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Author(s)	Nakayama, Shouta M. M.; Nakata, Hokuto; Ikenaka, Yoshinori; Yabe, John; Oroszlany, Balazs; Yohannes, Yared B.; Bortey-Sam, Nesta; Muzandu, Kaampwe; Choongo, Kennedy; Kuritani, Takeshi; Nakagawa, Mitsuhiro; Ishizuka, Mayumi
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1 **Title**

2 One year exposure to Cd- and Pb-contaminated soil causes metal accumulation and

3 alteration of global DNA methylation in rats

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5 Shouta M.M. Nakayama[1, a], Hokuto Nakata[1, a], Yoshinori Ikenaka[1, 2], John Yabe[3],

6 Balazs Oroszlany[1], Yared B. Yohannes[1, 4], Nesta Bortey-Sam[1], Kaampwe Muzandu[3],

7 Kennedy Choongo[3], Takeshi Kuritani[5], Mitsuhiro Nakagawa[5], Mayumi Ishizuka[1]

8

9 (1) Laboratory of Toxicology, Department of Environmental Veterinary Sciences,

10 Faculty of Veterinary Medicine, Hokkaido University, Kita 18 Nishi 9, Kita-ku,

11 Sapporo 060-0818, Japan, (2) Water Research Group, School of Environmental

12 Sciences and development, North-West University, South Africa, (3) The University of

13 Zambia, School of Veterinary Medicine, P.O. Box 32379. Lusaka, Zambia, (4)

14 Department of Chemistry, Faculty of Natural and Computational Science, University of

15 Gondar, P.O. Box 196, Gondar, Ethiopia (5) Graduate School of Science, Hokkaido

16 University, Japan

17 **(a) Both authors equally contributed to this study.**

18

19 **Corresponding author:**

20 Mayumi Ishizuka

21 E-mail: ishizum@vetmed.hokudai.ac.jp

22 Address: Laboratory of Toxicology, Department of Environmental Veterinary Sciences,

23 Faculty of Veterinary Medicine, Hokkaido University, N18, W9, Kita-ku, Sapporo

24 060-0818, Japan

25 Tel: +81-11-706-6949; Fax: +81-11-706-5105

26

27 **Abstract**

28 Metal pollution has been associated with anthropogenic activities, such as effluents and

29 emissions from mines. Soil could be exposure route of wild rats to metals, especially in

30 mining areas. The aim of this study was to verify whether soil exposure under

31 environmentally relevant circumstances results in metal accumulation and epigenetic

32 modifications. Wistar rats were divided to three groups: 1) control without soil exposure,

33 2) low-metal exposure group exposed to soil containing low metal levels (Pb: 75 mg/kg;

34 Cd: 0.4), and 3) high-metal exposure group exposed to soil (Pb: 3750; Cd: 6). After 1

35 year of exposure, the metal levels, Pb isotopic values, and molecular indicators were

36 measured. Rats in the high-group showed significantly greater concentrations of Pb and

37 Cd in tissues. Higher accumulation factors (tissue/soil) of Cd than Pb were observed in

38 the liver, kidney, brain, and lung, while the factor of Pb was higher in the tibia. The

39 obtained results of metal accumulation ratios (lung/liver) and stable Pb isotope ratios in

40 the tissues indicated that the respiratory exposure would account for an important share

41 of metal absorption into the body. Genome-wide methylation status and DNA

42 methyltransferase (Dnmt 3a/3b) mRNA expressions in testis were higher in the

43 high-group, suggesting that exposure to soil caused metal accumulation and epigenetic

44 alterations in rats.

45

46 **Keywords**: Cd, DNA methylation, epigenetics, Pb isotope, soil exposure

47

48 **Capsule**: Soil exposure caused metal accumulation and DNA hypermethylation in rats.

1. Introduction

Lead (Pb) and cadmium (Cd) are toxic metals that co-exist ubiquitously in the environment. Mining and smelting activities are among the major sources of these metals, and metal pollution is a matter of worldwide concern. Recently, more than 400 children died of Pb poisoning in Zamfara state, Nigeria, where long-term neurological impairment, including blindness and deafness, were also documented (Blacksmith Institute, 2014; Dooyema *et al*., 2012; Lo *et al*., 2012). Children in polluted areas are vulnerable to metal exposure because of their inclination to ingest Pb through pica behavior and to assimilate relatively larger amounts of inhaled and ingested Pb than adults (Calabrese *et al*., 1997; Manton *et al*., 2000). With regard to Cd, one of the most severe forms of chronic toxicity is *itai itai* disease (a Japanese term meaning "ouch-ouch"), which is characterized by nephrotoxicity, osteoporosis, and cardiovascular disease (Jarup and Akesson, 2009; Uno *et al*., 2005).

To evaluate the toxic effects of Cd and Pb exposure and their mechanisms, many laboratory studies have been performed using *in vitro*, *in vivo*, as well as *in silico* techniques in rodent animal models, such as mice and rats. In addition, in field studies, wild rats (e.g., *Rattus norvegicus*, *Rattus rattus*) have frequently been used as sentinel animals to monitor metal pollution around mining areas (Nakayama *et al*., 2011; Nakayama *et al*., 2013). These studies showed that fairly high concentrations of metals were accumulated in the tissues of wild rats collected from mining sites compared to those from control sites, resulting in biological reactions such as metallothionein (MT) upregulation. The authors suggested that soil may be major route of exposure to toxic metals in wild rats, especially in mining areas where soil possesses abundant mineral deposits. However, to our knowledge, there have been no reports of laboratory

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experiments to verify whether soil exposure under environmentally relevant conditions (i.e., not as oral/gavage administration) could result in metal accumulations in rats. As it is difficult to control the experimental conditions in field studies, laboratory soil exposure experiments should be performed to examine this issue. Many studies have been conducted using earthworms as a model animal to characterize metal accumulation patterns and accumulation factors between soil and terrestrial animals (Qiu *et al*., 2014), but there have been no such laboratory studies in mammals. To provide new knowledge on soil exposure in terrestrial mammals, we used the laboratory rat (*R. norvegicus*) because of the wealth of toxicological knowledge as well as genomics and epigenetics methodological strategies for this species.

We performed prolonged (1-year) exposure of Wistar rats to soil containing Cd and Pb collected in the Kabwe mining area, Zambia (Nakayama *et al*., 2011), to estimate accumulation factors in tissues of rats. Soil samples from Kabwe were used in this study because high concentrations of Cd and Pb were reported previously in soil, rat, chicken, goat, cattle, and children in this area (Nakata et al., 2016; Nakayama *et al*., 2011; Yabe et al., 2011; Yabe et al., 2015; Yabe et al., 2018). As inhalation of soil and metal accumulation were expected, we collected lung tissue from rats in addition to tissues known to accumulate Cd and Pb, such as the liver and kidney. Neurological effects, including decreased intelligence quotient (IQ), are serious problems associated with Pb exposure in humans, especially children (Manton *et al*., 2000). Therefore, brain tissues were also collected. The tibiae were collected as Pb accumulation targets because of the very long half-life of this metal in bone (Gerhardsson *et al*., 1993).

Pb isotope ratios of the $^{208}Pb/^{206}Pb$ and $^{207}Pb/^{206}Pb$ were also measured to clarify the change of those values by accumulation level and the differences among the

4

97   tissues. A large number of studies has utilized the method of Pb isotopic analysis since

98   an identification of Pb pollution source is highly required to prevent and mitigate the

99   further Pb exposure from the environment. Pb isotopic compositions which consist of

100   four main stable isotopes: [208]Pb, [207]Pb, [206]Pb, and [204]Pb are not affected to a measurable

101   extent by physico-chemical fractionation processes (Bollhöfer and Rosman, 2001;

102   Veysseyre et al., 2001). It is thus well known that isotopic ratios of the [208]Pb/[206]Pb and

103   [207]Pb/[206]Pb can be used as natural tracers and open up another possibility for tracking

104   the Pb source and pathway. Nevertheless, some previous studies revealed large

105   differences in the isotopic composition of Pb among biological samples within rats

106   (*Rattus norvegicus*), goats and humans (Liu et al., 2014; Nakata et al., 2016; Smith et al.,

107   1996; Wu et al., 2012). It was also suggested the possible biological fractionation

108   system of Pb isotopes and its threshold in the body (Nakata et al., 2016). Given these,

109   we verified the change of Pb isotopic compositions in rat tissues in case of exposure

110   from soil via inhalation.

111   Biological reactions, such as MT elevation as well as epigenetic alterations

112   regarding global DNA methylation, were examined to provide new insight into

113   epigenetic events associated with chronic metal exposure. This study is significant due

114   to the environmentally relevant soil exposure conditions used to evaluate metal

115   accumulation and biological alterations in rats. In addition, global DNA methylation

116   analysis was performed because DNA 5-methylcytosine (5-mC) modification is

117   increasingly recognized as a key process in the pathogenesis of complex disorders,

118   including cancer, diabetes, and cardiovascular disease (Feinberg 2010; Ordovas and

119   Smith, 2010). This is another significant point of the present study because a recent

120   review (Ray *et al*., 2014) noted that there have been few studies to assess associations

121   between DNA methylation and Cd or Pb exposure. Alterations of the DNA

122   methyltransferase (Dnmt) family were also examined because these molecules mediate

123   cytosine methylation through the transfer of a single methyl group from S-adenosine

124   methionine (SAM) to cytosine (Feinberg 2010; Ordovas and Smith, 2010).

2. Materials and Methods

2.1. Soil sampling

We collected soil samples in Kabwe, Zambia (May 2009), because soil in this area is highly polluted with Pb (9 – 51188 mg/kg) and Cd (0.01 – 139 mg/kg) (Nakayama *et al.*, 2011). Soil samples were passed through a 2 mm sieve and transported to the Laboratory of Toxicology, Graduate School of Veterinary Medicine, Hokkaido University, Japan, for laboratory exposure experiments as described in the following section. Details on soil sampling method are mentioned in supporting information of Materials and Methods section.

2.2. Animals and experimental design

All animal experiments were performed under the supervision and with the approval of the Institutional Animal Care and Use Committee of Hokkaido University (approval number 09-0220).

Thirty male Wistar rats (*R. norvegicus*, 7 weeks old) were purchased from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan). The rats (8 weeks of age) were divided into three groups (*n* = 10 for each group): 1) control without soil exposure, 2) low-metal exposure group exposed to soil containing low metal levels (Pb: 75 mg/kg; Cd: 0.4 mg/kg), and 3) high-metal exposure group exposed to soil containing high metal levels (Pb: 3750 mg/kg; Cd: 6 mg/kg) (Supplementary Table S1). Concentrations of Cd and Pb in the test soil samples were determined prior to exposure experiments by atomic absorption spectrometry (AAS) (Z-2010; Hitachi High-Technologies Corporation, Tokyo, Japan) according to the method described previously (Nakayama *et al.*, 2011). Soil samples were spread at the bottom of the cage and rats were exposed to

149 the soil for 1 year (Supplementary Fig. S1). The rats were housed in cages containing

150 either soil for the exposure groups or a bedding of paper chips (Paper Clean; Japan SLC,

151 Hamamatsu, Japan) for the control group (Supplementary Fig. S1). Body weight of the

152 individual rats was measured once every 2 weeks, and no differences were observed

153 among the groups (Tukey's test) during the 1-year exposure period (Supplementary Fig.

154 S2). Details on animal experiment design are also shown in supporting information of

155 Materials and Methods section.

156   To evaluate the effects of metal exposure, behavioral activity of rats was

157 monitored using a Scanet MV-10 (Matys Co., Tokyo, Japan) before starting exposure

158 (day 0) and after 2, 6, and 12 months of exposure. Rats were placed in a box measuring

159 480 mm × 480 mm that had infrared ray detectors set 12.5 cm above the floor. Larger

160 (MOVE 1) and smaller (MOVE 2) horizontal movements and vertical movement

161 (rearing) were recorded every 2 minutes for 20 minutes (Supplementary Fig. S3). This

162 instrument allowed monitoring of the rat behavior by one examiner without special

163 training. Behavioral experiments were performed at night (20:30 – 23:00) as rats are

164 nocturnal animals.

165   After 1 year of soil exposure, rats were euthanized by CO_2 inhalation, and

166 heparinized total blood, liver, kidney, lung, brain, testis, and tibia were collected. Tissue

167 samples other than the tibia were immediately frozen in liquid nitrogen. Plasma was

168 collected after centrifugation (2000 × g, 15 min at room temperature) of total blood with

169 heparin for blood biochemistry analysis. The collected samples were stored at –80°C

170 until analyses.

171

172 *2.3. Blood biochemistry*

8

173   A conventional blood chemical analyzer (COBAS Ready; Roche Diagnostic

174   Systems, Basel, Switzerland and Spotchem panels I and II; Arkray, Kyoto, Japan) was

175   used to analyze the levels of alanine aminotransferase (ALT), aspartate

176   aminotransferase (AST), gamma glutamyltranspeptidase (GGT), lactase dehydrogenase

177   (LDH), alkaline phosphatase (ALP), total bilirubin (T-Bil), total protein (TP), blood

178   urea nitrogen (BUN), albumin (Alb), urea acid (UA), and creatinine (Cre).

179

180   *2.4. Cd and Pb extraction and concentration analysis*

181   Extraction of metals in tissues were performed as described previously (Yabe

182   *et al.*, 2015) with slight modifications. Details on sample digestion and metal extraction

183   procedures are also described in supporting information of Materials and Methods

184   section.

185   The concentrations of Cd and Pb were determined using an inductively coupled

186   plasma – mass spectrometer (ICP-MS 7700 series; Agilent Technologies, Tokyo, Japan).

187   Analytical quality control was performed using the DORM-3 (fish protein, National

188   Research Council of Canada, Ottawa, Canada) and DOLT-4 (dogfish liver, National

189   Research Council of Canada) certified reference materials. Replicate analysis of these

190   reference materials showed good recoveries (95% – 105%). The instrument detection

191   limit was 0.001 µg/L. The accumulation factor for the high-metal exposure group was

192   calculated using the equation: [metal concentration in rat tissue/metal concentration in

193   soil].

194

195   *2.5. Sample purification and Pb stable isotope analysis*

196   Sample dissolution procedure was similar to the method described by Kuritani

197   and Nakamura (2002). The extracted solutions of liver, kidney, lung, brain, and blood,

198   except for one kidney each of control and low-metal exposure groups whose solution

199   volumes were not enough, were transferred into Teflon tubes after the analyses of Cd

200   and Pb levels. The Pb isotopic data of one kidney sample of each control and low-metal

201   exposure group were not analyzed due to insufficient volume of the solution. Details on

202   sample dissolution and purification procedures are also shown in supporting

203   information of Materials and Methods section.

204   Pb isotopic ratios of the ^{208}Pb/^{206}Pb and ^{207}Pb/^{206}Pb were determined on a

205   multiple collector (MC)-ICP-MS (Neptune Plus, Thermo Finnigan, California, USA) in

206   static mode with the Faraday cup configuration. Other general parameters were

207   described in Supplementary Table S2. Details on corrections of fractionation are

208   indicated in supporting information of Materials and Methods section.

209

210   *2.6. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)*

211   Total RNA was extracted using Nucleospin RNA II kit (Takara Bio, Otsu,

212   Japan) from approximately 100 mg of the liver and kidney according to the

213   manufacturer's instructions. Total RNA concentration was measured using NanoDrop

214   ND-1000 (Thermo-Scientific, Newark, DE). A260/280 and A260/230 were generally ≥

215   2. Total RNA (1 µg) was reverse transcribed using ReverTra Ace (Toyobo, Tokyo,

216   Japan) in a final volume of 40 µL, according to the manufacturer's instructions.

217   Gene-specific qRT-PCR primers for MT-1, MT-2, Dnmt 1, Dnmt 3a, Dnmt 3b, and

218   peptidylprolyl isomerase (cyclophilin) genes (Supplementary Table S3) were

219   synthesized by Sigma-Aldrich (Tokyo, Japan). qRT-PCR was performed using the

220 StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA). The PCR

221 mixtures consisted of Fast SYBR Green Master Mix (Applied Biosystems), forward and

222 reverse primers (200 nM each), and cDNA derived from 10 ng of total RNA in a total

223 volume of 10 µL. Details on PCR profile, primer specificity confirmation, internal

224 control and comparative quantification method are presented in supporting information

225 of Materials and Methods section. Eight rats selected at random from each group were

226 used for the qRT-PCR assay, whereas 10 rats from each group were used for all of the

227 other experiments.

228

229 *2.7. Genomic DNA extraction and LUminometric Methylation (LUMA) Assay*

230 Genomic DNA was extracted from the liver, kidney, and testis samples using a

231 GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich) according to the

232 manufacturer's instructions. DNA concentration was measured spectrophotometrically

233 (NanoDrop ND-1000; Thermo-Scientific). LUminometric Methylation Assay (LUMA)

234 assays were performed according to the method of Pilsner *et al*. (2009b). Briefly,

235 methylation-sensitive and methylation-insensitive enzymatic digestion of 300 ng

236 genomic DNA at CCGG sites was completely performed using *Hpa*II and *Msp*I

237 restriction enzymes (Invitrogen, Carlsbad, CA), respectively. *Eco*RI (Invitrogen) was

238 also used for complete digestion as an internal control. Annealing buffer (Qiagen,

239 Valencia, CA) was added after digestion, and the products were analyzed using the

240 PyroMark Q96 MD system (Qiagen). The *Msp*I/*Hpa*II ratios were calculated relative to

241 the *Eco*RI control, and the percent methylation of each sample was calculated using the

242 equation: $[1 - (HpaII/EcoRI)/(MspI/EcoRI)] \times 100$.

243

11

2.8. Statistical analysis

245   Tukey's test was used to identify significant differences among the groups and

246 tissues. Principal component analysis (PCA) was performed to characterize the

247 relationships among metal concentrations, methylation status, and mRNA expression of

248 Dnmt 1, Dnmt 3a, and Dnmt 3b in rat testis. JMP ver. 13.0 (SAS Institute Inc., Raleigh,

249 NC) was used for statistical analysis, and $P < 0.05$ was taken to indicate statistical

250 significance.

251 **3. Results**

252 *3.1. Blood biochemistry*

253   Significantly elevated levels of ALP and BUN were detected in the rats from

254 the high-metal exposure group compared to the other two groups (Supplementary Table

255 S4).

256

257 *3.2. Cd and Pb concentrations and accumulation factors*

258   The concentrations of Cd and Pb in rat tissues were determined (Table 1).

259 Significantly higher concentrations of Cd were observed in the blood, testis, lung, liver,

260 and kidney in rats from the high-metal exposure group compared to control and

261 low-metal exposure groups. No significant differences were found in Cd concentrations

262 in the tibia samples. Cd levels in brain samples were below the detection limit (BDL).

263 Significantly higher concentrations of Pb were observed in the blood, testis, brain, lung,

264 liver, kidney, and tibia of rats from the high-metal exposure group compared to the

265 control and low-metal exposure groups.

266   We calculated the accumulation factor between soil and rat tissues

267 (Supplementary Table S5). The unit of blood metal concentration was converted with

268 1.0 of conveniently set the blood specific gravity. The rank order of accumulation factor

269 ($\times 10^{-4}$) for Cd in rat tissues (except the brain) was as follows: kidney (850) > liver (50)

270 > lung (20) > testis (11) > tibia (8.0) > blood (0.2). The rank order of the accumulation

271 factor ($\times 10^{-4}$) for Pb was as follows: tibia (145) > kidney (7.2) > liver (1.4) > lung (0.6)

272 > brain (0.4) > testis (0.3) > blood (0.1).

273

3.3. Pb isotopic compositions

275   Geographical trends and the values of the Pb isotope ratios ($^{208}Pb/^{206}Pb$ and

276     $^{207}Pb/^{206}Pb$ ratios) from various tissues of rats are described in Fig. 1 and

277   Supplementary Table S6, respectively. The tissues of control group which was not

278   exposed to Pb recorded large variation in the mean values of $^{208}Pb/^{206}Pb$ and $^{207}Pb/^{206}Pb$

279   ratios among the different tissues. The average isotopic ratios of low-metal exposure

280   group exhibited relatively small variety among the tissues compared to control group.

281   Moreover, both ratios of $^{208}Pb/^{206}Pb$ and $^{207}Pb/^{206}Pb$ in the tissues of low-metal exposure

282   group tended to show the values closer to those in Kabwe galena (2.1342 ± 0.0009 of

283     $^{208}Pb/^{206}Pb$ and 0.8731 ± 0.0003 of $^{207}Pb/^{206}Pb$) reported by Kamona et al. (1999).

284   Compared with those two groups, high-metal exposure group indicated surprisingly

285   small differences of the isotopic compositions in the tissues other than liver, with quite

286   similar isotopic values to those in galena from the deposits of Kabwe. The standard

287   deviation values of isotopic ratios in tissues from high-metal exposure group were also

288   quite small, indicating small individual differences of the isotope ratios in the group.

289

290   *3.4. Behavioral activity*

291   No differences were observed among the groups in MOVE1, MOVE2, or

292   rearing before exposure (Table 2). After 2, 6, and 12 months of exposure, the numbers

293   of horizontal movements (MOVE1 and MOVE2) were significantly reduced in the

294   high-metal exposure group compared to the other two groups, whereas no differences

295   were observed in the number of rearing behaviors (Table 2).

296

297 *3.5. MT-1 and MT-2 mRNA expression in the liver and kidney*

298   Metallothionein (MT)-1 and MT-2 mRNA expression in the kidneys from rats

299 in the high-metal exposure group were significantly higher than those in controls (Fig.

300 2A, 2B). MT-1 mRNA expression in the kidneys of the high-metal exposure group

301 tended to be higher than that the low-metal exposure group, although the difference was

302 not statistically significant. No significant differences were observed among the three

303 groups in MT-1 or MT-2 mRNA expression in the liver (Fig. 2C, 2D).

304

305 *3.6. LUMA assay in the liver, kidney, and testis*

306   Global DNA methylation status was analyzed by LUMA assay. Significantly

307 high methylation level (%) was observed in the testis of the high-metal exposure group

308 compared to the other two groups (Fig. 3A). No significant differences were observed

309 among the groups in the liver or kidney (Fig. 3B, 3C).

310

311 *3.7. Dnmt 1, Dnmt 3a, and Dnmt 3b mRNA expression in the testis*

312   Dnmt 1 mRNA expression in the testis did not differ among the groups (Fig.

313 4A). Dnmt 3a mRNA expression levels in the testis of low- and high-metal exposure

314 groups were significantly higher than those in the control group (Fig. 4B). The

315 high-metal exposure group showed significantly elevated Dnmt 3b mRNA expression

316 compared to the control group and this tended to be higher than that in the low-metal

317 exposure group although the difference was not statistically significant (Fig. 4C).

318

319   *3.8. PCA*

320   Positive associations between metal concentrations, global DNA methylation

321   level, and methyltransferase (Dnmt 1, Dnmt 3a, and Dnmt 3b) mRNA expression in the

322   testis were observed by PCA (Fig. 5).

4. Discussion

323

324   In the present study, we exposed laboratory rats to soil containing Cd and Pb

325 via inhalation as well as through ingestion (e.g., grooming, soil adsorbed to food) to

326 represent environmentally relevant conditions. Metals were accumulated in the tissues

327 of rats after 1 year of exposure. To our knowledge, this is the first study to estimate the

328 accumulation factor between soil and rat tissues under conditions of natural exposure

329 and to analyze the biological reactions and epigenetic modifications.

330   Analysis of the tissue distributions of Cd and Pb among the three groups

331 indicated that soil exposure causes metal accumulation in the tissues of rats. As

332 expected, relatively high levels of Cd and Pb were accumulated in the liver and kidneys.

333 The liver and kidneys have been defined as metal accumulating tissues because they

334 express high levels of metal binding proteins (e.g., metallothionein), which play

335 important roles in detoxification of metals and metal elimination (Waalkes and Klaassen,

336 1985). On the other hand, it is well known that the Cd and Pb accumulation levels in the

337 lung are relatively smaller than those in the liver and kidneys because of the low

338 expression levels of metal binding proteins. A recent research reported that the ratio of

339 Cd and Pb accumulation in the lung as compared to the liver were 0.024 and 0.055,

340 respectively, in case of adult male Wister rat which was exposed to both Cd and Pb with

341 solid feed for 12 weeks (Winiarska-Mieczan, 2014). In the sense of those metal

342 accumulation ratios, our results indicated different trends; namely, the accumulation

343 ratios of Cd and Pb in the lung of high-metal exposure group as compared with the liver

344 were 0.401 and 0.432, respectively, whereas those ratios of low-metal exposure group

345 were 0.158 and 0.023, respectively. These greater ratios in the present study suggested

346 that respiratory exposure would account for a large fraction of Cd and Pb accumulation

17

347 in rats living at highly contaminated soil environment rather than oral exposure

348 although the distribution of absorbed metals through bloodstream should be taken into

349 consideration as well. In fact, the concentration ratios of low- and high-metal exposure

350 groups for Cd and Pb in the lungs were 5.9 and 35.8 times, respectively, which were

351 higher than the values for other tissues except Pb in the tibia for which a ratio of 130

352 was observed.

353   With the intent to consider the exposure route of metals, we also analyzed Pb

354 isotopic ratios of $^{208}Pb/^{206}Pb$ and $^{207}Pb/^{206}Pb$ in rat tissues. The variety of Pb isotope

355 ratios in rat tissues decreased with an increase in Pb accumulation level. Moreover, as

356 greater the Pb level is, as closer the isotopic compositions of tissues to those of Kabwe

357 galena which is considered as the origin of Pb pollution source in Kabwe (Kamona et al.,

358 1999). These findings are quite similar with those which were indicated by previous

359 studies of rat and goat (Liu et al., 2014 and Nakata et al., 2016). Among the rat tissues

360 of high-metal exposure group, lung tissues showed the isotopic values closer to those of

361 galena compared with other tissues except for kidney, suggesting the possibility that

362 lung could be the tissue absorbing Pb from outside of the body. After the absorption

363 from lung, the isotopic compositions of Pb could be fractionated during the distribution

364 to other tissues as the former research suggested although the exact mechanism is still

365 unknown (Nakata et al., 2016). These findings also support the hypothesis of a large

366 contribution of inhalation to the Pb absorption which was indicated by the result of

367 metal accumulation ratios of lung to liver. Considering the route of exposure in the

368 present study, the contribution of inhalation of soil particles cannot be neglected. Indeed,

369 Cd was accumulated in the lungs of rats due to inhalation of both soluble and insoluble

370 forms (Takenaka *et al*., 2004). Our results proposed meteorological factors could play

371   an important role of exposure level via inhalation in the field although the

372   meteorological factor was not verified in the present study of a laboratory experiment.

373   When the amount of precipitation is small and soil moisture level is low, the soil

374   containing Pb could easily scatter. Similarly, the strong wind could also increase the

375   amount of scattered dust. The increased amount of dust would contribute to the increase

376   of Pb exposure via inhalation. On the other hand, the wet surface soil could decrease the

377   exposure level via inhalation. Climate change, which is one of the major concerns in the

378   world in recent years, could also affect the environmental exposure level.

379   Although Cd was not detected in any individual rat, significantly higher

380   concentrations of Pb were observed in the brain in the high-metal exposure group. Pb is

381   known to pass through the blood/brain barrier and accumulate in the brain (Struzynska

382   *et al.*, 1997), and this phenomenon was also confirmed here in the case of soil exposure.

383   Among the tissues examined, quite different patterns of accumulation were observed

384   between Cd and Pb in the tibia. Cd in the tibia did not differ among the groups, whereas

385   quite high concentrations of Pb (average: 54.4 mg/kg) were detected. This value was

386   comparable to that reported previously by de Figueiredo *et al*. (2014) who demonstrated

387   that newborn rats exposed to 30 mg Pb/L in drinking water for 60 days after birth

388   accumulated 43.4 mg/kg of Pb in the tibia (Supplementary Table S7). Similarly, rats

389   exposed to 500 mg Pb/L in drinking water for 84 days had 58.2 mg/kg of Pb in the

390   femur (Li *et al*., 2013). Notably, in the study by Li *et al*. (2013), rats showed

391   hippocampal damage associated with Pb exposure. This accumulation pattern in the

392   tibia can be explained by competition of Pb for Ca^{2+} and deposition in the bone as more

393   than 90% of total Pb burden in the body accumulates in bone tissues. The accumulation

394   factor supports this difference between Cd and Pb; i.e., the highest accumulation factor

395 for Pb and the second lowest accumulation factor for Cd were observed in the tibia. The

396 half-life of Pb in the tibia is estimated as $20 – 30$ years in humans (Gerhardsson *et al.*,

397 1993) and lacteal as well as transplacental transfer is one of the most serious exposure

398 routes in infants (Chen *et al.*, 2014). Although the present study used male rats, similar

399 soil exposure experiments using pregnant females to clarify the effects on neonates

400 would be interest because an earlier study indicated that rat pups of dams exposed to Pb

401 via drinking water showed neurochemical alterations in the cerebellum and striatum

402 (Antonio *et al.*, 2002).

403   As we found that soil exposure can cause Cd and Pb tissue accumulation, we

404 further analyzed blood biochemistry and behavioral changes in the present study. Some

405 of the items examined suggested that the levels of functional damage in the liver and

406 kidney were not severe. In contrast, decreases in behavioral activity were observed in

407 the soil-exposed groups, suggesting effects of Pb on the central nervous system. In

408 accordance with our study, Pb was reported to cause a significant decrease of locomotor

409 activity in Wistar rats chronically administered 0.5% (v/v) Pb acetate in drinking water

410 for 3 months, with concomitant astrocytic and dopaminergic changes involved in

411 controlling many aspects of brain function (Sansar *et al.*, 2011). Although the molecular

412 mechanism of Pb neurotoxicity is not elucidated well, however some possible pathways

413 such as alterations in genetic regulation and protein synthesis have been reported so far.

414 For instance, the expression, synthesis and conformational maturation of the neuronal

415 cell adhesion molecule (NCAM) are affected by Pb exposure (Breen *et al.*, 1988; Davey

416 *et al.*, 1998). Additionally, voltage-gated calcium channels, which allow the flow in a

417 number of mono- and polyvalent cations, is also affected by Pb. It was observed that Pb

418 is capable of blocking some types of calcium channels, including potassium currents in

419   neurons (Peng *et al*., 2002; Dai *et al*., 2001). Oxidative stress is also associated with

420   neurotoxicity of Pb. The former exposure study of rat discovered higher levels of brain

421   2-thiobarbituric acid-reactive substances, an indicator of lipid oxidation, and higher

422   activities of glutathione reductase and glutathione peroxidase compared with controls

423   (Adonaylo and Oteiza; 1999). In the exposure study of the rat to Pb which was done by

424   Villeda-Hernandez *et al*. (2001), revealed Pb accumulation was related with high levels

425   of lipid oxidation products in different brain regions, such as the parietal cortex,

426   striatum, hippocampus, thalamus and cerebellum. Pb is known to be neurotoxic in

427   humans, especially children, because of its ability to compete with Ca^{2+} in nerve

428   functioning (Crosby, 1998). Recently, the Centers for Disease Control and Prevention

429   (CDC, 2012) revised the blood lead "level of concern" from 10 to 5 μg/dL in response

430   to reports that Pb levels of < 10 μg/dL can cause neurological abnormalities, such as

431   decreased IQ in children (Canfield *et al*., 2003). Therefore, a threshold below which Pb

432   does not result in neurological deficits has not been determined (Needleman *et al*.,

433   2004). The present results were consistent in that behavioral changes were detected at

434   an earlier stage before tissue dysfunctions were observed. In general, biological

435   responses precede the appearance of tissue damage. Therefore, we analyzed the

436   alterations of MT and confirmed the induction of MT-1 as well as MT-2 mRNA in the

437   kidneys of rats in the high-metal exposure group. On the other hand, no significant

438   difference of MT-1 and MT-2 expression in the liver samples was observed among the

439   groups, supporting the former study which revealed the expression levels of MT-1 and

440   MT-2 expression in the livers of rats from Kabwe, where the soil used in the present

441   study was collected, had no significant difference with those from control site

442   (Nakayama *et al*., 2013). However, in case of acute exposure, the induction of MT-1

443   and MT-2 in kidney of Cd-exposed rat was much lower than in the liver (Vasconcelos *et*

444   *al*.). The major difference between the current study and the former acute exposure

445   study is the accumulated hepatic metal levels. Our study showed approximately 5 times

446   and 2 times greater levels of Cd and Pb in liver of high-metal exposure group compared

447   with those of control, respectively, whereas almost 100 times elevation of the hepatic

448   Cd level were discovered within 24 hours after exposure in the acute exposure

449   experiment. Such the difference of exposure period and accumulation level could be the

450   reason why the significant difference in the hepatic MT expression levels was not

451   observed in our study.

452   Interestingly, global DNA methylation was altered in the testis in the

453   high-metal exposure group, although no changes were detected in the liver or kidneys in

454   the present study. This finding was supported by the higher mRNA expression levels of

455   *de novo* DNA methyltransferases, Dnmt 3a and Dnmt 3b, in the testis and the positive

456   associations among metals, methylation levels, and methyltransferases characterized by

457   PCA. It is unclear why the only testis showed a significant difference in DNA

458   hypermethylation status; however, one of the possible reasons is the rapid cell division

459   in the testis compared to in other organs. The short cycle of cell division could

460   contribute to the high sensitivity to molecular alteration. Ikeda *et al*. (2013) reported

461   testis- or germ cell-specific hypomethylated DNA domains with unique epigenetic

462   features on the mouse X chromosome, suggesting the unique molecular character of the

463   testis. Acute exposure (1 week) of TRL 1215 rat liver cells to Cd inhibited DNA

464   methyltransferase activity and induced global DNA hypomethylation, whereas a

465   relatively longer exposure period (10 weeks) resulted in DNA hypermethylation and

466   enhanced DNA methyltransferase activity, suggesting that the effects of prolonged Cd

467 exposure on DNA methylation may be responsible for its carcinogenicity (Arita and

468 Costa, 2009; Takiguchi *et al*., 2003). Another study also showed that 10-week exposure

469 to Cd induced malignant transformation associated with global DNA hypermethylation,

470 higher Dnmt 3b protein expression, and increased Dnmt activity, without any change in

471 Dnmt 1 (Arita and Costa, 2009; Benbrahim-Tallaa *et al*., 2003). In fact, previous studies

472 suggested that Dnmt 3a and Dnmt 3b, but not Dnmt 1, are responsible for *de novo*

473 methylation *in vivo*, as embryonic stem (ES) cells of mice lacking Dnmt 1 are still

474 capable of methylating retroviral DNA *de novo* (Lei et al., 1996; Okano *et al*., 1999).

475 Taken together, our results and those of these previous reports indicate that chronic Cd

476 exposure can cause global DNA hypermethylation in relation to the elevation of Dnmt

477 3a and Dnmt 3b mRNA expression and activities. It should be noted that Cd induced

478 global DNA hypomethylation, and this could be attributed to the potential facilitator of

479 Cd-stimulated cell proliferation in the chronic myelogenous leukemia K562 cell line

480 (Arita and Costa, 2009; Huang *et al*., 2008).

481   In a human epidemiological study, significant associations were observed

482 between urinary Cd concentrations and global DNA methylation as well as between

483 arsenic metabolism (measured as percentage of dimethylarsinate) and global DNA

484 hydroxymethylation (Tellez-Plaza *et al*., 2014). The major limitation of the present

485 study was that we did not measure hydroxymethylation levels. Histone modification is

486 also one of the key factors of epigenetics while the limited number of studies has

487 reported an effect of Cd and Pb on histone modification. It was suggested that Cd

488 exposure could make heritable change in chromatin structure for rapid transcription

489 activation, resulting the establishment and maintenance of a bivalent chromatin domain

490 at the MT-3 promoter as well as histone modifications (Martinez-Zamudio and Ha,

491 2011). In Cd-transformed urothelial cells, levels of H3K4me3, H3K27me3 and

492 H3K9me3 occupancy at the MT-3 promoter were increased compared to untransformed

493 cells, indicating chronic Cd exposure may alter transcriptional responses through

494 histone modification (Somji *et al*., 2011). Relating to Pb, Cantone *et al*. (2011) reported

495 that the levels of H3K4me2 and H3K9ac on histones from blood leukocytes of steel

496 production plant workers were not significantly associated with the Pb exposure level.

497 Another factor of epigenetic alteration is miRNA expression. After 3 days exposure to

498 particulate matter (PM) containing Cd, the expression of miR-146a in peripheral blood

499 leukocytes from electric furnace steel plant workers was not statistically increased

500 whereas miR-146a was negatively associated with exposure (Bollati et al., 2010). Same

501 research group also reported that miR-222 expression showed a positive association

502 with Pb exposure, while miR-146a expression was negatively correlated with Pb

503 (Bollati et al., 2010). Further studies of the association between metal exposure and

504 hydroxymethylation status, histone modification and miRNA expression are required to

505 reveal the molecular alteration mechanisms.

506   In contrast to the elevated methylation observed in the present study, the

507 methylation status of Long Interdispersed Nuclear Element 1 (LINE-1) in

508 pheochromocytoma (PC12) cells decreased after acute exposure to 500 nM Pb for 2 and

509 7 days (Li *et al*., 2012). In addition, a dose-dependent decrease in global DNA

510 methylation in PC12 cells was observed with decreasing Dnmt 1 mRNA expression (Li

511 *et al*., 2012). These discrepancies may be explained by differences in exposure duration,

512 as we used 1-year prolonged chronic exposure. Similar phenomena (i.e.,

513 hypomethylation with acute exposure and hypermethylation with chronic exposure)

514 observed in the case of Cd exposure were discussed above (Arita and Costa, 2009;

515   Takiguchi *et al*., 2003). Long-term chronic exposure experiments are necessary to

516   elucidate the effects of Pb on epigenetics.

517   Another interesting observed in the present study was that we found alterations

518   in DNA methylation in the male testis as a recent study indicated that aberrant DNA

519   methylation of the H19-DMR (differentially methylated region) and the DAZL (deleted

520   in azoospermia-like) gene promoters is associated with defects in sperm

521   production/function in infertile men (Li *et al*., 2013). Recently, our research team

522   reported severe Pb accumulation in the blood of children in Kabwe mining site, Zambia,

523   from which our soil samples were collected, and all children examined under the age of

524   7 years ($n = 246$) had blood Pb levels exceeding 5 µg/dL with a maximum of 427.8

525   µg/dL (Yabe *et al*., 2015). An earlier report showed that maternal bone Pb levels in

526   humans were inversely associated with cord blood methylation levels in LINE-1 and a

527   short interspersed element (Alu-1), which are frequently analyzed in genomic DNA

528   methylation studies (Pilsner *et al*., 2009a). However, little is known about the

529   relationship between Pb exposure and DNA methylation (and hydroxymethylation) in

530   humans. Therefore, the effects of prolonged Pb exposure on epigenetics modifications

531   in children at this site should be examined in future studies.

532

533   **5. Conclusions**

534   The aim of this study was to verify whether soil exposure under

535   environmentally relevant circumstances results in metal accumulation and epigenetic

536   modifications in the rats. Our present results suggested that soil contaminated with Cd

537   and Pb caused tissue metal accumulation and epigenetic alterations, such as elevation of

538   global DNA methylation, in rats. From the view of metal accumulation ratios

539 (lung/liver) and Pb isotopic ratios in the tissues, it was suggested that the respiratory

540 exposure would make up a significant proportion of metal absorption into the body.

541 Although we found elevation of methylation status in the rat testis, contradictory results

542 have also been documented in the literature. Further studies are required to gain a better

543 understanding of this issue.

544

562

563 **Declarations of interest**

564　　　None.

565

27

Figure Legends

Fig. 1. Analysis of Pb isotope ratios (^{208}Pb/^{206}Pb and ^{207}Pb/^{206}Pb) in the tissues of rats

and Kabwe galena (Kamona et al., 1999). The mean values and SD values are shown

with error bars. Bold dash = blood of control group, x mark = blood of low-metal

exposure group, astersisk = blood of high-metal exposure group, white diamond = brain

of control group, grey diamond = brain of low-metal exposure group, black diamond =

brain of high-metal exposure group, white square = lung of control group, grey square =

lung of low-metal exposure group, black square = lung of high-metal exposure group,

white triangle = liver of control group, grey triangle = liver of low-metal exposure

group, black trinagle = liver of high-metal exposure group, white circle = kidney of

control group, grey circle = kidney of low-metal exposure group, black circle = kidney

of high-metal exposure group, white cross in black square = Kabwe galena.

Fig. 2. Analysis of mRNA expression levels ($n = 8$ for each group) for kidney MT-1 (A),

kidney MT-2 (B), liver MT-1 (C), and liver MT-2 (D). Data are shown in box and

whiskers plots: box limits represent 25 and 75 percentiles; lines within the boxes

indicate the medians; whisker ends indicate minimum and maximum values. Different

characters (a, b) indicate significant differences (Tukey's test).

Fig. 3. Analysis of global methylation levels ($n = 10$ for each group) in the testis (A),

liver (B), and kidney (C). Data are shown in box and whiskers plots: box limits

represent 25 and 75 percentiles; lines within the boxes indicate the medians; whisker

589   ends indicate minimum and maximum values. Different characters (a, b) indicate

590   significant differences (Tukey's test).

591

592   Fig. 4. Analysis of mRNA expression levels ($n = 8$ for each group) of Dnmt 1 (A),

593   Dnmt 3a (B), and Dnmt 3b (C) in the testis. Data are shown in box and whiskers plots:

594   box limits represent 25 and 75 percentiles; lines within the boxes indicate the medians;

595   whisker ends indicate minimum and maximum values. Different characters (a, b)

596   indicate significant differences (Tukey's test).

597

598   Fig. 5. Principal component analysis among metal concentrations, global DNA

599   methylation, and of Dnmt 1, Dnmt 3a and Dnmt 3b mRNA expression in the rat testis.

600   The letters C, L, and H indicate individual rats of control, low-, and high-metal

601   exposure groups, respectively.

602

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912 Table 1. Comparison of Cd and Pb concentrations (mean ± standard deviation) in rat

913 tissues.

Tissue	Group	Cd		Pb	
Blood	Control	0.005 ± 0.001	b	0.24 ± 0.03	b
(μg/dL)	Low	0.005 ± 0.002	b	0.37 ± 0.14	b
	High	0.009 ± 0.002	a	4.22 ± 0.82	a
	Ratio *	1.7		11.3	
Testis	Control	2.1 ± 0.5	b	19.1 ± 11.3	b
(μg/kg)	Low	2.0 ± 0.3	b	13.1 ± 4.9	b
	High	7.2 ± 0.7	a	98.2 ± 79.5	a
	Ratio *	3.6		7.5	
Brain	Control	BDL		40.2 ± 39.1	b
(μg/kg)	Low	BDL		20.8 ± 9.9	b
	High	BDL		128.0 ± 100.4	a
	Ratio *	-		6.1	
Lung	Control	1.9 ± 1.0	b	4.7 ± 2.3	b
(μg/kg)	Low	2.1 ± 1.0	b	6.4 ± 4.8	b
	High	12.1 ± 2.8	a	227.7 ± 132.7	a
	Ratio *	5.9		35.8	
Liver	Control	5.9 ± 5.0	b	252.1 ± 139.4	b
(μg/kg)	Low	13.1 ± 5.2	b	279.7 ± 89.5	b
	High	30.2 ± 16.2	a	527.2 ± 97.0	a
	Ratio *	2.3		1.9	
Kidney	Control	155.3 ± 19.8	b	833.6 ± 150.3	b
(μg/kg)	Low	122.8 ± 16.9	b	1016.5 ± 242.1	b
	High	508.0 ± 88.0	a	2690.7 ± 464.2	a
	Ratio *	4.1		2.6	
Tibia	Control	5.6 ± 2.0	a	12.6 ± 165	b
(μg/kg)	Low	5.0 ± 0.6	a	419.5 ± 56.3	b
	High	5.2 ± 1.5	a	54444 ± 5831	a
	Ratio *	1.0		130	

Note: * and BDL indicate the concentration ratio (High/Low) and the below
detection limit, respectively.

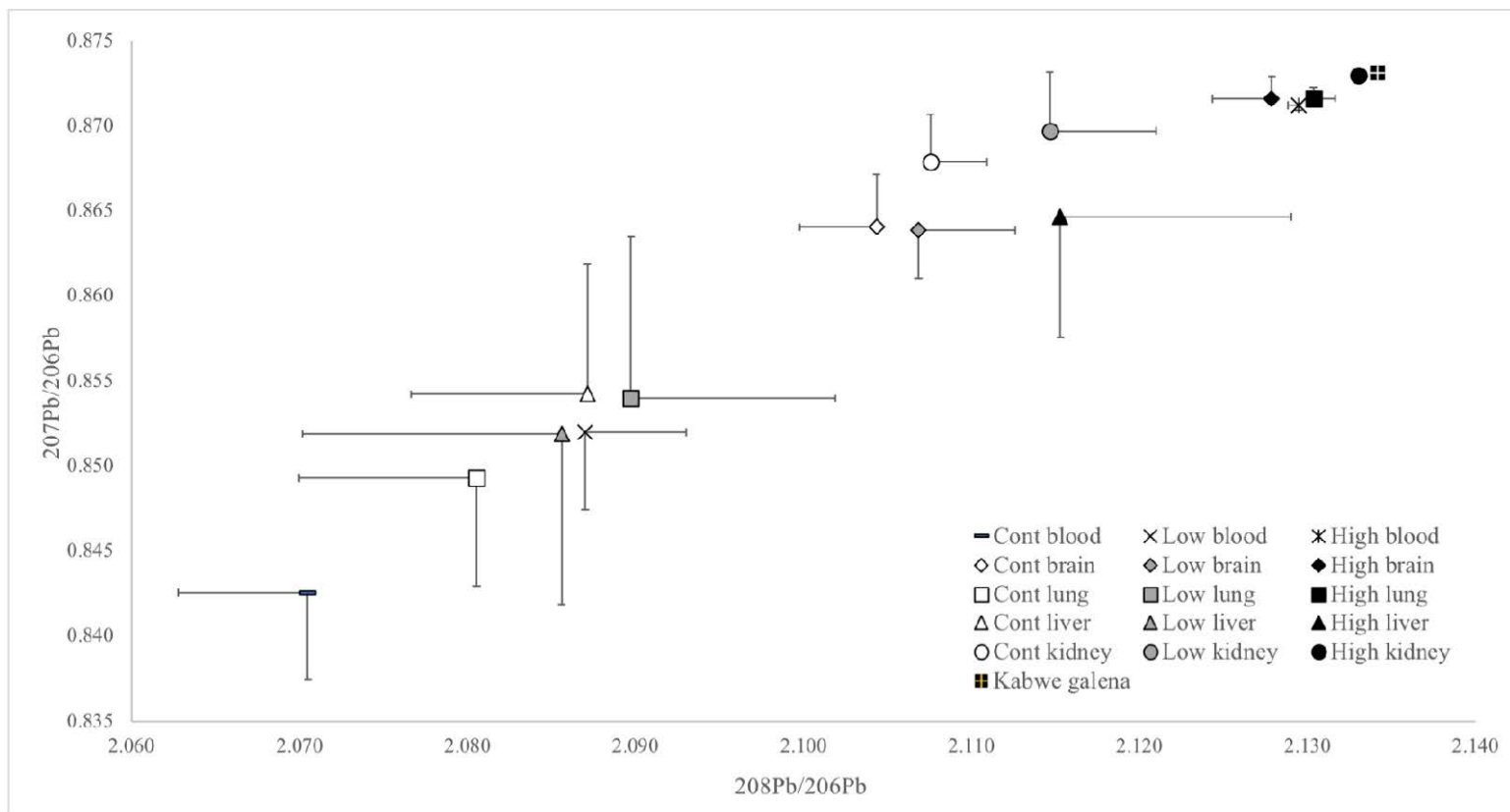Note: Different characters (a, b) indicate significant difference (Tukey's test).

914 Table 2. Comparison of behavioral activity (mean ± standard deviation) among the groups.

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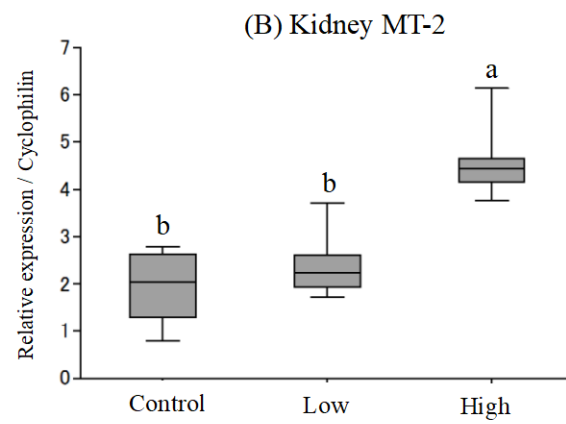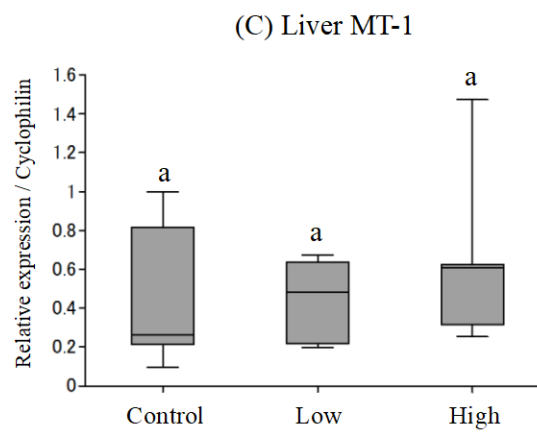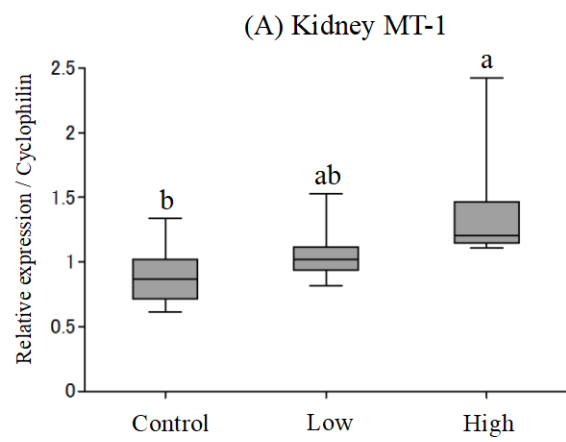| Period	Group	MOVE 1		MOVE 2		Rearing	
Before exposure	Control	5535 ± 1678	a	3745 ± 1264	a	164 ± 43	a
	Low	5263 ± 1385	a	3557 ± 1022	a	161 ± 42	a
	High	4313 ± 690	a	2861 ± 491	a	159 ± 32	a
2 month	Control	10522 ± 1535	a	6647 ± 1193	a	200 ± 37	a
	Low	7465 ± 1461	b	4795 ± 1036	b	163 ± 24	b
	High	8244 ± 1548	b	5452 ± 1145	ab	203 ± 17	a
6 month	Control	9159 ± 1782	a	5663 ± 1219	a	164 ± 28	a
	Low	7852 ± 989	ab	4852 ± 702	ab	165 ± 10	a
	High	7268 ± 1821	b	4379 ± 1249	b	151 ± 33	a
12 month	Control	6852 ± 1582	a	4281 ± 1174	a	117 ± 24	a
	Low	4894 ± 1755	b	2967 ± 1119	b	98 ± 30	a
	High	4798 ± 1442	b	2928 ± 988	b	104 ± 16	a

Note: MOVE 1 and MOVE 2 indicate large and small horizontal movement, respectively.

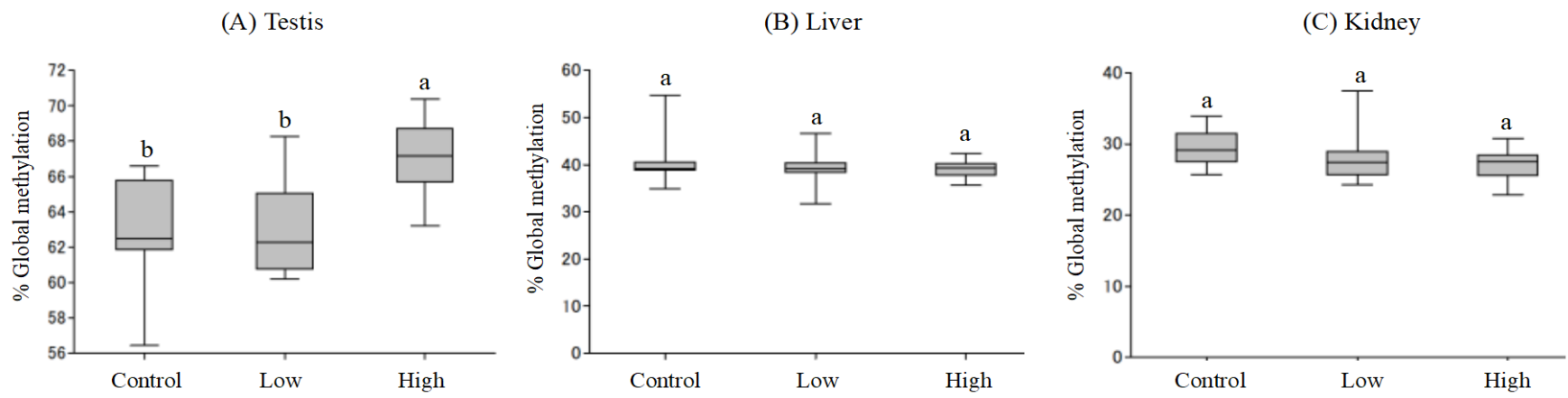Note: Different characters (a, b) indicate significant difference (Tukey's test).
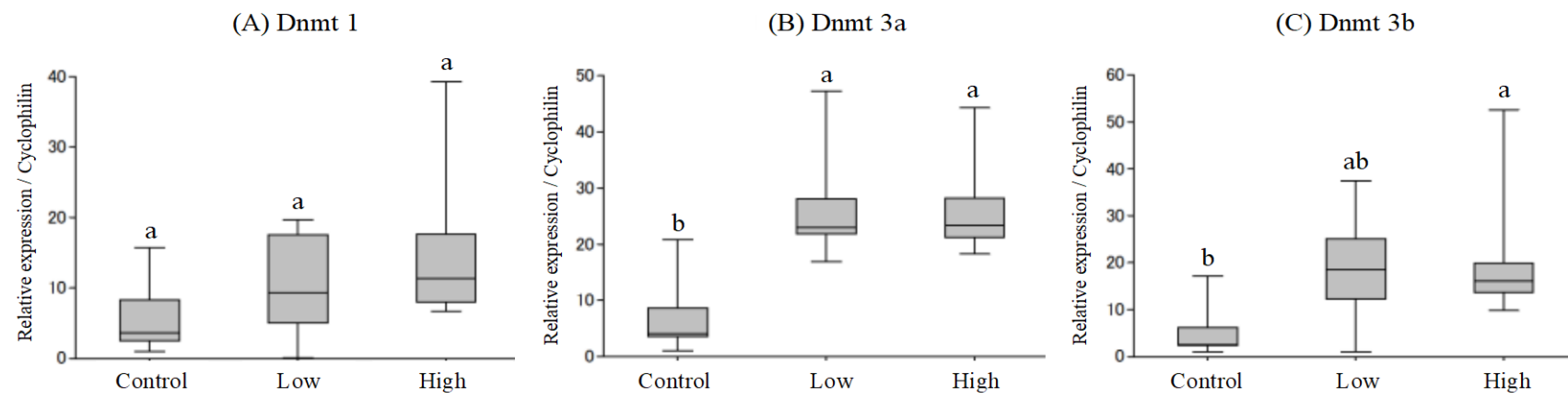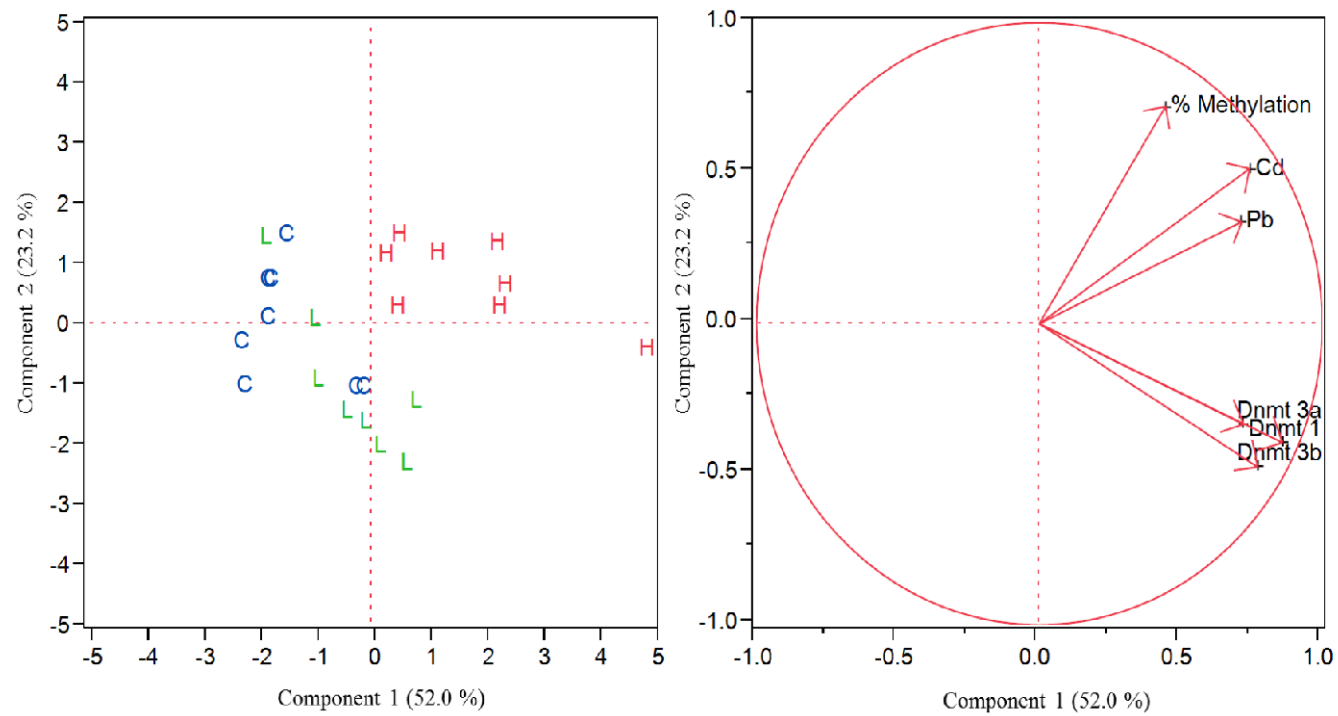
916

917   Fig. 1.

(A) Kidney MT-1

(B) Kidney MT-2

(C) Liver MT-1

(D) Liver MT-2

918

919   Fig. 2.

44

(A) Testis   (B) Liver   (C) Kidney

920

921   Fig. 3.

45

(A) Dnmt 1   (B) Dnmt 3a   (C) Dnmt 3b
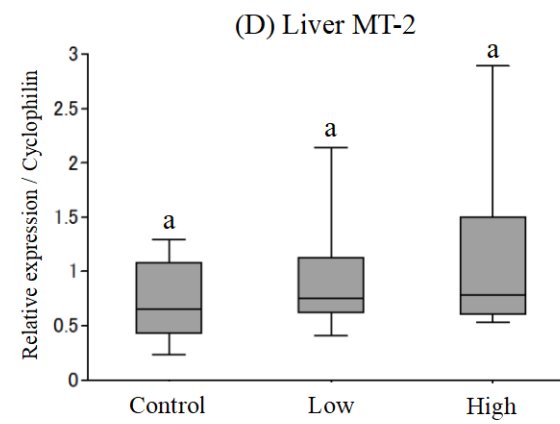
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924   Fig. 4.

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926   Fig. 5.

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