



Title	The effect of copper on the mRNA expression profile of xenobiotic-metabolizing enzymes in cultured rat H4-II-E cells
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1 **Title page**

2 **The effect of copper on the mRNA expression profile of xenobiotic-metabolizing**  
3 **enzymes in cultured rat H4-II-E cells**

4

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20

21 **Running head:**

22 Effects of Copper on XMEs in rat H4-II-E cells

23

24

25 **Abstract**

26 Copper ( $\text{Cu}^{2+}$ ) is an essential element that plays important roles in physiological  
27 functions of the body. However, high  $\text{Cu}^{2+}$  levels can have toxic implications. This study  
28 aims to investigate the constitutive response to  $\text{Cu}^{2+}$  exposure of xenobiotic-metabolizing  
29 enzymes in cultured rat liver (H4-II-E) cell lines. Rat cells were exposed to copper sulfate  
30 (0–500  $\mu\text{M}$ ) for 24 h. The effects of  $\text{Cu}^{2+}$  on the mRNA expressions of phase I and II  
31 enzymes and regulatory elements were examined using real-time PCR. Metallothionein  
32 mRNA expression was induced in a dose-dependent manner after treatment with  $\text{Cu}^{2+}$ .  
33 mRNA expressions of phase I enzymes such as cytochrome P450 1A1 and 1A2  
34 (CYP1A1 and CYP1A2) were slightly induced after exposure to low concentrations of  
35  $\text{Cu}^{2+}$ ; however, CYP1A1 and CYP1A2 mRNA expressions were significantly down-  
36 regulated at higher  $\text{Cu}^{2+}$  concentrations. These effects corresponded with expression of  
37 aryl hydrocarbon receptor mRNA. The mRNA expressions of phase II enzymes were  
38 reduced upon exposure to  $\text{Cu}^{2+}$ . In conclusion, phase I and II enzyme expressions were  
39 significantly modulated upon  $\text{Cu}^{2+}$  exposure. These results indicated that  $\text{Cu}^{2+}$  exposure  
40 had toxicological implications for cultured H4-II-E cells.

41

42

### 43 **Introduction**

44 Copper ( $\text{Cu}^{2+}$ ) is one of the essential trace elements that plays important roles in the  
45 biochemistry and physiology of all living organisms. As it is a cofactor for various  
46 enzymes such as cytochrome oxidase and superoxide dismutase,  $\text{Cu}^{2+}$  is also an essential  
47 element for cellular respiration and free radical defense [1]. Copper can also be toxic to  
48 cells when a significant quantity of its ions exist in a free, uncoordinated form [2]. This  
49 toxicity appears to cause oxidative damage to single macromolecules such as  
50 lipoproteins, DNA, or thiol-containing enzymes. Copper can therefore damage  
51 membranes, organelles, and intact cells, including hepatocytes [3]. Metals such as  $\text{Cu}^{2+}$   
52 are often persistent and accumulated in the environment and food; therefore, potential  
53 exposure of living organisms to these metals has biological consequences. Such effects  
54 involve the xenobiotic-metabolizing enzyme (XME) system.

55 Rats, particularly those in the wild, have been reported to be exposed to high levels of  
56 heavy metals and trace elements such as copper, lead, arsenic, chromium, nickel, zinc,  
57 and cadmium in lead–zinc mining areas in Kabwe, Zambia [4]. However, the biological  
58 response of rats to elevated levels of  $\text{Cu}^{2+}$  has not been thoroughly investigated.

59 Metallothionein (MT) is a cysteine-rich, metal-binding protein that has a significant role  
60 in the metabolism and detoxification of heavy metals such as cadmium and mercury [5].  
61 Thus, the induction of MT gene expression is considered as a valuable marker for heavy-  
62 metal exposure (particularly with respect to cadmium, mercury, and zinc). However, the  
63 relationship between  $\text{Cu}^{2+}$  exposure and MT gene expression has been less well defined.

64 Cytochrome P450 (CYP)1A1 and 1A2 are major XMEs in metabolizing procarcinogenic  
65 and environmental pollutants such as polycyclic aromatic hydrocarbons [6]. The resultant

66 metabolites undergo conjugation, elimination, and detoxification reactions via phase II  
67 metabolizing enzymes, including UDP-glucuronosyltransferases (UGT), glutathione *S*-  
68 transferases (GST), NAD(P):quinone oxidoreductase 1 (NQO1), and sulfotransferases  
69 (SULT) [7].

70 Regulation and expression of above XMEs are mainly mediated through a cytosolic  
71 receptor known as the aryl hydrocarbon receptor (AhR) [8]. The constitutive effects of  
72  $\text{Cu}^{2+}$  on the regulation and expression of the AhR-regulated genes in rats are still unclear.  
73 Therefore, the objectives of this study were firstly, to prove that MT1 is an ideal  
74 biomarker for  $\text{Cu}^{2+}$  exposure in the rat liver cells. Secondly, to investigate the cross-talks  
75 between  $\text{Cu}^{2+}$  and XMEs CYP1A1 and 1A2, UGT1A6, GST1A, NQO1, and SULT1C1,  
76 in cultured rat H4-II-E cells exposed to  $\text{Cu}^{2+}$ . Thirdly, the effect of  $\text{Cu}^{2+}$  on the regulatory  
77 element, AhR, mRNA expression was also examined.

78

79 **Materials and Methods**

80 Chemicals and reagents

81 All test reagents used were of reagent grade. TRI reagent was purchased from Sigma  
82 Chemical Co. (St. Louis, MO, USA). Oligo(dT) primers, reverse transcriptase (RT)-  
83 buffer and ReverTra Ace were purchased from Toyobo Co. (Osaka, Japan). Primer sets  
84 were purchased from Invitrogen (Carlsbad, CA, USA). All other reagents were of  
85 analytical grade or the highest quality available; they were purchased from Wako Pure  
86 Chemical Industries (Tokyo, Japan).

87

88 Cell line and culture conditions

89 All experiments were performed according to the guidelines of the Hokkaido University  
90 Biosafety Committee. H4-II-E rat hepatoma cells obtained from the American Type  
91 Culture Collection (Manassas, VA), were cultured in Dulbecco's modified Eagle's  
92 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum and  
93 antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin) at 37°C in a humidified  
94 incubator with 5% CO<sub>2</sub>. Cells were seeded in 60 mm collagen-coated dishes, sub-cultured  
95 twice a week, and subsequently grown to 80–90% confluence.

96

97 Exposure of cells to Cu<sup>2</sup>

98 Cells were exposed to copper sulfate (0, 30, 60, 125, 250 and 500 µM) in serum-free  
99 medium for 24 h. Five dishes represent each treatment. After 24 h exposure, the medium  
100 was removed and the cells were washed twice with phosphate-buffered saline. This  
101 experiment was repeated three times at different days.

102

### 103 Cell viability assay

104 Cell viability was determined through the CCK-8 assay (Sigma-Aldrich) by measuring  
105 the reduction of WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-  
106 disulfophenyl)-2*H*-tetrazolium, monosodium salt), which produces a water-soluble  
107 formazan dye.

108

### 109 RNA extraction

110 Total RNA was extracted using TRI reagent (Sigma-Aldrich) according to the  
111 manufacturer's instructions. Total RNA concentration and quality were checked with a  
112 Nanodrop ND-1000 spectrophotometer (DYMO, Stamford, USA). The RNA quality was  
113 estimated by the 260/280 nm and 260/230 nm absorbance ratios and confirmed by  
114 denaturing agarose gel electrophoresis.

115

### 116 cDNA synthesis

117 cDNA was synthesized as described previously [9]. In brief, a solution of 5 µg of total  
118 RNA and 0.5 ng of oligo(dT) primers in a total volume of 24 µl of sterilized ultrapure  
119 water was incubated at 70°C for 10 min and then removed from the thermal cycler. The  
120 volume was increased to 40 µl with a mixture of 4 µl (5×) RT-buffer, 8 µl 10 mM dNTP,  
121 2 µl water, and 2 µl RT (Toyobo Co., Ltd). The mixture was then re-incubated in the  
122 thermal cycler at 42°C for 45 min and at 90°C for 10 min to prepare the cDNA.

123

### 124 Quantitative real-time RT polymerase chain reaction (PCR)

125 Quantitative real-time RT PCR using TaqMan gene expression assays (Applied  
126 Biosystems, CA, USA) and a StepOne real-time PCR system (Applied Biosystems) were  
127 performed to determine rat mRNA levels. The primer and probe sets for each specific  
128 gene were as follows: Rn00487218\_m1 (*CYP1A1*), Rn00561082\_m1 (*CYP1A2*),  
129 Rn00565750\_m1 (*AhR*), Rn00756113\_AH (*UGT1A6*), Rn00755117\_A1 (*GSTA1*),  
130 Rn00566528\_m1 (*NQO1*), Rn00581955\_m1 (*SULT1C1*), and Rn99999916\_s1  
131 (*GAPDH*). The reaction underwent 40 cycles of the following: initial activation at 95°C  
132 for 20 s, denaturation at 95°C for 1 s, and annealing and extension at 60°C for 20 s.  
133 Measurements of specific enzyme and receptor genes, as well as *GAPDH*, were  
134 performed in duplicate and repeated three times. The expression of each gene was  
135 normalized with respect to the expression of *GAPDH*, and was calculated relative to  
136 control levels using the comparative threshold cycle (Ct) method. In addition, the  
137 following specific primers were used to measure the expression of the MT-1 gene:  
138 forward primer 5'-caccgttgctccagattcac-3', reverse 5'-aggagcagcagctcttcttg-3' (accession  
139 number: NG\_006919.3); and *GAPDH*, forward 5'-gtttcaccaccacggagaaggc-3', reverse  
140 5'-atgccagtgagcttcccgttcagc-3' (accession number: XR086293.2). To measure the  
141 expression levels of MT-1 and *GAPDH* genes, PCR reactions were run in a total volume  
142 of 10 µl. The PCR reaction mixture was prepared with SYBR qPCR Mix (Toyobo), 10  
143 µM of each primer, 600 ng cDNA, 50× ROX reference dye, and RNase-free water. The  
144 mixture was made up to a final volume of 10 µl. The reaction cycle comprised an initial  
145 holding stage at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s,  
146 annealing at 60°C for 1 min, and extension at 72°C for 30 s. Melting curve analysis and  
147 agarose gel electrophoresis confirmed the amplification of a single amplicon of the



148 expected size as well as the absence of primer dimers and genomic DNA amplification.

149 GAPDH was used for normalization in the comparative Ct method.

150

151 Statistical analysis

152 Statistical significance was evaluated using the Tukey–Kramer honestly significant

153 difference test (JMP statistical package, SAS Institute Inc., Cary, NC, USA). A *P* value

154 of  $<0.05$  was considered to be significant.

155

156 **Results**

157 Treatment of H4-II-E cells with various concentrations of  $\text{Cu}^{2+}$  did not significantly  
158 affect the cell viability, as demonstrated in Figure 1.

159 MT mRNA expression was clearly induced in a dose-dependent manner in cultured rat  
160 cells after exposure to various concentrations of  $\text{Cu}^{2+}$  (Fig. 2).

161 Figure 3 summarizes the effects of the various concentrations of  $\text{Cu}^{2+}$  on phase I and II  
162 metabolizing enzymes. Treatment of the cultured rat cells with  $30\ \mu\text{M}$   $\text{Cu}^{2+}$  significantly  
163 increased CYP1A1 mRNA expression by 2.5-fold compared with non-treated cells,  
164 although exposure to higher  $\text{Cu}^{2+}$  concentrations ( $60\text{--}250\ \mu\text{M}$ ) did not significantly alter  
165 CYP1A1 mRNA expression. However,  $500\ \mu\text{M}$   $\text{Cu}^{2+}$  reduced CYP1A1 mRNA levels, as  
166 shown in Figure 3a. The cells treated with  $\text{Cu}^{2+}$  concentration of  $30\ \mu\text{M}$  induced  
167 CYP1A2 mRNA expression level twofold of that of control cells, whereas treatment with  
168 higher concentrations had no effect (Fig. 3b). Phase II enzymes such as UGT1A6, GSTP1,  
169 NQO1, and SULT1C1 showed a similar pattern of mRNA expression after exposure to  
170 various concentrations of  $\text{Cu}^{2+}$ . Low concentrations of  $\text{Cu}^{2+}$  ( $30\text{--}60\ \mu\text{M}$ ) had no effect on  
171 the mRNA expression for UGT1A6, GSTP1, NQO1, or SULT1C1 in the cells.  
172 Meanwhile, treatment of H4-II-E cells with higher concentrations of  $\text{Cu}^{2+}$  resulted in a  
173 clearly dose-dependent reduction of mRNA expression (Fig. 3c, d, e, and f).

174 Copper induced AhR mRNA expression in H4-II-E cells, only when it was present in low  
175 concentrations ( $30\ \mu\text{M}$ ). However, at higher concentrations of copper, there was either no  
176 clear effect ( $60\text{--}250\ \mu\text{M}$   $\text{Cu}^{2+}$ ) or a reduction in mRNA expression ( $500\ \mu\text{M}$   $\text{Cu}^{2+}$ ) (Fig.  
177 4).

178

179 **Discussion**

180 Copper is an essential nutrient that is incorporated into a number of metalloenzymes for  
181 hemoglobin formation, carbohydrate metabolism and catecholamine biosynthesis, etc.  
182 These copper-dependent enzymes function by reducing molecular oxygen [10]. However,  
183 exposure to high doses of  $\text{Cu}^{2+}$ , especially in highly polluted environments, may result in  
184 several biological consequences, especially on XMEs.

185 Exposure of H4-II-E cells to various concentrations of  $\text{Cu}^{2+}$  did not affect cell viability.  
186 This result corresponds with those obtained by Jonsson and coworkers [11], who found  
187 that  $\text{Cu}^{2+}$  concentrations of 0 to 1000  $\mu\text{M}$  did not affect the cell viability of primary fish  
188 epithelial cells.

189 Few studies have examined copper chelation by MT since cadmium is considered as a  
190 principal MT inducer. Notwithstanding, the results in the present study demonstrate that  
191 MT in rat H4-II-E cells was also affected by exposure to  $\text{Cu}^{2+}$  in a dose-dependent  
192 manner (Fig. 2). The same physiological trend has been highlighted in other studies  
193 investigating dose–response relationships between injected cadmium and MT in toadfish  
194 liver [12]. Other published reports from our laboratory confirm that MT is also induced in  
195 cattle blood cells in a dose-dependent fashion after lead treatment [13] and even in  
196 cultured rat cells after exposure to various concentrations of lead [14]. Thus, MT could be  
197 used as a biomarker for  $\text{Cu}^{2+}$  exposure.

198 Interestingly, expressions of both CYP1A1 and CYP1A2 mRNA were induced after  
199 exposure to the low concentration of  $\text{Cu}^{2+}$  (30  $\mu\text{M}$ ). These inductions were markedly  
200 decreased to even below that of the control at exposure to higher concentrations of  $\text{Cu}^{2+}$   
201 (60–500  $\mu\text{M}$ ) (Fig. 3a, b). These results are in agreement with the reduction of CYP1A-

202 dependent ethoxyresorufin-*O*-deethylase activity in trout gill primary cells treated with a  
203 high concentration of Cu<sup>2+</sup> (1000 μM) [11].

204 CYP1A mRNA expression corresponded highly with that of AhR mRNA in treated rat  
205 cells. Treatment with Cu<sup>2+</sup> at the low concentration (30 μM) induced AhR mRNA  
206 expression, but high Cu<sup>2+</sup> concentrations (250–500 μM) downregulated AhR mRNA  
207 expression under (Fig. 4). AhR is located in the cytoplasm as an inactive form that is  
208 bound to other proteins, such as heat shock protein 90. AhR activates upon binding to  
209 AhR ligand-like dioxins, leading to its translocation to the nucleus, where this complex  
210 dimerizes with the AhR nuclear translocator, which in turn binds to xenobiotic-  
211 responsive elements located in the promoter region of each AhR-regulated gene, resulting  
212 in mRNA transcription and protein translation [8]. Regulation of the CYP1A subfamily  
213 (1A1 and 1A2) has been shown to be involved in the activation of the AhR-dependent  
214 pathway by direct binding of AhR ligands to the AhR [15]. Interestingly, Cu<sup>2+</sup> is not  
215 similar to and does not have the structural properties of classical AhR ligands, suggesting  
216 that it could be a novel non-classical inducer of AhR, inducing CYP1A expression  
217 without binding to the AhR. The exposure to higher copper concentrations seems to cause  
218 suppression of AhR gene battery in H-4-II-E cells. Heme oxygenase-1 (HO-1) mRNA,  
219 which is used as a biomarker for oxidative stress, is significantly induced in murine  
220 Hepa1c1c7 cells exposed to lead, copper, and mercury [16]. The inhibition of CYP1A1  
221 and AhR expression by high Cu<sup>2+</sup> concentrations may be explained by the induction of  
222 oxidative stress.

223 Phase II enzymes (UGT1A6, GST1A, NQO1, and SULT1C1) showed higher sensitivity  
224 to Cu<sup>2+</sup> exposure, as their expression were significantly reduced upon exposure to even

225 low concentrations of  $\text{Cu}^{2+}$ , especially in the cases of GST1A and SULT1C2 (Fig. 3c–f).  
226 These results were in agreement with those of Korashy and El-Kadi [16], who reported  
227 that the induced GST- and NQO1-dependent activities in murine Hepalclc7 cell lines  
228 exposed to AhR ligands such as 3-methylcholanthrene and benzo[a]pyrene were  
229 significantly reduced after co-exposure with increasing doses of  $\text{Cu}^{2+}$ . In addition to AhR,  
230 the functional suppression of Nrf2, which is one of regulators of phase II enzymes,  
231 should also be investigated in H4-II-E cells. The clear downregulation of phase II  
232 enzymes, especially at increasing doses of  $\text{Cu}^{2+}$ , may be attributed to metal-mediated  
233 oxidative stress, such as the production of reactive oxygen species and lipid peroxidation  
234 [17, 18]. This interference with phase I and II enzyme expression may have toxicological  
235 implications for H4-II-E cells, particularly in cases of co-exposure to  $\text{Cu}^{2+}$  and other  
236 xenobiotics.

237 In conclusion, the present study declared that XMEs are significantly modulated upon  
238 exposure to  $\text{Cu}^{2+}$  in the rat H4-II-E cells in a dose-dependent fashion.  $\text{Cu}^{2+}$  induced  
239 CYP1A family mRNA expression; however, phase II enzymes mRNA expression was  
240 reduced. These biological changes produce a state of imbalance between bioactivation  
241 and detoxification pathways, resulting in several toxicological implications upon  
242 exposure to other xenobiotics, especially in highly polluted areas. This modulation may  
243 be attributed to mechanistic pathways. Furthermore, MT is induced after  $\text{Cu}^{2+}$  treatment,  
244 suggesting that it could be a major detoxification enzyme during exposure to higher  
245 concentrations of  $\text{Cu}^{2+}$ . Thus, it could be used as a biomarker for  $\text{Cu}^{2+}$  exposure in rats.  
246 Further approaches are still needed to elucidate the exact mechanisms mediating the  
247 effects of  $\text{Cu}^{2+}$  on XMEs in rats.



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256

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- 315
- 316
- 317

318 **Figure legends**

319

320 Figure 1: Effect of copper on H4-II-E cell viability.

321

322 Figure 2: MT-1 mRNA expression in H4-II-E rat cells treated with Cu<sup>2+</sup>.

323 The effect of Cu<sup>2+</sup> treatments on MT-1 mRNA expression in H4-II-E rat cells, as  
324 analyzed using real-time RT-PCR. Data are presented as the mean ± standard deviation  
325 (SD). Identical letters represent expression levels that are not significantly different from  
326 each other ( $P < 0.05$ ).

327

328 Figure 3: Expression of phase I and II enzyme mRNA in H4-II-E rat cells treated with  
329 Cu<sup>2+</sup>.

330 The effect of Cu<sup>2+</sup> treatment on a) CYP1A1, b) CYP1A2, c) UGT1A6, d) GST1A, e)  
331 NQO1, and f) SULT1C1 mRNA expression in H4-II-E rat cells as determined by real-  
332 time RT-PCR. Data are presented as the mean ± SD. Identical letters represent expression  
333 levels that are not significantly different from each other ( $P < 0.05$ ).

334

335 Figure 4: AhR mRNA expression in H4-II-E rat cells treated with Cu<sup>2+</sup>.

336 The effect of Cu<sup>2+</sup> treatment on AhR mRNA expression in H4-II-E rat cells as  
337 determined using real-time RT-PCR. Data are presented as the mean ± SD. Identical  
338 letters represent expression levels that are not significantly different from each other ( $P <$   
339  $0.05$ ).

340

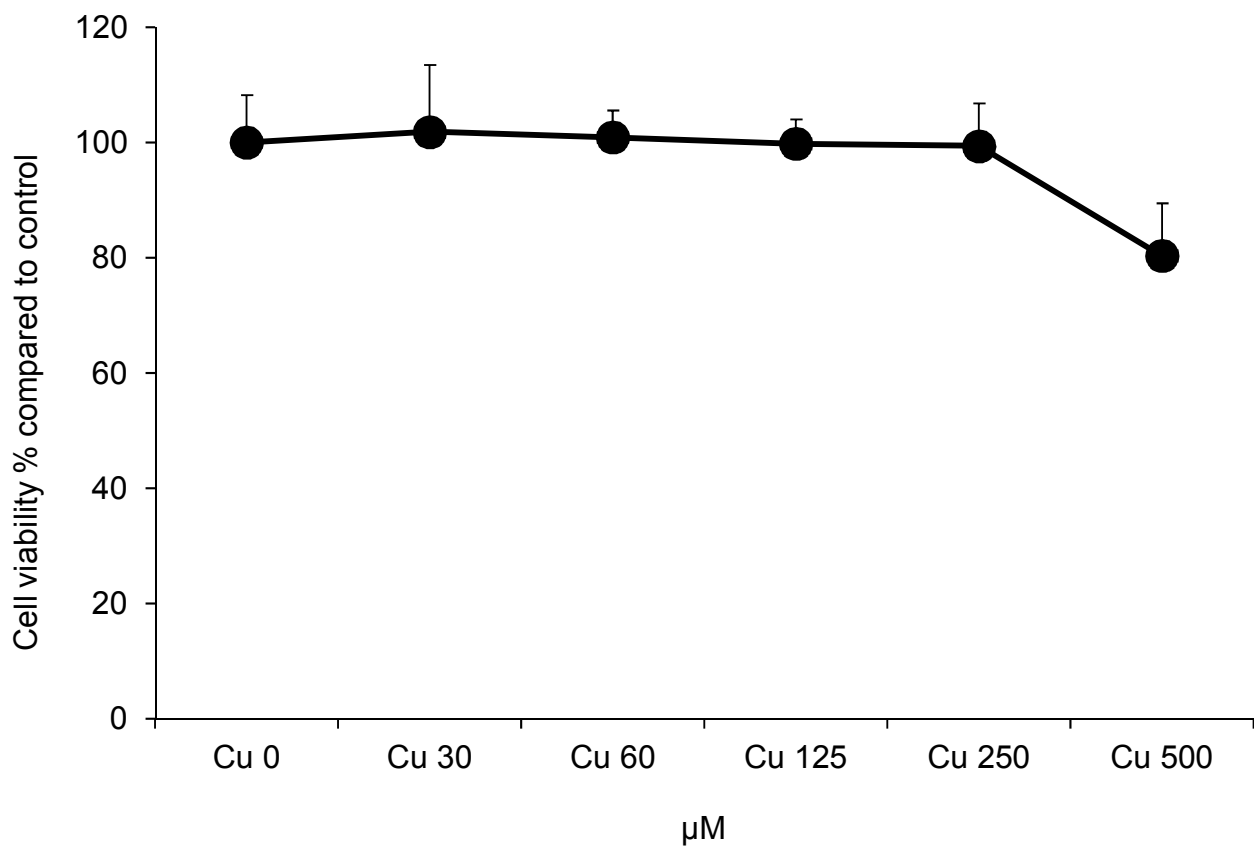


Fig. 1

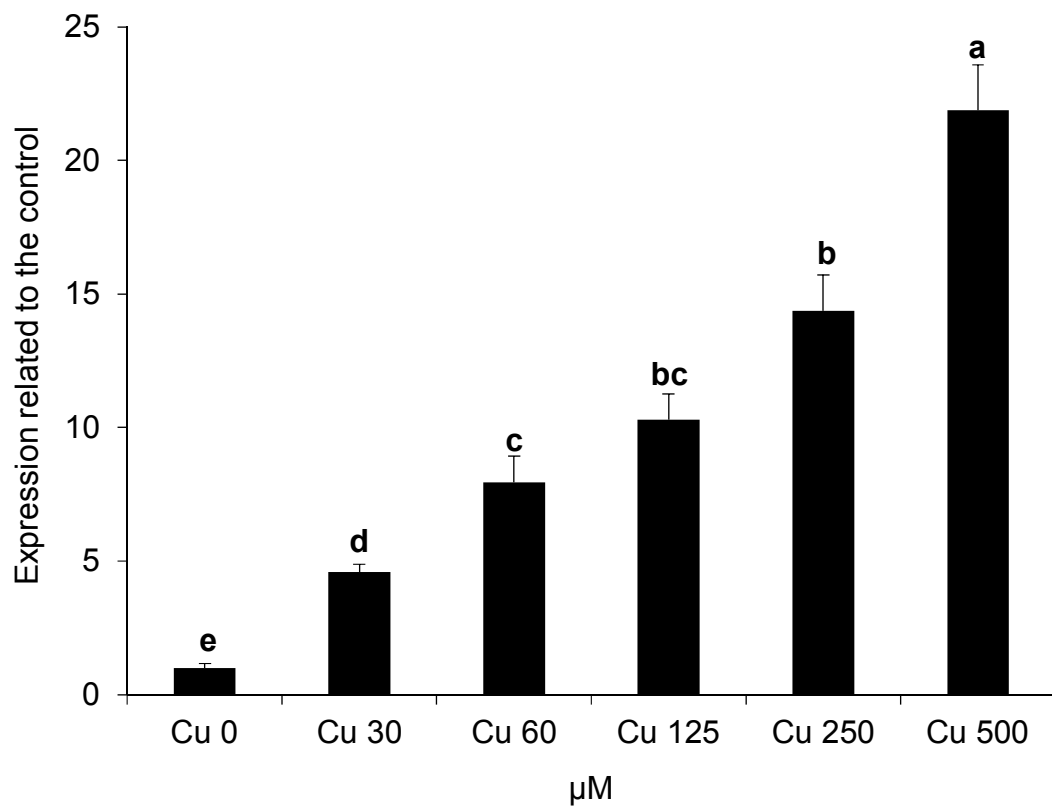


Fig. 2

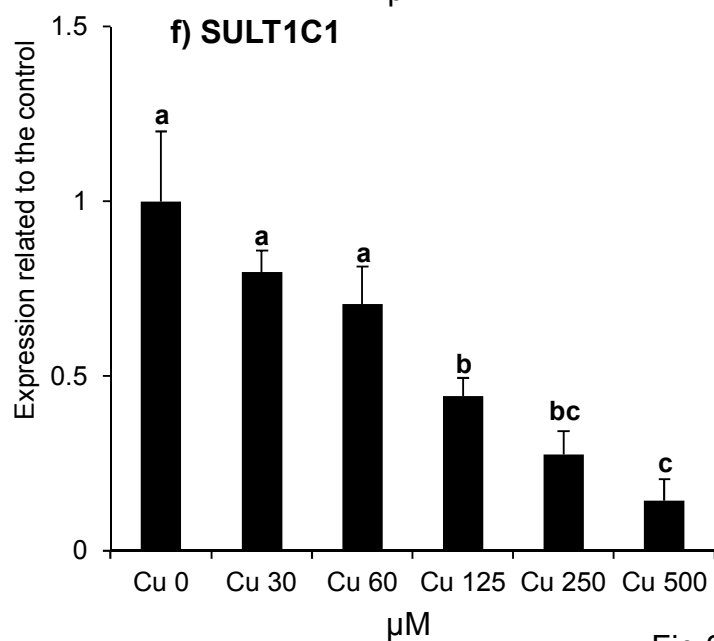
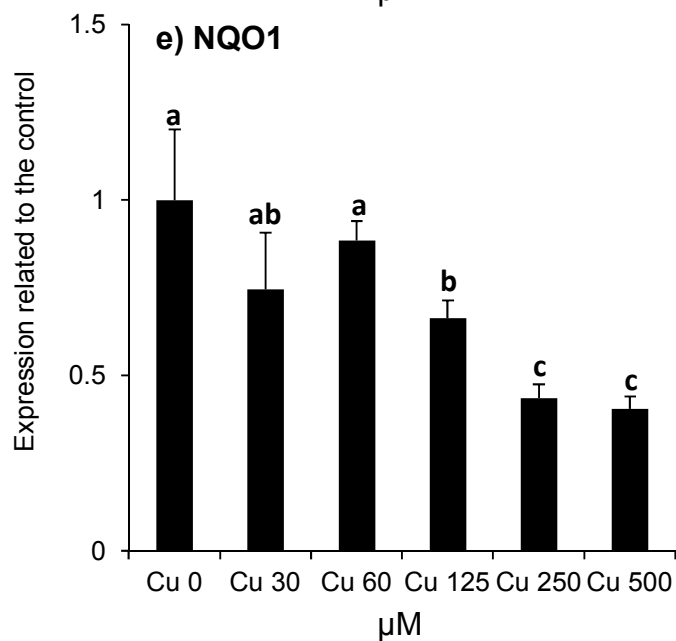
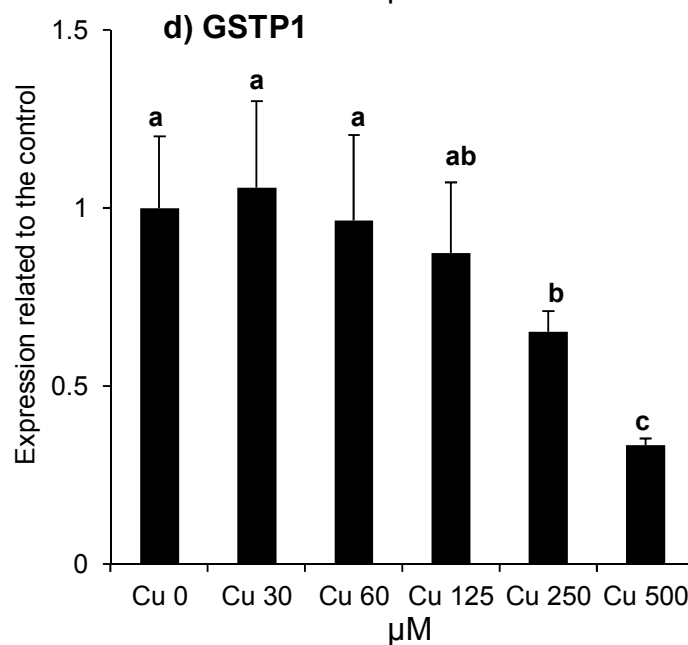
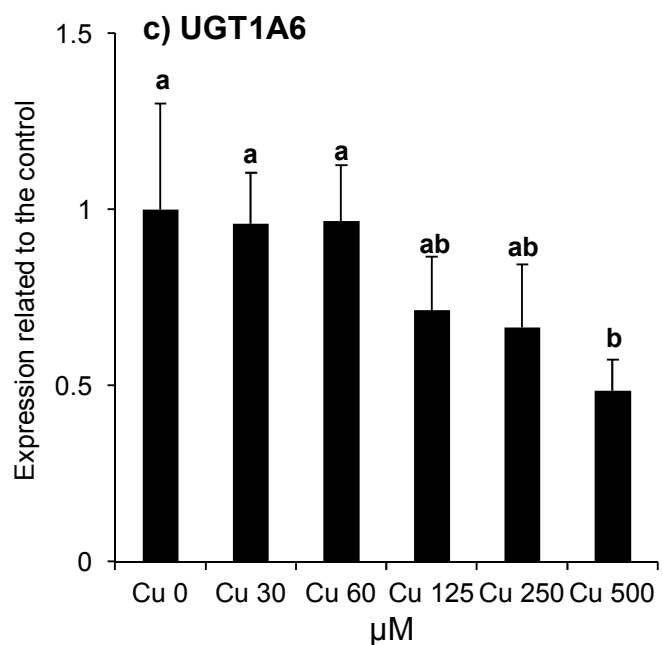
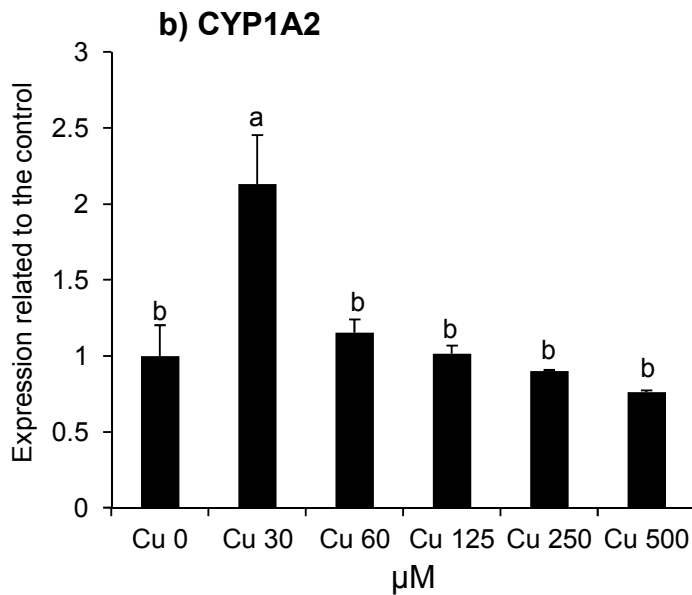
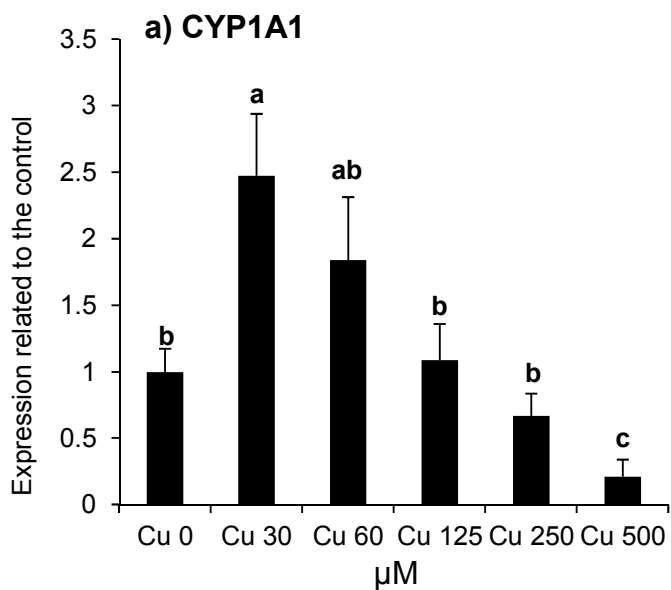


Fig.3

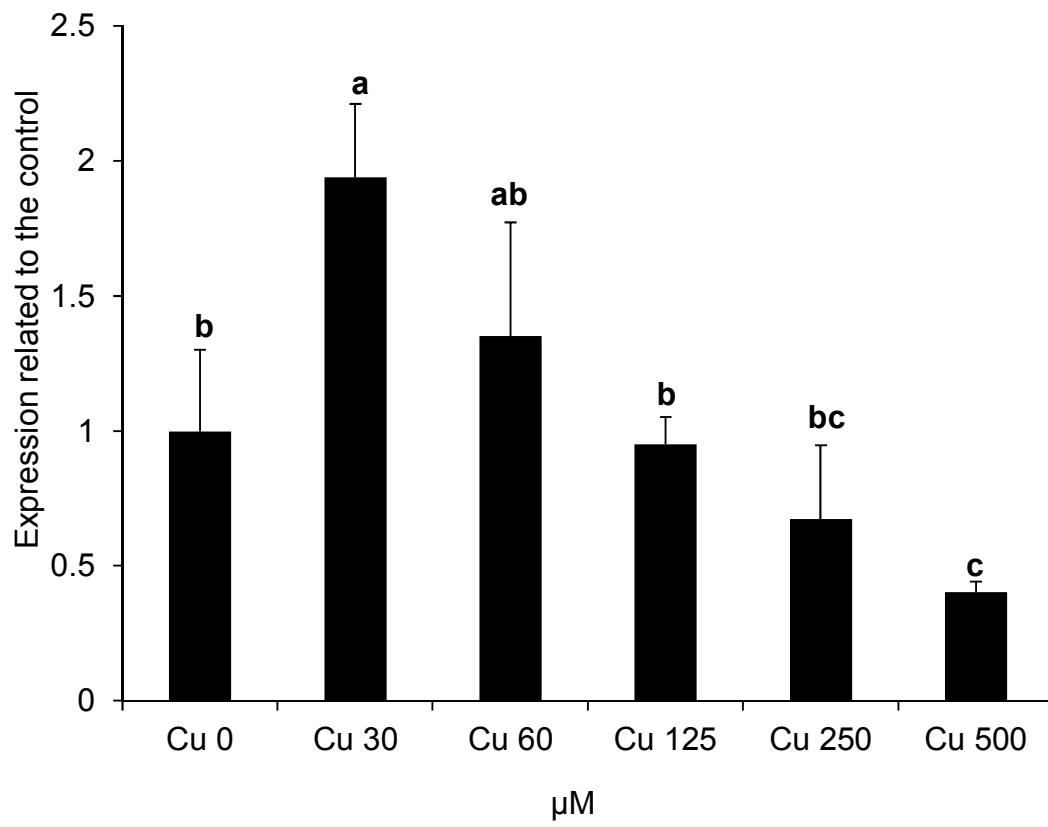


Fig. 4