in September 2008. Samples from Lusaka were used as controls (samples from a noncontaminated area) because UNZA is very far away from any obvious sources of pollution such as general roads, industrial facilities, and mining activities.

We used gauze cage traps with food as bait to capture live wild rats. After euthanasia of captured rats by anesthesia overdose, body weight and sex were determined. Livers, kidneys, brains, and spleens were collected from each rat and stored at −20 °C for metal analysis. Livers stored at −20 °C were also used for genome assays. Small portions of liver and kidney were collected and stored in RNAlater (Sigma-Aldrich, St. Louis, MO, USA) for mRNA study. Treatment of all animals was performed according to the policies of the Institutional Animal Care and Use Committee of Hokkaido University.

Extraction and measurement of metals

We used the same method as in our previous study (Nakayama et al. 2011) for metal extraction and measurement in the soil and rat tissue samples. Briefly, 1 g of each soil sample was placed in a 200-mL flask. Nitric acid (15 mL, atomic absorption spectrometry grade, 60 %, Kanto Chemical Corp., Tokyo, Japan) was then added. The mixture was heated at 180 °C for 5 h on a hot plate. After the mixture cooled, 1 g of ammonium chloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added. The samples were reheated at 180 °C for 1 h and evaporated to approximately 5 mL. After the samples cooled, they were filtered into plastic bottles through ashless filter paper 5B (Advantec, Tokyo, Japan). The sample volume was standardized to 50 mL using 2 % HNO_3. A reagent blank was produced following the same process. For metal extraction from each tissue, 1 g of fresh tissue was placed in a 200-mL flask and nitric acid (20 mL) was added. The samples were gradually heated to 225 °C on a hot plate and left for 12 h to evaporate to approximately 5 mL. The volume was then made up to 20 mL with 2 % HNO_3. A reagent blank was produced using the same procedure. We measured the concentrations of eight elements (Cr, Co, Cu, Zn, Cd, Pb, Ni, and As) in the soil and rat tissue samples using an atomic absorption spectrophotometer (AAS, Z-2010, Hitachi High-Technologies Corporation,

Tokyo, Japan) with an acetylene flame or argon non-flame after preparation of the calibration standard. Each metal concentration was converted from milligram per kilogram wet weight (wt) to milligram per kilogram dry weight using the water content of each sample. Analytical quality control was performed using four certified reference materials as follows: BCR-320R (channel sediment, Community Bureau of Reference of European Commission, Brussels, Belgium), SRM 1944 (New York/New Jersey Waterway Sediment, National Institute of Standards and Technology, New York, USA), DOLT-4 (Dogfish liver, National Research Council of Canada, Ottawa, Canada), and DORM-3 (Fish protein, National Research Council of Canada, Ottawa, Canada). Recovery rates (percent) of soil and sediment using certified reference materials (BCR-320R and SRM 1944) were acceptable: Cr (80–115), Co (96–111), Cu (88–95), Zn (90–95), Cd (113–120), Pb (91–114), Ni (85–87), and As (113–121). For fish liver and muscle, recovery rates (percent) of all elements were acceptable: Cr (91–108), Co (96–111), Cu (88–90), Zn (78–83), Cd (91–108), Pb (89–98), and Ni (98–111), except for As (50–67 %). The detection limits (microgram per kilogram) of Cr, Co, Cu, Zn, Cd, Pb, Ni, and As were 0.5, 0.5, 1.0, 0.1, 0.2, 1.0, 0.5, and 2.0, respectively.

Extraction of genomic DNA and identification of *Rattus* species

Genomic DNA was extracted from frozen liver samples using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). This was used to identify *Rattus* species. Species of wild rat were identified through the cytochrome b (*cyt-b*) gene using a method modified from a previous study (Robins et al. 2007). The primers used to amplify 762 bp of *cyt-b* are shown in the Electronic Supplementary Material Table S1. The PCR amplification was performed on an iCycler (Bio-Rad, Hercules, CA, USA) using Ex Taq (Takara Bio Inc., Shiga, Japan), with a 2-min initial denaturing step of 94 °C; followed by 35 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min; and a final extension step of 72 °C for 5 min. Sequencing was done with the BigDye Terminator version 1.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA), with a 7-min initial denaturing step of 94 °C, followed by 35 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. Nucleotide sequences were determined by

an automated DNA sequencer (ABI PRISM 310 genetic analyzer, Applied Biosystems, CA, USA).

The *cyt-b* gene sequences for the major *Rattus* species, *Mus musculus*, and *Homo sapiens* were retrieved from the GenBank database. Accession numbers of the genes are shown in the Electronic Supplementary Material Table S2. Sequences of *cyt-b* of the samples were aligned with these mammalian sequences using Molecular Evolutionary Genetics Analysis (MEGA) (Tamura et al. 2007). A phylogenetic tree was constructed by the neighbor-joining method based on the MEGA program (Tamura et al. 2007).

Age estimation of wild rats

We determined the age of each rat by paired eye-lens weight (Tanikawa 1993). Both eyes were removed immediately after euthanasia and kept in 10 % formalin. We fixed eyes in formalin at room temperature for 4 weeks. After fixation, tissues around the lens were removed, and the lenses were washed in distilled water. The lenses were dried at 60 °C for 2 days in an oven and were weighed to estimate the rat's age according to the formula described previously (Tanikawa 1993).

Measurement of MT-1, MT-2, and HO-1 mRNA expression levels using reverse transcription real-time PCR

Since MT-1 and MT-2 mRNA expression levels were used to assess biological responses for metal exposure (Swiergosz-Kowalewska et al. 2007), mRNA expression levels of these genes were measured with reverse transcription real-time PCR. HO-1 mRNA expression levels were also measured on the assumption of being a stress marker for metal exposure. Total RNA was extracted from liver and kidney samples stored in RNAlater using TRI reagent (Sigma-Aldrich). First-strand cDNA was synthesized from total RNA with ReveTra Ace (Toyobo, Osaka, Japan) and oligo-dT primers (Toyobo).

The primers used for measurement of MT expression levels are shown in the Electronic Supplementary Material Table S1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene. The assay was conducted on a StepOnePlus Real-Time PCR System (Applied Biosystems) using THUNDERBIRD

SYBR qPCR Mix (Toyobo). Each sample was analyzed in duplicate. The cycling parameters were one initial denaturation step of 95 °C for 10 m, 40 cycles of 95 °C for 15 s, and 61 °C for 1 min. Melting curve analysis was performed to verify that only a single product with no primer–dimers were amplified.

To measure HO-1 mRNA expression level, TaqMan Gene Expression Assays (Applied Biosystems) were used. GAPDH was used as a reference gene. Assay IDs of TaqMan Gene Expression (Applied Biosystems) are Rn00561387_m1 for HO-1 and Rn99999916_s1 for GAPDH. The THUNDERBIRD Probe qPCR Mix (Toyobo) was used for the assays. The reaction was a 40-s initial denaturing step followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Relative quantity (RQ) of gene expression was calculated from real-time PCR data (cycle threshold, CT, value) by the comparative CT method. The sequences of the PCR products were confirmed using the ABI PRISM 310 genetic analyzer (Applied Biosystems).

Statistical analysis

The data were shown to be not normally distributed and to have heterogeneity of variance by the Shapiro–Wilk test and Levene's test. Therefore, the data were log-transformed and fitted normal distribution. We treated *Rattus rattus* and *Rattus tanezumi* as the same group because there were no difference in metal levels and mRNA expression levels between these rats from Kabwe, where sample size is not unbalanced. Since there were no sex differences in metal levels and mRNA expression levels in wild rats, we treated these samples as the same group. Differences among the three regions, Kabwe, Chingola, and Lusaka, were analyzed by the Tukey–Kramer HSD (for age and body weight) test and Dunnett's test (metal and mRNA expression levels). A generalized linear model (GLM) was used with body weight as response variable and age, sex, species, sampling site, season, and metal levels as explanatory variables. GLM analysis was also performed with MT-1, MT-2, and HO-1 mRNA expression levels as response variables and age, body weight, sex, species, sampling site, season, and metal levels as explanatory variables. Tests applied were considered statistically significant when $p < 0.05$. All statistical

analyses were performed using JMP 9 (SAS Institute, Cary, NC, USA).

Results

Metal concentrations in soil samples

Concentrations of eight elements were measured in soil samples from Chingola and compared with metal levels in soil from Kabwe and Lusaka (Table 1). High concentrations of Cu, Co, Cd, and Pb were recorded in soil samples from Chingola, while high concentrations of Cu, Zn, Cd, Pb, and As were detected in Kabwe. Metal concentrations in soil from Lusaka were generally lower compared to those from Chingola and Kabwe.

Species identification and physiological data of wild rats

Phylogenetic analysis of the *cyt-b* gene showed that the wild rats we collected were *R. rattus* and *R. tanezumi* (data not shown). The number of rats (males and females), species (*R. rattus* and *R. tanezumi*), body weight, ages, and sampling period are summarized in Table 2. These two species are not only closely related but are also commonly classified as black rats and considered as synonyms (*R. tanezumi* belongs to the *R. rattus* groups). Also, metal accumulation levels and mRNA expression levels were not significantly different between these rat species from

Kabwe where sample size is not much different (*R. rattus*: $n=13$, *R. tanezumi*: $n=7$, data not shown). Therefore, we treated them as a single group in the data analysis. Body weights of wild black rats from the mining areas, Kabwe and Chingola, were significantly lower than those from the noncontaminated control area (Lusaka). There were no regional differences in age.

Metal concentrations in tissues of wild rats

Metal concentrations in the liver, kidney, brain, and spleen of wild rats are shown in Table 3. Metal concentrations in each tissue were compared among the three rat populations (Kabwe, Chingola, and Lusaka as control). Concentrations of Pb in liver, kidney, brain, and spleen in rats from Kabwe were significantly higher than those from Lusaka. Concentrations of Pb in liver and kidney in Chingola rats were also significantly higher than those of rats from Lusaka. Concentrations of Cd in liver and kidney were significantly higher in rats from both Kabwe and Chingola, compared with those of rats from Lusaka. Cu concentrations in liver and kidney and Co concentrations in liver, brain, and spleen were significantly higher in rats from Chingola compared to rats from Lusaka. Zn concentrations in kidney, brain, and spleen in rats from Kabwe, and brain and spleen from Chingola were significantly lower than in rats from Lusaka. Ni concentrations in liver and brain of rats from the two mining areas

Table 1 Metal concentrations (range and median, mg/kg dry wt) in soil from Chingola, Kabwe, and Lusaka [combined results of this study (soil in Chingola) and the reference (Nakayama et al. 2011)]

Element	Chingola ($n=40$)	Kabwe[a] ($n=101$)	Lusaka[a] ($n=7$)	Recommended[b]	World range in nonpolluted soils[c]
Cr	3–191 (14)	3–110 (42)	27–52 (39)	240	5–120
Co	4–1,868 (35)	2–93 (13)	3–31 (9)	–	0.1–20
Cu	45–16,540 (790)	2–5,727 (37)	19–1,873 (31)	70	6–60
Zn	5–554 (33)	5–9,1595 (607)	17–777 (48)	50–120	17–125
Cd	0.01–7 (0.05)	0.01–139 (1)	0.03–0.33 (0.08)	1.6	0.07–1.1
Pb	16–8,008 (54)	9–51,188 (282)	8–134 (26)	120	10–70
Ni	3–52 (6)	2–74 (12)	7–31 (21)	30–200	1–200
As	0.26–24 (2)	0.04–141 (7)	1–5 (4)	18	1–15

[a] Nakayama et al. (2011)

[b] Soil cleanup criteria and Eco-Soil Screening Levels (US EPA, 2003; 2004)

[c] Kabata-Pendias and Pendias (1992)

Table 2 Information on samples of wild black rats

Region	Number	Gender		Species		Body weight (g)	Age (day)	Sampling period
		Male	Female	*R. rattus*	*R. tanezumi*	Average ± SD	Average ± SD	
Kabwe	20	11	9	13	7	110.9±32[B]	132.64±69[A]	2009 May
Chingola	13	5	8	10	3	113.2±20[B]	140.28±57[A]	2008 Sep
Lusaka	18	12	6	1	17	139.2±27[A]	141.73±41[A]	2008 Sep
Total	51	28	23	24	27			

Different letters indicate a significant difference (Tukey–Kramer HSD test, $p<0.05$)

were significantly lower compared with rats from Lusaka. Liver Cr concentrations in rats from both Kabwe and Chingola were significantly lower than in rats from Lusaka.

mRNA expression levels of MT-1, MT-2, and HO-1 in liver and kidney of wild rats

We compared mRNA expression levels of MT-1, MT-2, and HO-1 among the three rat populations (Figs. 1 and 2). While we did not find a significant difference in liver MT-1 mRNA expression levels (Fig. 1a), liver MT-2 mRNA expression in the rats from Kabwe was significantly higher than in the rats from Lusaka (Dunnett's test, $p<0.05$) (Fig. 1b). Expression levels of kidney MT-1 and MT-2 mRNA in the Kabwe rat population were significantly higher than those from Lusaka ($p<0.001$; $p<0.05$) (Fig. 2a, b). There were no differences in HO-1 mRNA expression levels between the three locations (Figs. 1c and 2c).

GLM analysis between body weight and age, sex, species, sampling site, season, and metal levels in rats

GLM analysis showed that age, concentrations of Cr in the liver, Co in the kidney, and Cu in the spleen had significant effects on body weight in the rat (Table 4). Concentrations of nonessential metals (Pb and Cd) and other metals (Zn, Ni, and As) did not show any significant effects on the body weight in the current study.

GLM analysis between MT-1, MT-2, and HO-1 mRNA expression levels and age, body weight, sex, species, sampling site, season, and metal concentrations in rats

For MT-1 mRNA expression levels in the liver, concentrations of Cu and Zn and body weight indicated

significant positive effects. Concentrations of Cu and Zn and site (Kabwe) had positive effects on the MT-1 mRNA expression levels in the kidney, while As and Co showed negative effects. For MT-2 mRNA expression levels in the liver, concentrations of As and Zn and body weight showed significant positive effects. In the kidney MT-2, Zn showed positive effects, whereas Cd and Co indicated negative effects. For HO-1 mRNA levels, concentrations of Cr both in the liver and kidney had positive effects. Sampling site (Chingola) and body weight showed positive effects on the HO-1 mRNA expression levels in the liver. Concentrations of Ni and Zn indicated significant negative effects on the HO-1 mRNA levels in the kidney.

Discussion

Regional differences in metal levels in soil and wild rats

We observed high concentrations of Cu and Co in Chingola soil. Interestingly, concentrations of Pb and Cd in Chingola soil were also high (Table 1). The presence of these metals in Copperbelt soils is likely to be a result of mining activities (Mihaljevic et al. 2011). In Kabwe, high concentrations of Pb, Zn, Cu, Cd, and As in soil have been previously reported (Nakayama et al. 2011). These values exceeded both the benchmark values from the US EPA (2003; 2004) and previously published results for global ranges in nonpolluted soils (Kabata-Pendias and Pendias 1992), indicating that soil in these two mining areas were contaminated probably by mining operations.

Compared with the control rats from Lusaka, concentrations of Cd and Pb were significantly higher in

Table 3 Metal concentrations (mg/kg dry wt) in the tissues of wild rats

Liver		As	Cd	Co	Cr	Cu	Ni	Pb	Zn
Kabwe	Average ± SD	0.50±1.7	0.12±0.14***	0.22±0.10	0.28±0.20***	10.2±1.8	0.11±0.1***	1.19±1.9***	151±45
	Range median	0.03–7.7 0.09	0.007–0.58 0.06	0.01–0.4 0.23	0.029–0.78 0.21	7.4–13.4 10.6	0.005–0.37 0.057	0.02–7.3 0.50	73–241 141
Chingola	Average ± SD	0.07±0.1	0.05±0.04*	1.72±1.50***	0.15±0.13***	29.5±23.9**	0.05±0.0**	0.41±0.2**	115±30
	Range median	0.04–0.3 0.04	0.012–0.15 0.04	0.20–4.8 1.23	0.045–0.49 0.08	8.0–83.4 16.6	0.013–0.09 0.044	0.09–0.9 0.37	79–189 103
Lusaka	Average ± SD	0.57±2.2	0.03±0.04	0.37±0.29	0.78±0.40	14.0±3.6	0.69±0.5	0.14±0.1	141±53
	Range median	0.03–9.2 0.06	0.003–0.17 0.02	0.08–1.1 0.27	0.047–1.55 0.77	7.0–20.0 14.0	0.026–1.92 0.604	0.03–0.5 0.10	58–304 132

Kidney		As	Cd	Co	Cr	Cu	Ni	Pb	Zn
Kabwe	Average ± SD	0.52±1.0	0.64±0.9***	0.26±0.12***	0.93±0.27	11.5±1.8	0.64±0.3	5.00±5.98***	91±24**
	Range median	0.07–4.5 0.22	0.06–3.9 0.28	0.12–0.6 0.23	0.52–1.4 0.93	7.4–14.1 12.0	0.24–1.39 0.51	0.3–22.1 2.01	65–167 82
Chingola	Average ± SD	0.28±0.4	0.71±0.7***	1.88±1.33	0.82±0.62	23.0±6.4***	0.63±0.7	2.85±2.03***	109±18
	Range median	0.07–1.5 0.14	0.05–2.0 0.42	0.27–4.2 1.74	0.27–2.5 0.57	12.9–38.0 22.0	0.20–2.46 0.36	1.2–8.9 2.32	83–148 107
Lusaka	Average ± SD	1.72±6.1	0.14±0.2	0.98±0.52	0.75±0.32	14.0±4.7	0.59±0.4	0.39±0.45	0.39±0.45
	Range median	0.07–26.3 0.18	0.01–0.8 0.09	0.23–2.5 0.92	0.20–1.3 0.76	10.0–28.0 12.7	0.19–2.07 0.52	0.1–1.9 0.21	54–137 111

Brain		As	Cd	Co	Cr	Cu	Ni	Pb	Zn
Kabwe	Average ± SD	ND	ND	0.079±0.10*	0.91±0.4	14.2±1.9	0.49±0.2***	0.22±0.16**	49±7.8**
	Range median			0.011–0.37 0.049	0.48–2.2 0.85	10.5–18.1 13.6	0.30–0.99 0.45	0.05–0.61 0.19	38–63 49
Chingola	Average ± SD	ND	ND	0.095±0.11**	0.90±0.4	18.3±4.3	0.60±0.2***	0.12±0.16	44±4.8***
	Range median			0.017–0.42 0.063	0.47–1.6 0.73	13.5–25.0 17.2	0.39–0.89 0.55	0.02–0.59 0.06	36–50 45
Lusaka	Average ± SD	ND	ND	0.029±0.04	1.37±0.9	16.0±2.4	1.51±0.9	0.10±0.12	56±6.3
	Range median			0.010–0.19 0.014	0.46–3.8 1.15	11.9–21.1 16.0	0.41–3.21 1.07	0.02–0.55 0.08	46–71 54

Spleen		As	Cd	Co	Cr	Cu	Ni	Pb	Zn
Kabwe	Average ± SD	1.0±1.5	0.035±0.027	0.13±0.10	3.34±2.4	18.6±7.5	6.98±6.4	1.18±0.7*	108±25**
	Range median	0.13–6.7 0.50	0.0048–0.11 0.031	0.01–0.35 0.11	0.55–11.2 2.62	8.6–40.0 18.0	0.55–25.4 5.28	0.11–2.7 1.34	75–182 102
Chingola	Average ± SD	0.8±0.8	0.026±0.017	0.36±0.21***	1.78±1.6	16.5±5.6	3.46±2.2	0.54±0.5	111±48**
	Range median	0.12–2.7 0.47	0.0050–0.06 0.020	0.12–0.82 0.32	0.49–6.8 1.61	10.0–30.0 14.0	1.17–8.7 2.39	0.16–2.2 0.40	75–262 96
Lusaka	Average ± SD	5.8±22.5	0.027±0.017	0.11±0.07	1.97±1.0	22.9±9.4	4.78±3.9	1.18±2.6	138±28
	Range median	0.10–96.1 0.49	0.0056–0.08 0.023	0.04–0.29 0.09	0.46–4.9 1.68	11.7–45.0 20.4	1.26–14.5 3.67	0.05–11.0 0.32	107–187 130

* $p<0.05$; ** $p<0.01$; *** $p<0.001$ (Dunnett's test, Lusaka as control)
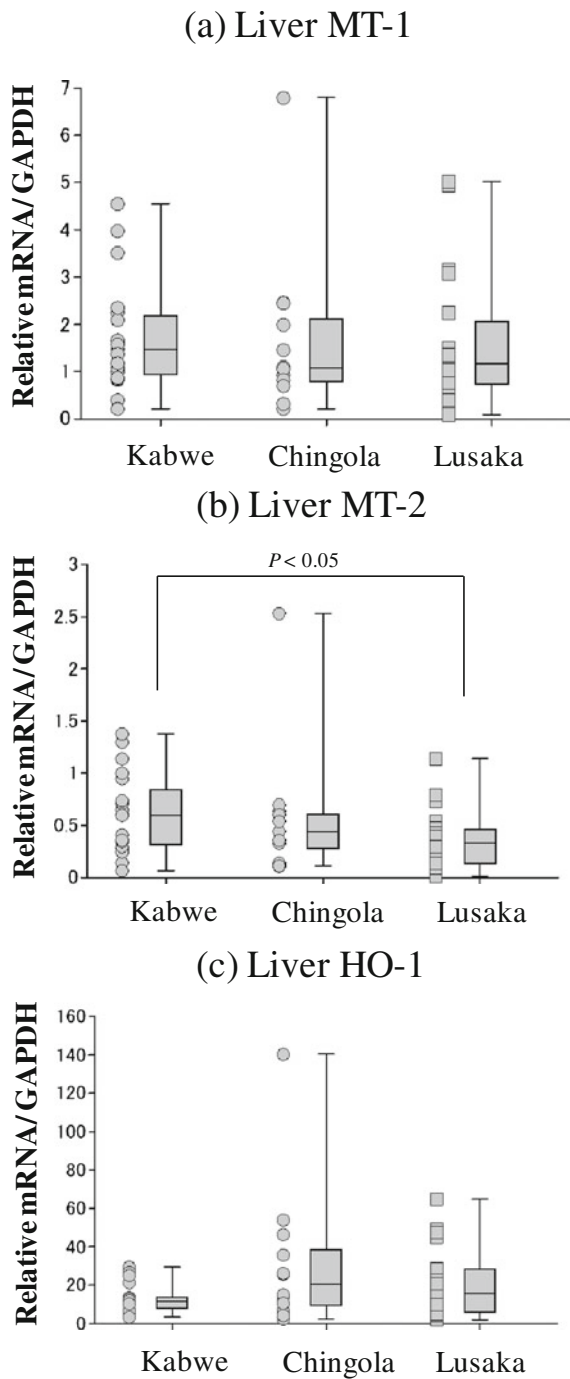
Fig. 1 *Box and vertical plot* of mRNA expression levels of metallothionein (MT)-1 (**a**), MT-2 (**b**), and heme oxygenase-1 (HO-1) (**c**) in rat livers. The expression levels were normalized using the *GAPDH* gene. Significant differences compared with the Lusaka rats were determined by Dunnett's test
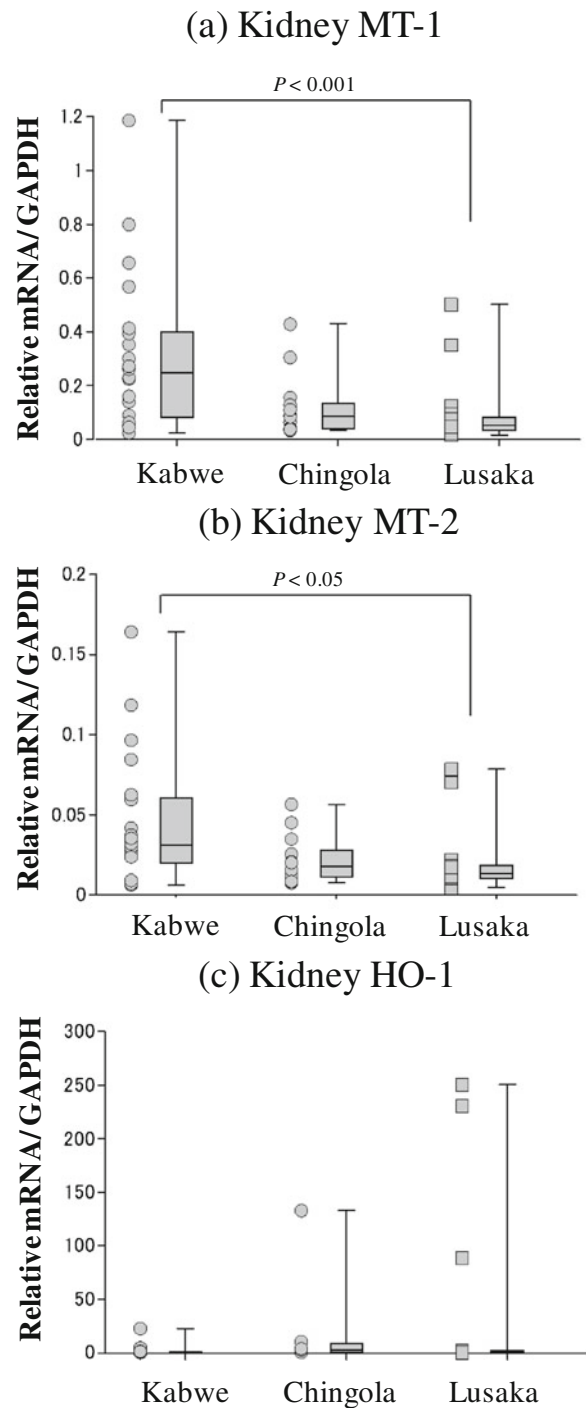

Fig. 2 *Box and vertical plot* of mRNA expression levels of metallothionein (MT)-1 (**a**), MT-2 (**b**), and heme oxygenase-1 (HO-1) (**c**) in rat kidneys. The expression levels were normalized using the *GAPDH* gene. Significant differences compared with the Lusaka rats were determined by Dunnett's test

Table 4 Generalized linear model with body weight as response variable in rats. Metal concentrations in each tissue, age, sex, species, sampling site, and season were analyzed as explanatory response. Only significant effects of explanatory variable were shown in the table

Tissue	Explanatory variable	Estimate	SE	p value
Liver	Age	0.380	0.054	<0.0001
	Cr	−0.092	0.039	0.021
Kidney	Age	0.395	0.055	<0.0001
	Co	−0.090	0.037	0.017
Spleen	Age	0.203	0.054	0.0004
	Cu	−0.238	0.112	0.0365
Brain	Age	0.261	0.061	<0.0001

Kabwe rats, while concentrations of Cu, Co, Cd, and Pb were higher in Chingola rats. These results suggest that metal accumulation patterns in wild rats are similar to those in soil and that soil pollution from mining activities is one of the exposure routes for metal accumulation in wild rats living in the mining areas.

Toxicological aspects of metal levels in wild rats

Although we found regional differences of metal levels in the tissues of wild rat, from toxicological points of view, accumulation levels of nonessential metals (Pb and Cd) in wild rats in the current study were generally low as mentioned below. This could be attributed to relatively young age of rats used for the current study.

Kidney Pb concentrations more than 15 mg/kg dry wt was needed for structural and functional kidney damage and Pb levels >120 caused body weight loss in adult rats (Ma 2011). Metal accumulation reportedly gives rise to histological changes (Shore and Douben 1994; Ceruti et al. 2002; Damek-Poprawa and Sawicka-Kapusta 2003). Kidney Pb concentration exceeding 2.5 mg/kg dry wt caused histological changes, such as karyocytomegaly and intranuclear inclusion bodies in the kidneys of wild brown rats (*R. norvegicus*) (Ceruti et al. 2002). In our present study, several of the Kabwe rats had Pb levels higher than the histopathological threshold (2.5 mg/kg dry wt), whereas only one rat exceeded toxic level of 15 mg/kg dry wt (22.1 mg/kg dry wt in Kabwe). This may be due to fact that most rats were captured in Northern Kabwe, while metal concentrations in soil were highest to the south (Nakayama et al. 2011). Pb

is known to accumulate in the brain with a half-life of 2 years and causes neurotoxicity (Barry 1975). An earlier study demonstrated that disorder in learning and memory and in spatial and visual recognition were observed in adult rats with Pb concentrations of 0.4–0.7 mg/kg dry wt in the brain (Ma 2011). Only a few rats (three rats from Kabwe and one rat from both Chingola and Lusaka) were within this range.

In the current study, ranges of Cd concentrations were 0.003–0.58 and 0.01–3.9 mg/kg dry wt in the liver and kidney, respectively. The average concentrations of Cd in the liver and kidney in small mammals (mice, rat, and voles) from noncontaminated reference sites were 0.2–0.5 and <0.1–5.6 mg/kg dry wt, respectively (Talmage and Walton 1991). Further, a recent comprehensive review indicated that Cd concentrations of 105–210 mg/kg dry wt in the kidney was taken as the critical level for causing serious adverse effects in wild small mammals (Cooke 2011). Taken together, Cd concentrations in the current study were within the range of reference sites and far below toxic levels.

In relation to metal levels with body weight, GLM analysis indicated that concentrations of Cr in the liver, Co in the kidney, and Cu in the spleen had negative effects on the weight of wild rats (Table 5). However, we believe that this result may be fortuitous because accumulation levels of these metals were very low to cause a toxicological effect such as growth suppression.

Effects of metals on expression levels of biomarkers in wild rats

Even though we did not observe toxicological levels of metal accumulation in wild rats, MT-1, MT-2, and HO-1 mRNA expression levels were determined in order to evaluate biological responses to metal exposure in the livers and kidneys of wild rats. Metallothioneins are cysteine-rich metal-binding protein of low molecular weight. In *Rattus* species, four MT isoforms (MT-1, MT-2, MT-3, and MT-4) are reported (Haq et al. 2003). In particular, MT-1 and MT-2 are expressed in many organs and tissues, and they play major roles in detoxification of toxic metals, homeostatic regulation of essential metals, and protection of tissue against oxidative injury (Haq et al. 2003). As MT-1 and MT-2 are mainly induced by metals such as Zn, Cd, and Cu, they have been used as biomarkers of metal pollution for aquatic organisms (Hylland et al.

Table 5 Generalized linear model with MT-1, MT-2, and HO-1 mRNA expression levels in the liver and kidney as response variable in rats. Metal concentrations in each tissue, age, body weight, sex, species, sampling site, and season were analyzed as explanatory response. Only significant effects of explanatory variable were shown in the table

Tissue	Response variable	Explanatory variable	Estimate	SE	p value
Liver	MT-1	Body weight	0.005	0.002	0.044
		Cu	0.614	0.301	0.046
		Zn	2.031	0.318	<0.0001
	MT-2	Body weight	0.006	0.002	0.003
		As	0.197	0.090	0.034
		Zn	2.098	0.287	<0.0001
	HO-1	Site (Chingola)	0.496	0.160	0.003
		Body weight	0.006	0.003	0.032
		Cr	0.646	0.239	0.009
Kidney	MT-1	Site (Kabwe)	0.233	0.112	0.043
		As	−0.318	0.107	0.004
		Co	−0.815	0.202	<0.001
		Cu	1.026	0.492	0.041
		Zn	2.465	0.612	<0.001
	MT-2	Cd	−0.265	0.120	0.031
		Co	−0.831	0.176	<0.0001
		Zn	2.702	0.535	<0.0001
	HO-1	Cr	2.790	0.928	0.004
		Ni	−1.571	0.647	0.019
		Zn	−3.512	1.509	0.024

1998; Amiard et al. 2006), birds (Vanparys et al. 2008), rodents (Rogival et al. 2007; Sanchez-Chardi et al. 2007; Marques et al. 2008), and other organisms. In the present study, we observed regional differences in MT-1 and MT-2 mRNA expression levels in wild rats as observed differences in metal accumulation.

In order to analyze relationships between MT mRNA expression levels and metal concentrations, GLM analysis was conducted. In the current study, sampling site (Kabwe) showed significant positive effect on MT-1 mRNA in kidney, probably reflecting regional differences in metal accumulation. Zn had significant positive effects on both MT-1 and MT-2 mRNA expression levels in the liver and kidney. This is not surprising because Zn is known to be a strong MT inducer (Coyle et al. 2002). Although the effect was less than Zn, Cu showed positive effect on MT-1 expression levels in the liver and kidney. This result is

also consistent with a previous report (Coyle et al. 2002). Surprisingly, Cd concentrations in the kidney showed a negative effect on the MT-2 mRNA expression levels despite the fact that Cd is one of the strongest MT inducer (Coyle et al. 2002). This is probably due to the fact that MT expression levels are not specific to metal pollution and also due to a more complicated situation in the field (e.g., multiple metal exposure). Interestingly, As had a positive effect on MT-2 in the liver, but the effect was negative for MT-1 in the kidney. Moreover, Co was a negative effecter for both MT-1 and MT-2 in the kidney. While induction ability is less than Cd and Zn, As and Co have also been known to induce MT (Haq et al. 2003; He and Ma 2009). In contrast, results from the present study suggested that MT was suppressed by As and Co. A recent laboratory study demonstrated that mice MT-1 mRNA expression levels in the liver were suppressed after 0.5 h to 1 h of subcutaneous injection of Co chloride, although the authors noted that expression levels should be examined at further late time (Matsumoto et al. 2010). Since a relationship between As and Co content and MT transcription is still unknown, further investigations are required from both laboratory and field studies.

Although there was no significant difference in HO-1 mRNA expression levels among sites, the result of GLM in the present study suggested that Cr had a positive effect on HO-1mRNA expression levels in both the liver and kidney. To our knowledge, information on relationships between Cr and HO-1 mRNA expression levels in the liver and kidney is scarce. Only a few studies demonstrated that Cr(VI), more toxic form, induced HO-1 mRNA in human dermal fibroblast cells and was considered to be a marker for stress induced by Cr(VI) (Joseph et al. 2008). At present, a useful biomarker for Cr exposure is not known. A current study showed that HO-1 mRNA expression could be one of the stress markers for Cr exposure.

Conclusions

In the present study, we concluded that wild black rats (*R. rattus* and *R. tanezumi*) from two mining areas in Zambia, Kabwe and Chingola, accumulated metals that had likely originated from mining activities. Although concentrations of metals were lower than the

levels that cause toxicological effects on rats, the accumulated metal levels caused biological responses such as MT induction in the livers and kidneys. We also observed that HO-1 mRNA expression levels could be a marker for Cr exposure. Further studies are required to clarify the relationships between multiple metal exposure and biological responses in wild rats.

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