Title	Changes in the expression of epigenetic factors during copper-induced apoptosis in PC12 cells
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Citation	Journal of Environmental Science and Health, Part A : Toxic/Hazardous Substances & Environmental Engineering, 49(9), 1023-1028 https://doi.org/10.1080/10934529.2014.894847
Issue Date	2014-07-29
Doc URL	http://hdl.handle.net/2115/59555
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Type	article (author version)
File Information	Ms #A-3686revised.pdf

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Changes in the expression of epigenetic factors during copper-induced apoptosis in PC12 cells

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Short title: Changes of epigenetic factors in apoptotic status

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Received November 16, 2013

25 **ABSTRACT**

26 Despite extensive research on copper toxicity the mechanisms involved are not fully characterized.
27 There have been many recent reports concerning the relationship between epigenetic factors and
28 cell metabolism, but the effects of copper exposure on epigenetic factor has not been investigated.
29 In this study, an *in vitro* culture system was employed to study the influence of copper on apoptosis
30 and epigenetic factors in PC12 cells. When PC12 cells were exposed to copper, DNA damage was
31 observed as DNA fragmentation. In addition, cytosolic cytochrome c levels were increased by
32 copper treatment. These results suggested that copper induced apoptosis via an oxidative stress
33 pathway. This was consistent with the observation that copper-induced apoptosis was enhanced by
34 further oxidative stress induced by exposing cells to H₂O₂. In addition, the epigenetic factors were
35 significantly increased in apoptotic cells following exposure to copper and oxidative stress.

36

37 **Keywords:** DNA methylation, Epigenetic, Sir2, Copper, Oxidative Stress

38

39 **INTRODUCTION**

40

41 Copper (Cu) is an essential trace element that requires careful homeostasis within mammalian cells
42 to provide the necessary level, whilst avoiding the toxic effects associated with excess Cu. The
43 induction of the formation of reactive oxygen species (ROS) played a major role on Cu toxicity in
44 trout hepatocytes.^[1] The concept of a lysosomal-mitochondrial axis of cell death induced by

oxidants has been described.^[2] According to this concept, oxidative damage of lysosomes could result in released lysosomal enzymes acting on the mitochondria, where ROS formation would be enhanced and/or cytochrome c and other proapoptotic factors would be released.^[3] Many related studies have produced data consistent with the above reports. For instance, dithiocarbamates (DCs) are sulfur-based metal chelators that are known to exert pro-oxidant and antioxidant effects in both cell-free and biological systems.^[4] Low concentrations of N,N-diethyldithiocarbamate (DEDTC) induce apoptosis by raising the intracellular Cu level, triggering cytochrome c release and caspase activation via the formation of DEDTC-Cu complexes in human SH-SY5Y neuroblastoma cells.^[5] However, the mechanisms underlying abnormal Cu metabolism, and the resultant oxidant status require further elucidation.

The term “epigenetic changes” refers to heritable alterations in gene expression that do not involve changes in the DNA sequence. Epigenetic changes mainly originate from DNA methylation, histone modification and non-coding RNAs, all of which can regulate gene expression patterns potentially leading to abnormal expression of multiple genes and inducing diseases.^[6-9] DNA methylation is catalyzed by three major DNA methyltransferases (DNMTs).^[10] DNMT1 maintains methylation status,^[11] while DNMT3a and 3b are likely involved in *de novo* methylation.^[12]

SIRT1 is a highly-conserved longevity-related gene which encodes sirtuin 1 (known as nicotinamide adenine dinucleotide-dependent deacetylase sirtuin-1), one of six members of SIRT gene family.^[13] SIRT1 and SIRT2 are considered to play important roles in cell survival, differentiation, and metabolism.^[14]

65 These epigenetic factors (DNMT1, DNMT3a, SIRT1 and SIRT2) can be influenced by various
66 environmental factors. We have previously established a useful tool for assessing the risk of
67 chemical toxicity by measuring the mRNA expression of the epigenetic factors in an *in vitro* cell
68 culture system.^[15] PC12 cells, a cell line derived from rat pheochromocytoma cells, provide a
69 mature neuroendocrine cell model for studying diseases of the nervous and endocrine systems.^[16,17]
70 In our previous study, we showed that expression of DNMT3a, DNMT3b, and SIRT1 during serum
71 deprivation-induced apoptosis in PC12 cells was influenced by an endocrine disrupter (diethyl
72 phthalate).^[15] However, the relationship between the effects of Cu on organisms, and epigenetic
73 changes, is still unclear. In this study, PC12 cells were used to study the influence of Cu on
74 apoptosis and epigenetic factors, and the effects of oxidative stress.

75

76 **MATERIALS AND METHODS**

77

78 **Materials**

79 PC12 cells were purchased from the American Type Culture Collection (Manassas, VA, USA).

80 Dulbecco's modified Eagle's medium (DMEM) was obtained from Sigma-Aldrich (St. Louis, MO,
81 USA). Fetal bovine serum (FBS) was bought from HyClone (Rockville, MD USA). The goat anti-
82 mouse IgG biotinylated whole antibody from goat and enhanced chemiluminescence (ECL) western
83 blotting detection reagents were from Amersham Pharmacia Biotech (Buckinghamshire, England).

84 The monoclonal antibody against cytochrome c was purchased from BD Biosciences Pharmingen

85 (San Jose, CA, USA). SV Total RNA Isolation System, and Access RT-PCR Introductory System
86 were purchased from Promega (Madison, WI, USA). The highly pure PCR template preparation kit
87 was bought from Roche Diagnostics (Mannheim, Germany). Agilent RNA 6000 Nano Reagents and
88 Agilent DNA 7500 Reagents were purchased from Agilent Technologies (Waldbronn, Germany). The
89 Rotor-Gene SYBR Green RT-PCR Kit was purchased from Qiagen (Pleasanton, CA, USA).
90 Cytochrome c Releasing Apoptosis Assay Kit was from the Medical & Biological Laboratory Co.,
91 Ltd (Nagoya, Japan). Other chemicals were of analytical reagent grade. CuSO₄ and H₂O₂ were
92 dissolved in distilled water and using a sterilizing-grade filter.

93

94 **Cell Culture**

95 PC12 cells were maintained in 25 cm² flasks in DMEM supplemented with 10% FBS in a humidified
96 incubator at 37°C and 5% CO₂. Inactivation of FBS was carried out at 56°C for 30 min prior to use.
97 The cells were preincubated overnight, and then the medium was replaced with serum-containing
98 DMEM with or without CuSO₄ (final CuSO₄ concentration: 200 or 500 µM) and H₂O₂ (final H₂O₂
99 concentration: 250 µM). Five µL of a 1000-fold concentration stock solution of CuSO₄ and/or H₂O₂
100 in distilled water were added to 5 mL cell medium. For the negative control, 5 µL of distilled water
101 was added to the DMEM supplemented with 10% FBS.

102

103 **Cell Viability**

104 Cell viability was measured by trypan blue exclusion assay. PC12 cells were incubated in serum-

105 containing DMEM with CuSO₄ and/or H₂O₂ for 72 hr. After the incubation, cells were stained with
106 0.25% trypan blue solution in phosphate-buffered saline. The numbers of total cells and trypan blue-
107 stained cells were then counted using an automated cell counter (Bio-Rad, USA). Cell viability was
108 expressed as a percentage of the total cell number in each experiment. Each experiment was repeated
109 four times.

110

111 **Electrophoresis of Genomic DNA**

112 PC12 cells were cultured in 5 mL DMEM containing 10% FBS with CuSO₄ and/or H₂O₂, or 5 µL
113 distilled water (negative control) for 72 hr. After this incubation, cells were harvested using a cell
114 scraper and centrifuged at 700 × g for 5 min to remove the supernatant. Genomic DNA was isolated
115 from the cell pellet using a high pure PCR template preparation kit, according to the manufacturer's
116 instructions. After RNAase incubation, ethanol precipitation was carried out. DNA (3-5 µg) was then
117 subjected to electrophoresis on a 1.5% agarose gel. After electrophoresis, the DNA was visualized by
118 staining with ethidium bromide for 15min, and the agarose gel was photographed with a ChemiDoc
119 XRS (Bio-Rad, USA) under UV illumination.

120

121 **Western Blotting**

122 The PC12 cells treated with CuSO₄ and/or H₂O₂ were harvested by cell scraper, washed with ice-
123 cold PBS and resuspended in 500 µL Cytosol Extraction Buffer Mix containing protease inhibitor
124 cocktail and 1 mM DTT. The mixture was allowed to stand on ice for 10 min. For analysis of

125 cytochrome c, leaked cytochrome c in the cytosol was isolated using the Cytochrome c Releasing
126 Apoptosis Assay Kit. The protein concentrations in each sample were measured by Bradford assay
127 (Protein Assay, BIO-RAD, USA). The obtained solution was heated for 5 min at 100°C, and 0.1%
128 BPB-glycerol was added.

129 Supernatant proteins (10 µg) was loaded and separated by SDS-PAGE (12.5% and 5%–20% e-
130 PAGEL, ATTO, Japan), and the electrophoresed proteins were transferred onto nitrocellulose
131 membranes using a semidry blotting system (type-AE6678, ATTO, Japan). The membranes were
132 then incubated with a monoclonal antibody against cytochrome c (dilution; 1:200) in 40 mM Tris-
133 HCl buffer, pH 7.4, containing 150 mM NaCl and 1% blocking reagent for 30 min at 37°C. Anti-
134 mouse IgG biotinylated was incubated with the membrane (dilution; 1:200). Finally, streptavidin-
135 conjugated peroxidase (1:400, diluted in the same blocking buffer) was added and the membrane was
136 incubated for 30 min at 37°C. Protein bands detected by the antibody were visualized using an
137 enhanced chemiluminescence system, after washing the membrane with washing buffer. The images
138 of the detected bands were analyzed using a ChemiDoc XRS (Bio-Rad, USA).

139

140

141 **mRNA Expression Analysis by Real-time Reverse Transcription-polymerase Chain Reaction** 142 **(Real-time RT-PCR)**

143

144 Total RNA was prepared from PC12 cells cultured in serum-containing medium with 500 µM CuSO₄,

145 500 μ M CuSO₄, and 250 μ M H₂O₂ for 72 hr was prepared using the SV Total RNA Isolation kit. The
146 PCR primers for DNMT1, DNMT3a, SIRT1, SIRT2, and β -actin were synthesized according to the
147 DNA sequence as described previously.^[15] The real-time RT-PCR analysis was carried out as
148 described in our previous study.^[18] Briefly, the RT reaction was carried out at 55°C for 10 min, and
149 subsequently 40 PCR cycles were executed with an initial activation step of 95°C for 5 min, a
150 denaturation of 95°C for 5 sec, and an annealing and extension step of 60°C for 10 sec. β -actin was
151 chosen as an internal control. PCR products were real-time monitored using Rotor-Gene Q (USA)
152 software. Real-time PCR specificity and identity were verified by melting curve analysis of the PCR
153 products. Furthermore, to improve the accuracy of these RNA expression analyses, standard curves
154 were always used to calculate the relative copy numbers of the target genes and β -actin using cycle
155 threshold (Ct) values. Relative values of target gene expression in cells exposed to Cu and H₂O₂
156 were obtained from the ratio of target-genes copy numbers/ β -actin, in comparison with that in cells
157 that were not exposed to Cu and H₂O₂. These values were defined as the relative values of mRNA
158 expression for each target gene.

159

160 **Statistical Analyses**

161 All values are presented as mean $\pm$ S.E.M. and treatment groups were compared using a one-way
162 analysis of variance (ANOVA), followed by Student's t-test.

163

164 **RESULTS**

165

166 **Cell Viability**

167 To investigate whether Cu and H₂O₂ showed cell toxicity, cell viability was measured by trypan blue
168 staining after exposure of PC12 cells to 200 or 500 µM CuSO₄, with or without 250 µM H₂O₂, for 72
169 hr. As shown in Figure 1a, significant decreases in cell viability were observed in the cells exposed
170 to 200 and 500 µM CuSO₄ in comparison with control cells. In addition, the cell viability following
171 exposure to both Cu and H₂O₂ treatment was significantly lower than that observed with exposure to
172 Cu alone. These results demonstrated that Cu toxicity was enhanced in the presence of H₂O₂.

173

174 **Detection of DNA Fragmentation by Agarose Gel Electrophoresis**

175 It has been reported that apoptosis can be induced by serum deprivation in PC12 cells.^[19] Apoptosis
176 is frequently accompanied by multiple cleavage of DNA into 180–200 base pair fragments. These
177 oligonucleosomal fragments can be visualized as a characteristic DNA ladder, following agarose gel
178 electrophoresis. DNA fragmentation was observed in PC12 cells cultured in serum-containing
179 medium with 500 µM CuSO₄ (Figure 1b). However, DNA laddering was low in the presence of Cu
180 and H₂O₂.

181

182 **Cytochrome c Release Detected by Western Blotting Analyses**

183 To investigate whether Cu affected apoptotic pathways via oxidative stress, western blotting analyses

184 using lysates from PC12 cells treated with Cu and/or H₂O₂ were performed using an anti-cytochrome
185 c monoclonal antibody. Apoptosis induced by oxidative stress is generally associated with release of
186 mitochondrial cytochrome c into the cytosol. After treatment with Cu, cytosolic cytochrome c levels
187 in PC12 cells were obviously increased in comparison with those in cells not treated with Cu (Figure
188 2). In addition, cytochrome c release in PC12 cells was increased by the addition of H₂O₂, an oxidant,
189 to Cu. It was suggested that Cu treatment caused oxidative stress, and H₂O₂ enhanced the oxidative
190 stress caused by Cu treatment.

191

192 **mRNA Expression of Epigenetic Factors in PC12 Cells Exposed to Cu and H₂O₂**

193 To examine whether expression of epigenetic factors was altered by exposure to CuSO₄ and H₂O₂,
194 DNMT1, DNMT3a, SIRT1 and SIRT2 were measured using real-time RT-PCR analysis.

195 As shown in Figure 3, there were significant differences between the levels of DNMT1 (Figure
196 3a) and SIRT1 (Figure 3b) mRNA in the PC12 cells cultured in the serum-containing medium with
197 500 μM CuSO₄. DNMT1 mRNA levels in PC12 cells also tended to increase when 250 μM H₂O₂
198 was added to Cu-containing medium (Figure 3a). When PC12 cells were exposed to 500 μM CuSO₄
199 and 250 μM H₂O₂, the relative expression level for SIRT1 had a wider variance than that of the
200 control. Although no significant differences were observed, PC12 cells exposed to 500 μM CuSO₄
201 showed higher levels of DNMT3a (Figure 3c) and SIRT2 (Figure 3d) mRNAs than control cells.
202 When 250 μM H₂O₂ was added to Cu-containing medium, expression of both DNMT3a and SIRT2
203 in PC12 cells was significantly increased as compared with that of control cells. There were no

204 significant H₂O₂-dependent differences in DNMT1, DNMT3a, SIRT1 or SIRT2 mRNA expression
205 in Cu-treated PC12 cells. These findings indicated that both DNMT1 and SIRT1 expression may
206 have been upregulated by induction of apoptosis by Cu in PC12 cells. It was interesting that
207 increased DNMT1, DNMT3a, SIRT1 and SIRT2 expression were observed in PC12 cells
208 simultaneously exposed to 500 µM CuSO₄ and 250 µM H₂O₂ showed, because all of these genes
209 may be involved in a Cu-induced apoptotic pathway.

210

211 **DISCUSSION**

212 The accumulation of Cu was reported to induce reversible oxidative stress in hepatocytes, leading to
213 cytotoxicity with oxidative damage, and finally resulting in apoptosis due to the enhancement of free
214 radical levels.^[20] From the results in this study, it was indicated that gene expression of DNMT1 and
215 SIRT1 in PC12 cells was activated by Cu (Figures 3a and b). In addition, this activation was
216 amplified by the addition of H₂O₂ (Figure 3). Cu has the redox properties of a transition metal and
217 has been reported to promote lipid peroxidation probably due to hemolytic scission of H₂O₂ and of
218 endogenous hydroperoxides (ROOH), the generating hydroxyl (HO•) and alcoxyl (RO•) radicals.^[21]
219 These findings were supported by the results presented here. Previously, DNMT3a expression was
220 reported to be significantly decreased following serum-deprivation in PC12 cells which induced
221 apoptosis that was dependent on oxidative stress.^[15] The reason for this conflict between previous
222 and present results is still unclear.

223 On the other hand, serum deprivation directly affects the essential factors. For this reason, *de novo*

224 synthesis ability of DNA methylation in PC12 cells may depend on the presence of essential factors.
225 DNMT3a gene expression in PC12 cells was decreased by serum deprivation and increased by Cu
226 treatment, although both actions induced apoptosis involving oxidative stress.

227 H_2O_2 accelerated the oxidative stress caused by Cu. The presence of Cu, H_2O_2 and ascorbic acid
228 in cells can trigger a Fenton-type reaction.^[22] As shown in Figure 1a and Figure 2, the cell viability
229 and released cytochrome c levels were significantly decreased in PC12 cells treated with Cu and
230 H_2O_2 , compared with those treated with Cu alone. However, DNA ladder was not observed in the
231 PC12 cells exposed to Cu and H_2O_2 (Figure 1b). This may have been due to complete DNA digestion
232 in the presence of Cu and H_2O_2 , with the smaller fragments running out of the bottom of the agarose
233 gel, or decreased DNA synthesis in these cells due to the severe damage caused by Cu and H_2O_2 .

234 As shown in Figure 3, DNMT1, DNMT3a, SIRT1 and SIRT2 gene expression in PC12 cells
235 treated with Cu and H_2O_2 had a tendency to increase. Previous reports have indicated that SIRT1
236 expression decreased under oxidative stress induced by H_2O_2 .^[23-25] In addition, resveratrol enhanced
237 SIRT1 expression via p53 deacetylation to depress oxidative stress.^[25] p53 expression after diethyl
238 phthalate (DEP) treatment was increased in PC12 cells, which protects the cell from DEP toxicity
239 due to the oxidative stress.^[15] The increased SIRT1 expression identified in the present study may
240 contribute to protecting cells against Cu and H_2O_2 induced-oxidative stress..

241 Furthermore, it has been reported that SIRT2 expression was increased by treatment with H_2O_2 .^[26]
242 Consistent with this, the present study found that SIRT2 was significantly increased by treatment of
243 PC12 cells with Cu and H_2O_2 . Under oxidative stress, the activity and expression of DNMT1 and 3a

244 were reportedly increased.^[27] In particular, arsenic-induced oxidative stress was associated with
245 decreased DNMT1 and DNMT3a expression.^[27] However, in this study, DNMT1 gene expression in
246 PC12 cells treated with Cu was significantly increased, as compared with control. The reason for this
247 discrepancy is unclear, but may relate to the activation of different oxidative stress pathways by
248 arsenic and Cu. On the other hand, analysis of H₂O₂-resistant cells (OC-14) showed increased
249 DNMT1 expression.^[28]

250 In conclusion, DNA damage related to oxidative stress was observed in PC12 cells exposed to Cu
251 and H₂O₂. Under conditions of oxidative stress in PC12 cells, the expression of epigenetic factors
252 (DNMT1, DNMT3a, SIRT1, and SIRT2) tended to be upregulated. These results indicated that
253 changes in epigenetic factors could be used as assessment tools for oxidative dysfunction in these
254 cells. Further investigation will be needed to clarify the mechanisms regulating expression of these
255 epigenetic factors in detail.

256

257 **ACKNOWLEDGMENTS**

258 This research was partly supported by Grants-in-Aid from the Japan Society for the Promotion of
259 Science (No. 23655139 for Kurasaki).

260

261 **REFERENCES**

262 [1] Krumschnabel, G.; Manzl, C.; Berger, C.; Hofer, B. Oxidative stress, mitochondrial
263 permeability transition, and cell death in Cu-exposed trout hepatocytes. *Toxicol. Appl.*

264 Pharmacol. **2005**, *209*, 62-73.

265 [2] Brunk, U.T.; Neuzil, J.; Eaton, J.W. Lysosomal involvement in apoptosis. Redox Rep. **2001**, *6*,
266 91-97.

267 [3] Zhao, M.; Antunes, F.; Eaton, J.W.; Brunk, U.T. Lysosomal enzymes promote mitochondrial
268 oxidant production, cytochrome c release and apoptosis. Eur. J. Biochem. **2003**, *270*, 3778-3786.

269 [4] Nobel, C.I.; Kimland, M.; Lind, B.; Orrenius, S.; Slater, A.F. Dithiocarbamates induce apoptosis
270 in thymocytes by raising the intracellular level of redox-active copper. J. Biol. Chem. **1995**, *270*,
271 26202-26208.

272 [5] Matias, A.C.; Manieri, T.M.; Cipriano, S.S.; Carioni, V.M.; Nomura, C.S.; Machado, C.M.;
273 Cerchiaro, G. Diethyldithiocarbamate induces apoptosis in neuroblastoma cells by raising the
274 intracellular copper level, triggering cytochrome c release and caspase activation. Toxicol. In
275 Vitro **2013**, *27*, 349-357.

276 [6] Wilkins, J.F. Genomic imprinting and methylation: epigenetic canalization and conflict. Trends
277 Genet. **2005**, *21*, 356-365.

278 [7] Maruyama, R.; Suzuki, H.; Yamamoto, E.; Imai, K.; Shinomura, Y. Emerging links between
279 epigenetic alterations and dysregulation of noncoding RNAs in cancer. Tumour Biol. **2012**, *33*,
280 277-285.

281 [8] Fraga, M.F.; Esteller, M. Epigenetics and aging: the targets and the marks. Trends Genet. **2007**,
282 *23*, 413-418.

283 [9] Flower, K.; Hellen, E.; Newport, M.J.; Jones, S.; Sinclair, A.J. Evaluation of a prediction

284 protocol to identify potential targets of epigenetic reprogramming by the cancer associated
 285 Epstein Barr virus. PLoS One **2010**, 5, e9443.

286 [10] Rodriguez-Osorio, N.; Wang, H.; Rupinski, J.; Bridges, S.M.; Memili, E. Comparative
 287 functional genomics of mammalian DNA methyltransferases. Reprod. Biomed. Online **2010**, 20,
 288 243-255.

289 [11] Chmurzynska, A. Fetal programming: link between early nutrition, DNA methylation, and
 290 complex diseases. Nutr. Res. **2009**, 68, 87-98.

291 [12] Li, S.; Chiang, T.C.; Richard-Davis, G.; Barrett, J.C.; McLachlan, J.A. DNA
 292 hypomethylation and imbalanced expression of DNA methyltransferases (DNMT1, 3A, and 3B)
 293 in human uterine leiomyoma. Gynecol. Oncol. **2003**, 90, 123-130.

294 [13] Lavu, H.; Klinge, M.J.; Nowcid, L.J.; Cohn, H.E.; Grenda, D.R.; Sauter, P.K.; Leiby, B.E.;
 295 Croker, S.P.; Kennedy, E.P.; Yeo, C.J. Perioperative surgical care bundle reduces
 296 pancreaticoduodenectomy wound infections. J. Surg. Res. **2012**, 174, 215-221.

297 [14] Wakeling, L.A.; Ions, L.J.; Ford, D. Could Sirt1-mediated epigenetic effects contribute to
 298 the longevity response to dietary restriction and be mimicked by other dietary interventions?
 299 Age **2009**, 31, 327-341.

300 [15] Sun, Y.; Guo, Z.; Iku, S.; Saito, T.; Kurasaki, M. Diethyl phthalate enhances expression of
 301 SIRT1 and DNMT3a during apoptosis in PC12 cells. J. Appl. Toxicol. **2013**, 33, 1484-1492

302 [16] Chestnut, B.A.; Chang, Q.; Price, A.; Lesuisse, C.; Wong, M.; Martin, L.J. Epigenetic
 303 regulation of motor neuron cell death through DNA methylation. J. Neurosci. **2011**, 31, 16619-

304 16636.

- 305 [17] De Simone, R.; Ajmone-Cat, M.A.; Tirassa, P.; Minghetti, L. Apoptotic PC12 cells
306 exposing phosphatidylserine promotes the production of anti-inflammatory and neuroprotective
307 molecules by microglial cells. *J. Neuropathol. Exp. Neurol.* **2003**, *62*, 208-216.
- 308 [18] Sun, Y.; Mukai, Y.; Tanaka, M.; Saito, T.; Sato, S.; Kurasaki, M. Green tea extract increases
309 mRNA expression of enzymes which influence epigenetic marks in newborn female offspring
310 from undernourished pregnant mother. *PLoS One* **2013**, *8*, e74559.
- 311 [19] Maroto, R.; Perez-Polo, J.R. BCL-2-related protein expression in apoptosis: oxidative
312 stress versus serum deprivation in PC12 cells. *J. Neurochem.* **1997**, *69*, 514-523.
- 313 [20] Boveris, A.; Musacco-Sebio, R.; Ferrarotti, N.; Saporito-Magriñá, C.; Torti, H.; Massot, F.;
314 Repetto, M.G. The acute toxicity of iron and copper: Biomolecule oxidation and oxidative
315 damage in rat liver. *J. Inorg. Biochem.* **2012**, *116*, 63-69.
- 316 [21] Repetto, M.G.; Ferrarotti, N.F.; Boveris, A. The involvement of transition metal ions on
317 iron-dependent lipid peroxidation. *Arch. Toxicol.* **2010**, *84*, 255-262.
- 318 [22] Cao, H.; Wang, Y. Quantification of oxidative single-base and intrastrand cross-link lesions
319 in unmethylated and CpG-methylated DNA induced by Fenton-type reagents. *Nucleic Acids Res.*
320 **2007**, *35*, 4833-4844.
- 321 [23] Furukawa, A.; Tada-Oikawa, S.; Kawanishi, S.; Oikawa, S. H₂O₂ accelerates cellular
322 senescence by accumulation of acetylated p53 via decrease in the function of SIRT1 by NAD⁺
323 depletion. *Cell Physiol. Biochem.* **2007**, *20*, 45-54.

- 324 [24] de Boer, V.C.; de Goffau, M.C.; Arts, I.C.; Hollman, P.C.; Keijer, J. SIRT1 stimulation by
325 polyphenols is affected by their stability and metabolism. *Mech. Ageing Dev.* **2006**, *127*, 618-
326 627.
- 327 [25] Cao, C.; Lu, S.; Kivlin, R.; Wallin, B.; Card, E.; Bagdasarian, A.; Tamakloe, T.; Wang,
328 W.J.; Song, X.; Chu, W.M.; Kouttab, N.; Xu, A.; Wan, Y. SIRT1 confers protection against
329 UVB- and H2O2-induced cell death via modulation of p53 and JNK in cultured skin
330 keratinocytes. *J. Cell Mol. Med.* **2009**, *13*, 3632-3643.
- 331 [26] Wang, F.; Nguyen, M.; Qin, F.X.; Tong, Q. SIRT2 deacetylates FOXO3a in response to
332 oxidative stress and caloric restriction. *Aging Cell* **2007**, *6*, 505-514.
- 333 [27] Han, Z.J.; Song, G.; Cui, Y.; Xia, H.F.; Ma, X. Oxidative stress is implicated in arsenic-
334 induced neural tube defects in chick embryos. *Int. J. Dev. Neurosci.* **2011**, *29*, 673-680.
- 335 [28] Mishra, M.V.; Bisht, K.S.; Sun, L.; Muldoon-Jacobs, K.; Awwad, R.; Kaushal, A.; Nguyen,
336 P.; Huang, L.; Pennington, J.D.; Markovina, S.; Bradbury, C.M.; Gius, D. DNMT1 as a molecular
337 target in a multimodality-resistant phenotype in tumor cells. *Mol. Cancer Res.* **2008**, *6*, 243-249.

338 **FIGURE CAPTIONS**

339

340 Figure 1. Cell viability (a) and DNA agarose gel electrophoresis (b) of PC12 cells treated with 0-
341 500 μ M Cu and 250 μ M H₂O₂ for 72 hr. Error bars indicate S.E.M. (n=4). *: there are significant
342 differences in cell viability between control cells and each treatment cell (p<0.01). a, b, c, d:
343 significant difference between the same letter (p<0.05).

344

345 Figure 2. Cytochrome c levels in PC12 cells with 0–500 μ M Cu and 250 μ M H₂O₂. *: there are
346 significant differences in cell viability between control cells and each treatment cell (p<0.05). a, b, c,
347 d: significant difference between the same letter (p<0.05)

348

349 Figure 3. mRNA expression of epigenetic factors determined by real-time RT-PCR for DNMT1 (a),
350 SIRT1 (b), DNMT3a (c) and SIRT2 (d) in PC12 cells treated with and without CuSO₄ and H₂O₂ for
351 72 hr. Values are mean $\pm$ S.E.M. (n = 6). DNMT, DNA methyltransferase; SIRT, Sirtuin; ^{a, b} P<0.05
352 compared with the same letter.

353

Fig. 1

Fig. 2

356

357 Fig. 3