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

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

Abstract

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

Introduction

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

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

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

Cytochrome P450 (CYP)1A1 and 1A2 are major XMEs in metabolizing procarcinogenic 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 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 Cu^{2+} exposure in the rat liver cells. Secondly, to investigate the cross-talks
75 between Cu^{2+} and XMEs CYP1A1 and 1A2, UGT1A6, GST1A, NQO1, and SULT1C1,
76 in cultured rat H4-II-E cells exposed to Cu^{2+}. Thirdly, the effect of Cu^{2+} on the regulatory
77 element, AhR, mRNA expression was also examined.
78

Materials and Methods

Chemicals and reagents

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

Cell line and culture conditions

All experiments were performed according to the guidelines of the Hokkaido University Biosafety Committee. H4-II-E rat hepatoma cells obtained from the American Type Culture Collection (Manassas, VA), were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) at 37ºC in a humidified incubator with 5% CO_2. Cells were seeded in 60 mm collagen-coated dishes, sub-cultured twice a week, and subsequently grown to 80–90% confluence.

Exposure of cells to Cu^2

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

Quantitative real-time RT PCR using TaqMan gene expression assays (Applied Biosystems, CA, USA) and a StepOne real-time PCR system (Applied Biosystems) were performed to determine rat mRNA levels. The primer and probe sets for each specific gene were as follows: Rn00487218_m1 (*CYP1A1*), Rn00561082_m1 (*CYP1A2*), Rn00565750_m1 (*AhR*), Rn00756113_AH (*UGT1A6*), Rn00755117_A1 (*GSTA1*), Rn00566528_m1 (*NQO1*), Rn00581955_m1 (*SULT1C1*), and Rn99999916_s1 (*GAPDH*). The reaction underwent 40 cycles of the following: initial activation at 95°C for 20 s, denaturation at 95°C for 1 s, and annealing and extension at 60°C for 20 s. Measurements of specific enzyme and receptor genes, as well as GAPDH, were performed in duplicate and repeated three times. The expression of each gene was normalized with respect to the expression of *GAPDH*, and was calculated relative to control levels using the comparative threshold cycle (Ct) method. In addition, the following specific primers were used to measure the expression of the MT-1 gene: forward primer 5'-caccgttgctccagattcac-3', reverse 5'-aggagcagcagctcttcttg-3' (accession number: NG_006919.3); and GAPDH, forward 5'-gtcttcaccaccacggagaaggc-3', reverse 5'-atgccagtgagcttcccgttcagc-3' (accession number: XR086293.2). To measure the expression levels of MT-1 and GAPDH genes, PCR reactions were run in a total volume of 10 µl. The PCR reaction mixture was prepared with SYBR qPCR Mix (Toyobo), 10 µM of each primer, 600 ng cDNA, 50× ROX reference dye, and RNase-free water. The mixture was made up to a final volume of 10 µl. The reaction cycle comprised an initial holding stage at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 1 min, and extension at 72°C for 30 s. Melting curve analysis and 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

Results

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

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

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

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

Discussion

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

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

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

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

dependent ethoxyresorufin-*O*-deethylase activity in trout gill primary cells treated with a high concentration of Cu^{2+} (1000 μM) [11].

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

Phase II enzymes (UGT1A6, GST1A, NQO1, and SULT1C1) showed higher sensitivity to Cu^{2+} exposure, as their expression were significantly reduced upon exposure to even

low concentrations of Cu^{2+}, especially in the cases of GST1A and SULT1C2 (Fig. 3c–f). These results were in agreement with those of Korashy and El-Kadi [16], who reported that the induced GST- and NQO1-dependent activities in murine Hepa1c1c7 cell lines exposed to AhR ligands such as 3-methylcholanthrene and benzo[a]pyrene were significantly reduced after co-exposure with increasing doses of Cu^{2+}. In addition to AhR, the functional suppression of Nrf2, which is one of regulators of phase II enzymes, should also be investigated in H4-II-E cells. The clear downregulation of phase II enzymes, especially at increasing doses of Cu^{2+}, may be attributed to metal-mediated oxidative stress, such as the production of reactive oxygen species and lipid peroxidation [17, 18]. This interference with phase I and II enzyme expression may have toxicological implications for H4-II-E cells, particularly in cases of co-exposure to Cu^{2+} and other xenobiotics.

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

248

Acknowledgments

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257 **References**

258 1. Linder MC, Hazegh-Azam M (1996) Copper biochemistry and molecular biology.
259 Am J Clin Nutr 63(5):797S-811S
260 2. Letelier ME, Faúndez M, Jara-Sandoval J, Molina-Berríos A, Cortés-Troncoso J,
261 Aracena-Parks P, Marín-Catalán R (2009) Mechanisms underlying the inhibition
262 of the cytochrome P450 system by copper ions. J Appl Toxicol 8:695-702
263 3. Goldstein S, Czapski G (1986) Mechanisms of the reactions of some copper
264 complexes in the presence of DNA with superoxide, hydrogen peroxide, and
265 molecular oxygen. J Am Chem Soc 108(9):2244-50
266 4. Nakayama SM, Ikenaka Y, Hamada K, Muzandu K, Choongo K, Teraoka H,
267 Mizuno N, Ishizuka M (2011) Metal and metalloid contamination in roadside soil
268 and wild rats around a Pb-Zn mine in Kabwe, Zambia. Environ Pollut 159(1):175-
269 81
270 5. Sato M, Kondoh M (2002) Recent studies on metallothionein: Protection against
271 toxicity of heavy metals and oxygen free radicals. Tohoku J Exp Med 196:9-22
272 6. Darwish WS, Ikenaka Y, Eldaly E, Ishizuka M (2010) Mutagenic activation and
273 detoxification of benzo*[a]*pyrene *in vitro* by hepatic cytochrome P450 1A1 and
274 phase II enzymes in three meat-producing animals. Food Chem Toxicol 48:2526-
275 2531
276 7. Darwish WS, Ikenaka Y, El-Ghareeb W, Ishizuka M (2010) High expression of
277 the mRNA of cytochrome P450 and phase II enzymes in the lung and kidney
278 tissues of cattle. Animal 4:2023-2029

279 8. Braeuning A, Köhle C, Buchmann A, Schwarz M (2011) Coordinate regulation of
280 cytochrome P450 1a1 expression in mouse liver by the aryl hydrocarbon receptor
281 and the beta-catenin pathway. Toxicol Sci 122(1):16-25
282 9. Darwish WS, Ikenaka Y, Ohno M, Eldaly E, Ishizuka M (2010) Carotenoids as
283 regulators for inter-species difference in Cytochrome P450 1A expression and
284 activity in food producing animals and rats. Food Chem Toxicol 48:3201-3208
285 10. Tchounwou PB, Newsome C, Williams J, Glass K (2008) Copper-induced
286 cytotoxicity and transcriptional activation of stress genes in human liver
287 carcinoma (HepG(2)) cells. Met Ions Biol Med 10:285-290
288 11. Jönsson ME, Carlsson C, Smith RW, Pärt P (2006) Effects of copper on CYP1A
289 activity and epithelial barrier properties in the rainbow trout gill. Aquat Toxicol
290 79(1):78-86
291 12. Campana O, Sarasquete C, Blasco J (2003) Effect of lead on ALA-D activity,
292 metallothionein levels, and lipid peroxidation in blood, kidney, and liver of the
293 toadfish Halobatrachus didactylus. Ecotoxicol Environ Safety 55: 116-125
294 13. Ikenaka Y, Nakayama SM, Muroya T, Yabe J, Konnai S, Darwish WS, Muzandu
295 K, Choongo K, Mainda G, Teraoka H, Umemura T, Ishizuka M (2012) Effects of
296 environmental lead contamination on cattle in a lead/zinc mining area: Changes
297 in cattle immune systems on exposure to lead in vivo and in vitro. Environ
298 Toxicol Chem 31 (10):2300-5
299 14. Darwish WS, Ikenaka Y, Ishizuka M (2013) Biological responses of xenobiotic
300 metabolizing enzymes to lead exposure in cultured H4IIE rat cells. Jap J Vet Res
301 61:S13-S22

15. Whitlock JP (1999) Induction of cytochrome P4501A1. Annu Rev Pharmacol Toxicol 39:103-125

16. Korashy H, El-Kadi A (2004) Differential effects of mercury, lead, and copper on the constitutive and inducible expression of aryl hydrocarbon receptor (AHR)-regulated genes in cultured hepatoma Hepa 1c1c7 cells. Toxicol 201:153-172

17. Kaliman PA, Nikitchenko IV, Sokol OA, Strel'chenko EV (2001) Regulation of heme oxygenase activity in rat liver during oxidative stress induced by cobalt chloride and Hg^{+2} chloride. Biochem 66:77-82

18. Vakharia DD, Liu N, Pause R, Fasco M, Bessette E, Zhang QY, Kaminsky LS (2001) Effect of metals on polycyclic aromatic hydrocarbon induction of CYP1A1 and CYP1A2 in human hepatocyte cultures. Toxicol Appl Pharmacol 170(2):93-103

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^{2+}.

323 The effect of Cu^{2+} 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^{2+}.

330 The effect of Cu^{2+} 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^{2+}.

336 The effect of Cu^{2+} 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

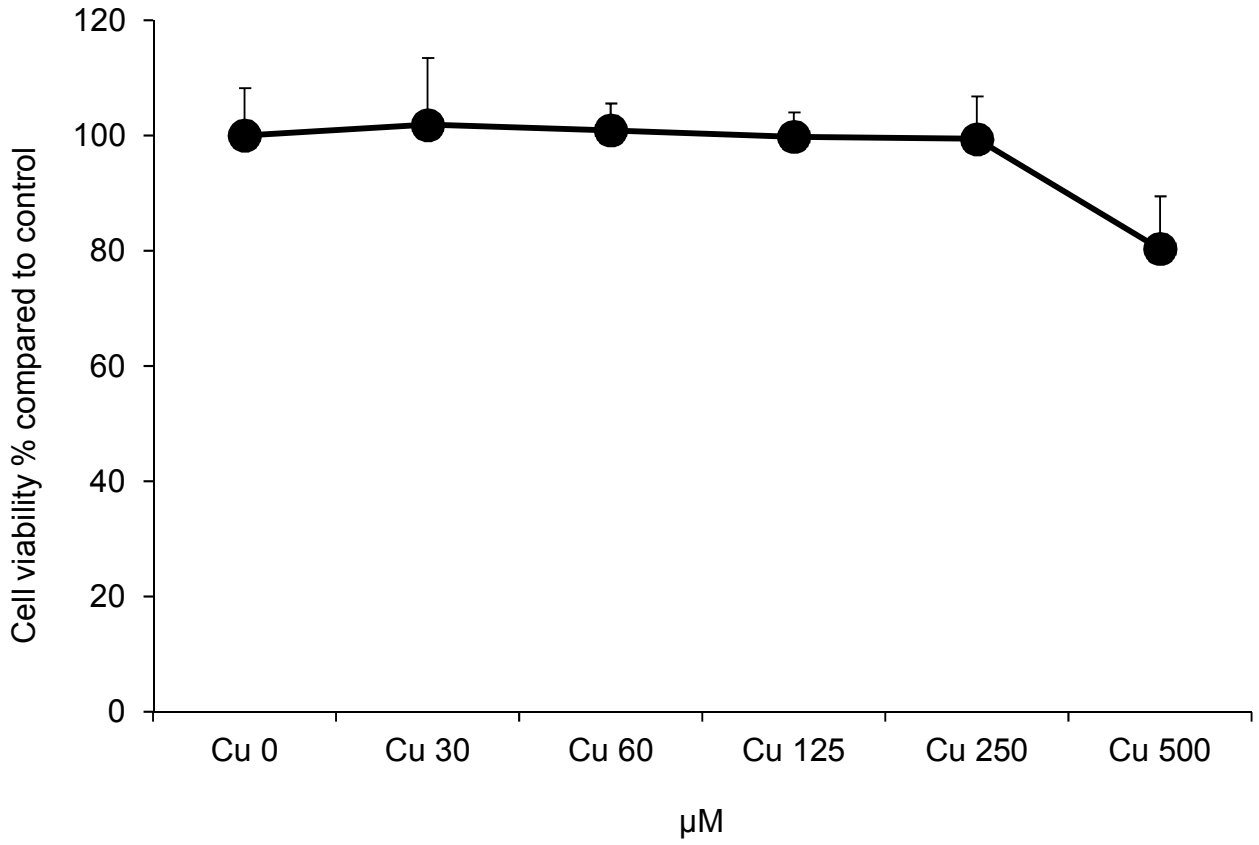
120
100
80
60
40
20
0
Cell viability % compared to control
Cu 0
Cu 30
Cu 60
Cu 125
Cu 250
Cu 500
µM

Fig. 1

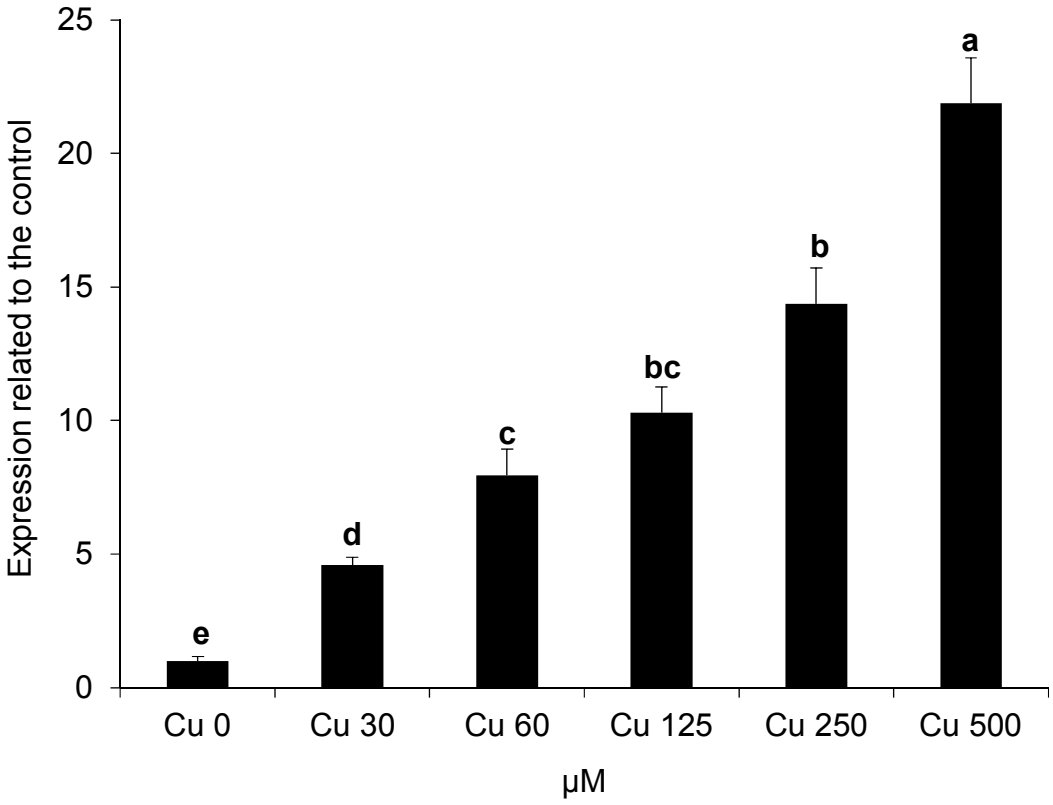
25
20
15
10
5
0
Expression related to the control
e
d
c
bc
b
a
Cu 0
Cu 30
Cu 60
Cu 125
Cu 250
Cu 500
µM

Fig. 2

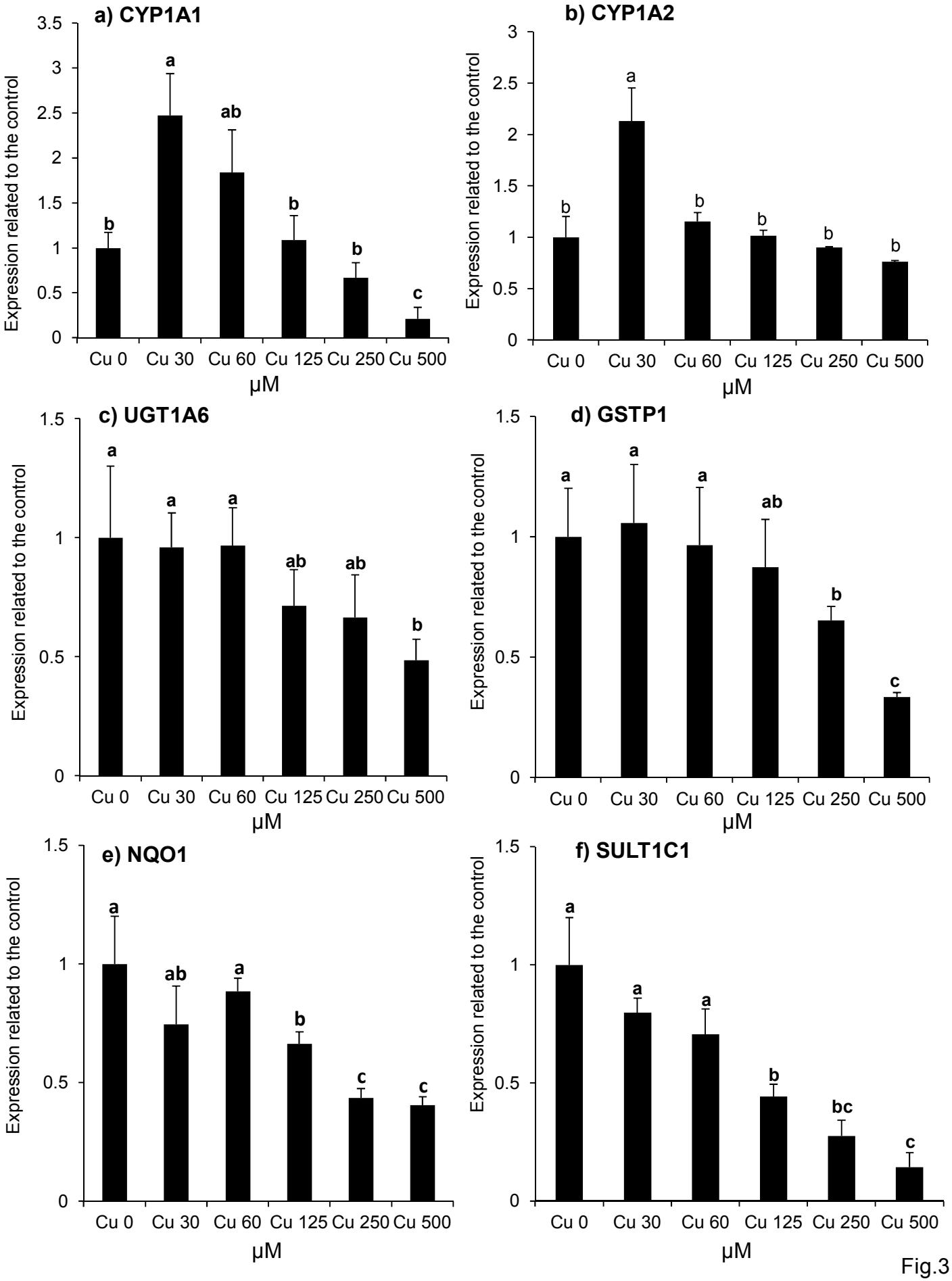
a) CYP1A1
Expression related to the control
3.5
3
2.5
2
1.5
1
0.5
0
a
ab
b
b
b
c
Cu 0
Cu 30
Cu 60
Cu 125
Cu 250
Cu 500
µM
b) CYP1A2
Expression related to the control
3
2.5
2
1.5
1
0.5
0
a
b
b
b
b
b
Cu 0
Cu 30
Cu 60
Cu 125
Cu 250
Cu 500
µM
c) UGT1A6
Expression related to the control
1.5
1
0.5
0
a
a
a
ab
ab
b
Cu 0
Cu 30
Cu 60
Cu 125
Cu 250
Cu 500
µM
d) GSTP1
Expression related to the control
1.5
1
0.5
0
a
a
a
ab
b
c
Cu 0
Cu 30
Cu 60
Cu 125
Cu 250
Cu 500
µM
e) NQO1
Expression related to the control
1.5
1
0.5
0
a
ab
a
b
c
c
Cu 0
Cu 30
Cu 60
Cu 125
Cu 250
Cu 500
µM
f) SULT1C1
Expression related to the control
1.5
1
0.5
0
a
a
a
b
bc
c
Cu 0
Cu 30
Cu 60
Cu 125
Cu 250
Cu 500
µM

Fig.3

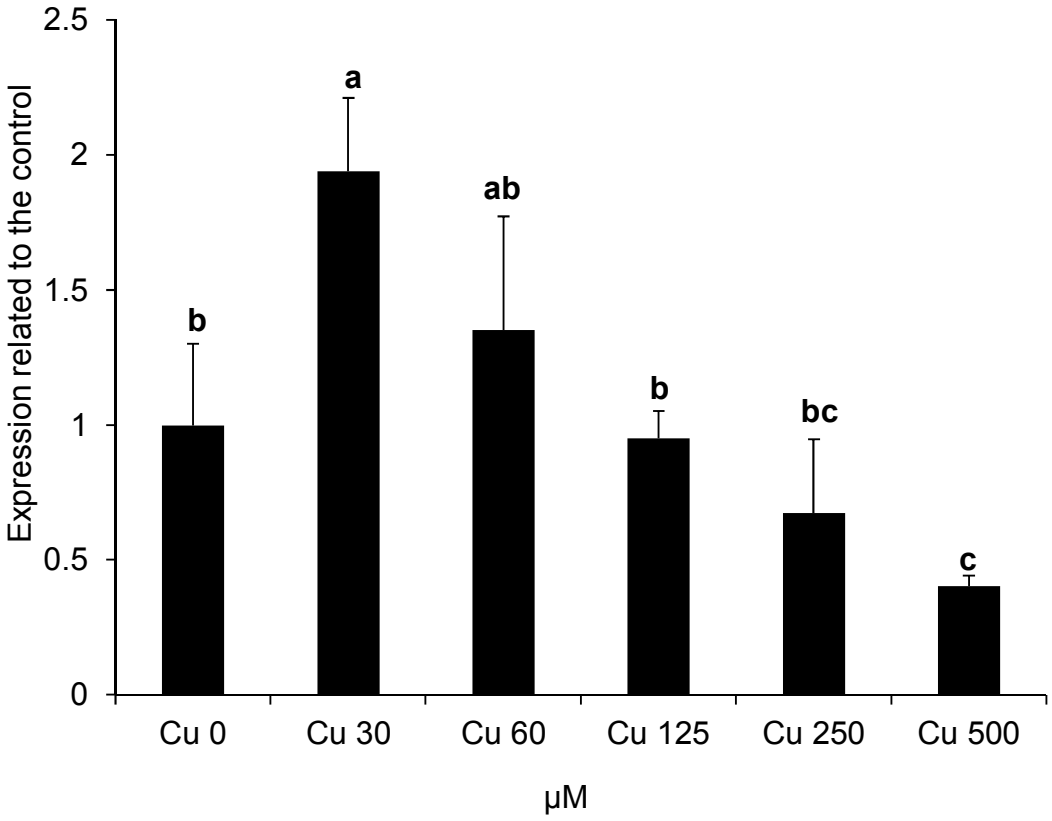
2.5
2
1.5
1
0.5
0
Expression related to the control
a
ab
b
b
bc
c
Cu 0
Cu 30
Cu 60
Cu 125
Cu 250
Cu 500
µM

Fig. 4