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Technical note

Hematoxylin assay of cupric chelation can give false positive results

Kateřina Macáková[a], Maria Carmen Catapano[b], Václav Tvrdý[b], Kateřina Klimková[b], Jana Karlíčková[a], Přemysl Mladěnka[b,*]

[a] Department of Pharmaceutical Botany, Faculty of Pharmacy in Hradec Králové, Charles University, Akademika Heyrovského 1203, 500 05, Hradec Králové, Czech Republic

[b] Department of Pharmacology and Toxicology, Faculty of Pharmacy in Hradec Králové, Charles University, Akademika Heyrovského 1203, 500 05, Hradec Králové, Czech Republic

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ABSTRACT

Some compounds without apparent chelation sites have been shown to chelate cupric ions using the hematoxylin assay. Since these compounds also have reduction potential (direct antioxidant effect), the aim of this study was to determine the possible interference of reducing agents with the hematoxylin assay. Four different known reducing agents (hydroxylamine, vitamin C, trolox – a water-soluble form of vitamin E and reduced glutathione /GSH/) were selected for the study together with oxidized glutathione (GSSG) for comparison.

All tested compounds behaved as cupric chelators in the spectrophotometric mildly competitive hematoxylin assay. In-depth analysis however showed that only GSH and GSSG were able to form complexes with both cupric and cuprous ions and only GSSG partly retained copper in its complexes in the more competitive bathocuproine assay. Further experiments showed that with the exception of GSSG, all other compounds reduce Cu^{2+} ions.

Conclusion: Compounds reducing copper such as antioxidants can give false positive results in the hematoxylin-screening assay. GSSG is a stronger Cu chelator than GSH and does not reduce Cu, in contrast to the latter and thus may be a protective element after oxidation of GSH.

1. Introduction

In 2013, our group introduced a method for screening of copper chelation based on two consecutive tests – the hematoxylin and the bathocuprione assays [1]. The first is mildly competitive since the indicator reacts with cupric ions in a ratio of 1:1. The second is highly competitive. It uses sodium bathocuproinedisulphonate (BCS), which is a selective indicator for cuprous ions, and is given in a 20× times excess over cuprous ions. Over the 5 years of our use of the former assay, we have noticed that some antioxidant compounds with no clear chelation site were partially active in the hematoxylin assay (unpublished results). Since these compounds were direct antioxidants, they may possess metal reducing activity [2]. To confirm the hypothesis that the hematoxylin assay can give false positive results with antioxidants, we selected 3 known reducing but not chelating agents with physiological and/or experimental importance: hydroxylamine, vitamin C and a water-soluble form of vitamin E – trolox. Reduced (GSH) and oxidized (GSSG) glutathione were also included. Both GSH and GSSG chelate copper [3], but GSH is also a strong reducer in contrast to GSSG [4].

Hydroxylamine is a very simple inorganic compound commonly used as a reducing agent in several organic and inorganic reactions. It can also act as an antioxidant for fatty acids [5]. Trolox /(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid/, a synthetic compound from the family of tocopherols, can prevent DNA fragmentation in cells, caused by H_2O_2 [6]. Vitamin C (L-ascorbic acid) is a water-soluble micronutrient required for multiple biological functions [7]; it plays a crucial role in neutralising endogenous and exogenous reactive oxygen species [8].

Glutathione (L-glutathione) is an important endogenous direct antioxidant found in micromolar concentrations in cells (1–10 mM) and in the blood (1–30 mM) [9]. Less is known about the fact that it also binds both cuprous and cupric ions. As mentioned, glutathione exists in both GSH and GSSG states. In the reduced state, the thiol group of cysteine is able to donate a reducing equivalent ($H^+ + e-$) to other molecules, such as reactive oxygen species to neutralize them, or to maintain cysteine in proteins in the reduced forms. By donating an electron, glutathione itself becomes reactive and readily reacts with another glutathione radical to form glutathione disulphide (GSSG). GSH has

Abbreviations: BCS, sodium bathocuproinedisulphonate; HA, hydroxylamine hydrochloride; GSH, reduced glutathione; GSSG, oxidized glutathione

* Corresponding author at: Department of Pharmacology and Toxicology, Faculty of Pharmacy in Hradec Králové, Charles University, Akademika Heyrovského, Hradec Králové, Czech Republic.

E-mail address: mladenkap@faf.cuni.cz (P. Mladěnka).

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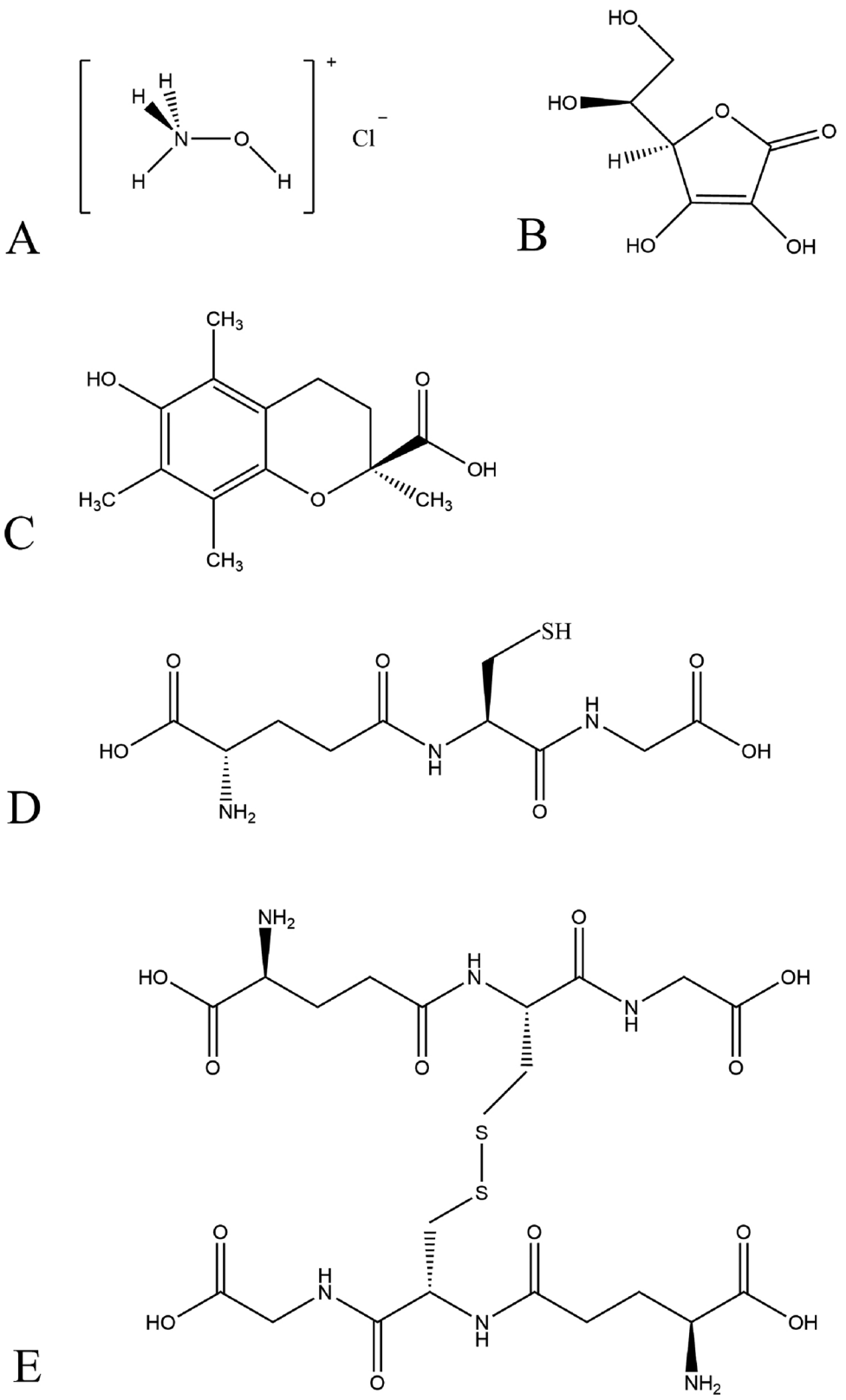

Fig. 1. Chemical structure of the tested compounds. **A**: hydroxylamine hydrochloride (HA), **B**: L-ascorbic acid (vitamin C), **C**: trolox, **D**: reduced L-glutathione (GSH) and **E**: oxidized L-glutathione (GSSG).

multiple functions. In particular, it is a major endogenous antioxidant that participates directly in neutralizing reactive oxygen species as well as maintaining exogenous antioxidants such as vitamins C and E in their reduced (active) forms. Interestingly, high GSH levels are found in cancer cells and appear to be an adaptive mechanism for elevated intracellular oxidative stress. Reducing the level by synthesis inhibition or prooxidation using redox active metal chelators sensitises the cancer cells leading to their death [10–13]. GSH is also involved in the

regulation of nitric oxide and has a decisive role in intracellular Cu kinetics [14,15]. In contrast, GSSG is considered to be a temporary intermediate with no direct biological function. It is generated via oxidation of GSH and reduced by glutathione reductase back to GSH [16].

GSH is known to be the crucial cellular antioxidant following oxidative stress insult but the involvement of metal chelation and the direct involvement of GSSG in the process are yet to be elucidated.

For these reasons, this short report aims at checking the possible pitfalls of the hematoxylin method by selection of a relevant mix of reducing agents together with the crucial physiological player in redox homeostasis, GSH and its oxidized product GSSG (Fig. 1).

2. Materials and methods

2.1. Reagents

L-ascorbic acid, cupric sulphate pentahydrate ($CuSO_4 \cdot 5\ H_2O$), cuprous chloride (CuCl), BCS, DMSO, hydroxylamine hydrochloride (HA), GSH, GSSG and trolox were purchased from Sigma-Aldrich (Germany). Methanol was purchased from J.T. Baker (Avantor Performance Materials, Inc., USA). Ultrapure water (Milli-Q RG, Merck Millipore, Massachusetts, USA) was used throughout this study. A stock solution of cuprous ions (5 mM) was prepared by the dissolution of CuCl in an aqueous solution of 1 M NaCl in 0.1 M HCl. Working solutions were prepared using ultrapure water. Cupric ions 5 mM ($CuSO_4 \cdot 5\ H_2O$) were dissolved directly in distilled water. Acetate buff ;ers (15 mM sodium acetate salt, and 27.3 and 2.7 mM acetic acid, respectively) were used for the two lower pH values (pH 4.5, 5.5) whereas HEPES buff ;ers (15 mM HEPES sodium salt, and 71.7 and 14.3 mM HEPES, respectively) for pH 6.8 and 7.5.

2.2. Methods

2.2.1. Copper competitive chelation assays

Hematoxylin, BCS chelation method and BCS reduction method were performed in 96-well microplates at room temperature using Synergy HT Multi-Detection Microplate reader (BioTec Instruments, Inc., USA) as described in [1]. In all competitive assays, the final concentration of cupric or cuprous ions was 50 μM. Hydroxylamine was added in cases when it was necessary to maintain cuprous ions in the reduced state or to reduce cupric to cuprous ions. The concentrations varied according to pH in line with the published methodology [1].

2.2.2. Hematoxylin method

Various concentrations of tested substances were mixed with cupric ions (50 μL, different concentrations) in a buffer (150 μL, pH 5.5, 6.8, 7.5) for 2 min. The mixture was stirred for the next 3 min after addition of the indicator hematoxylin (50 μL, final concentration 50 μM). The absorbance was measured immediately and after 4 min. The wavelengths were set according to pH: 595 nm (pH 5.5), 590 nm (pH 6.8), and 610 nm (pH 7.5).

2.2.3. BCS method

Various concentrations of tested substances were mixed with cupric or cuprous ions (50 μL, different concentrations) in a buffer (100 μL, 4.5, 5.5, 6.8, 7.5) for 2 min. Hydroxylamine (50 μL) was added before the copper solution in the case of cuprous ions to retain copper in the reduced state. The final concentration varied according to pH: 1 mM at pH 6.8 and 7.5 but 10 mM at pH 4.5 and 5.5 according to our methodological paper [1]. For determination of cupric ions, hydroxylamine (the same volume and concentration as above) was added after mixing for reduction of non-chelated cupric ions. Finally, the BCS indicator (50 μL, in a final concentration of 1 mM) was added and the absorbance was measured immediately and after 5 min.

The BCS method was also used for determination of cupric ion reducing potential. Cupric ions were mixed with various concentrations of tested substances in a buffer without hydroxylamine for 2 min (the volumes and concentrations as above). The reduced copper ions were evidenced by BCS (50 μL, in a final concentration of 1 mM). Hydroxylamine (the concentrations varied according to pH and were the same as for competitive chelation assays) was used as a positive control (100% copper reduction).

2.2.4. Non-competitive copper assays

Determination of the stoichiometry was performed according to a previously established protocol [17]. First, we checked whether the metal formed complexes with the substances. The absorption spectra of the compounds and their mixtures with Cu ions ranging from 220 to 800 nm were scanned in order to check if the complex was formed under all selected pathophysiologically relevant pH conditions (4.5, 5.5, 6.8 and 7.5). In initial measurements, the blank was composed of 850 μL of buffer, 500 μL of the solvent (methanol for trolox and water for all other compounds) and 150 μL of water. Since both copper ions absorb in the 220–300 nm range, additional experiments were performed with a blank which also contained a copper water solution (150 μL) in a final concentration of 500 μM instead of 150 μL of water. If a shift or a new absorption maximum(a) was found, stoichiometry was determined by two methods: Job's method [18] and a complementary approach [17].

2.2.4.1. Job's method. In Job's method, the concentrations of both components (metal and glutathione) change while the total concentration is kept constant (100 μM). An aqueous solution of metal ion (ranging from 14 to 80 μM) was mixed with a water solution of GSH or GSSG (ranging from 20 to 86 μM) at diff ;erent molar concentration ratios ranging from 1:4 to 6:1 (substance: copper) at all tested pH values, and absorption spectra were immediately measured. The blank was composed of a buff ;er (1000 μL) and ultrapure water (500 μL).

2.2.4.2. Complementary method. For the complementary approach, the molar concentration of GSH/GSSG was continuously changed, while the final concentration of copper (10 μM) was maintained constant in all samples with diff ;erent molar concentration ratios of substance and copper ranging from 1:3 to 6:1. The composition of the blank was analogous to Job's method (1000 μL of buff ;er and 500 μL of ultrapure water).

2.3. Statistical analysis

Data are shown as means ± SD. The dose-dependent metal chelation curves corresponding to the equation $y = 100 / (1 + 10^{(\log EC - x) * slope})$, where y is the percentage of metal chelation and x is the decadic logarithm of concentration ratio tested substance/Cu^{n+}, and reduction lines with a 95% confidence (prediction) interval were constructed in GraphPad Prism 6 for Windows (GraphPad Software, USA). The reduction lines were created from the linear regression of the experimental data (non-significant reduction points *vs.* DMSO as the solvent /negative blank/ and complete reduction points *vs.* hydroxylamine /positive control/ were excluded from the analysis). All experiments were performed with at least two fresh solutions and each point was measured at least twice. When the coefficient of non-linear or linear regression was below 0.95, a third fresh solution was prepared and additional experiments were performed.

3. Results

Initially, all compounds were tested in DMSO as the solvent. All compounds "chelated copper" according to the hematoxylin assay under all tested pH conditions (Fig. 2). Very similar results were obtained when the selected compounds were dissolved in water (Fig. S1).

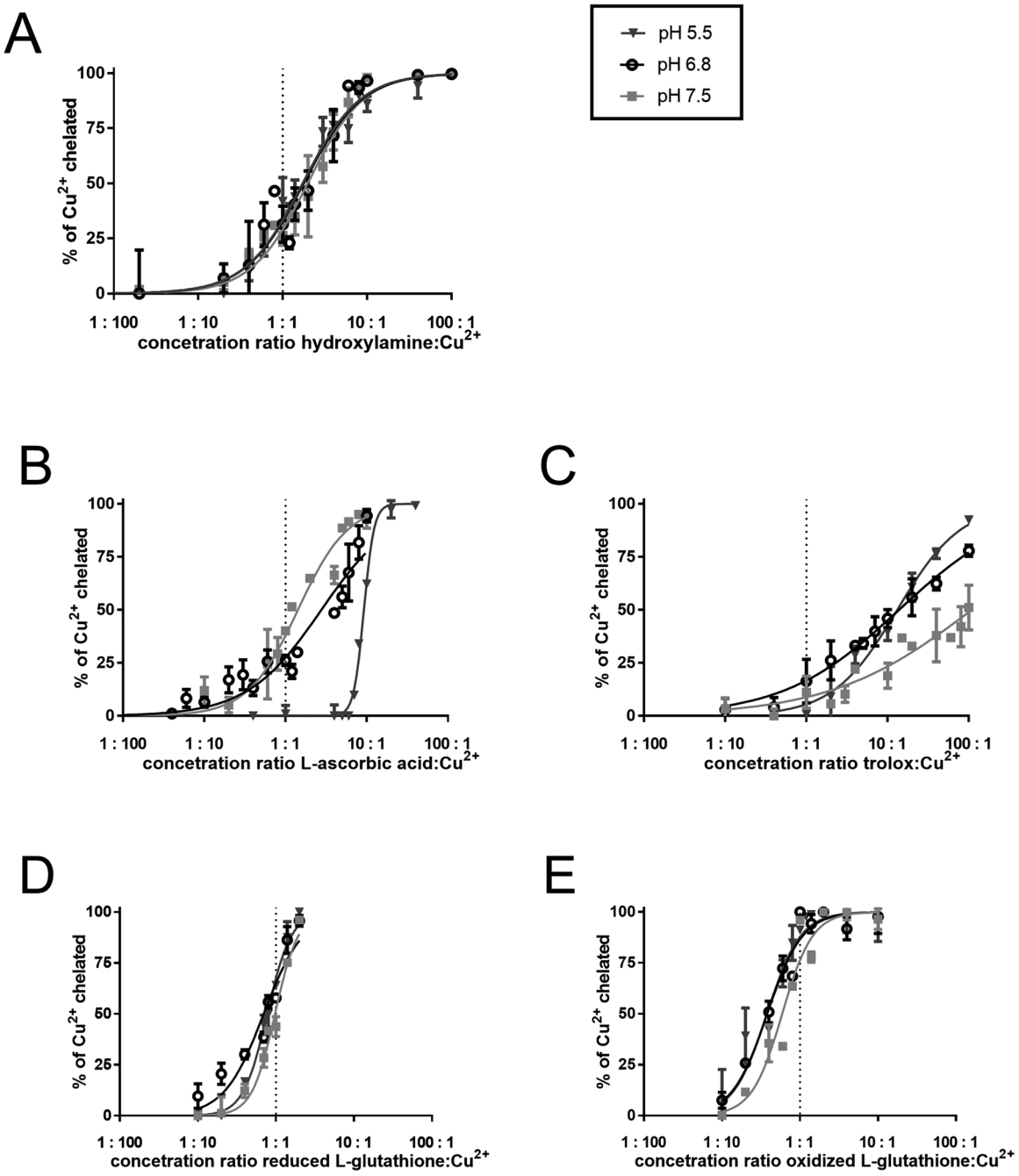

Fig. 2. Cupric chelation according to the hematoxylin assay. Tested compounds were dissolved in DMSO. **A**: hydroxylamine, **B**: L-ascorbic acid, **C**: trolox, **D**: reduced L-glutathione and **E**: oxidized L-glutathione.

There were no significant differences in "hematoxylin Cu^{2+}-chelation results" between identical compounds dissolved in DMSO and water, with the exception of HA at pH 5.5 (Fig. S2). Comparing the potency of all compounds, the most active were always GSH and GSSG without any significant difference between them. HA dissolved in water reached their activity at pH 5.5 but was less active at higher pH. Vitamin C had the same effect as HA and was more active than trolox at pH 6.8 and 7.5. At pH 5.5, it had a similar effect as trolox but was less effective than HA (Fig. S3).

In the next step, Cu^{2+} and Cu^{+} chelation was tested by the more competitive BCS method. Only GSSG was able to chelate copper under these conditions but in relatively high ratios and the complexes were unstable (Fig. 3). All other compounds failed to chelate Cu in all ratios (Fig. S4). Since GSH is a known Cu chelator, we also tested this compound with lower concentrations of the indicator BCS to decrease the competitive environment. The results were similar (not shown). This confirmed that GSH is unable to retain Cu ions, even under mildly competitive conditions.

Since no chelation was observed under competitive conditions with the exception of GSSG, further analysis was targeted to testing whether the tested compounds could or could not form copper complexes under non-competitive conditions. In the case of HA and trolox, no modifications of the spectra of the tested compounds were observed after addition of copper ions in either valence state. Hence, it appears they do not form complexes with either copper ion at any of the tested pH levels. In the case of vitamin C, the absorption band disappeared after addition of copper ions (Fig. S5). On the other hand, both GSH and GSSG formed complexes with both Cu^{+} and Cu^{2+} ions as can be seen in

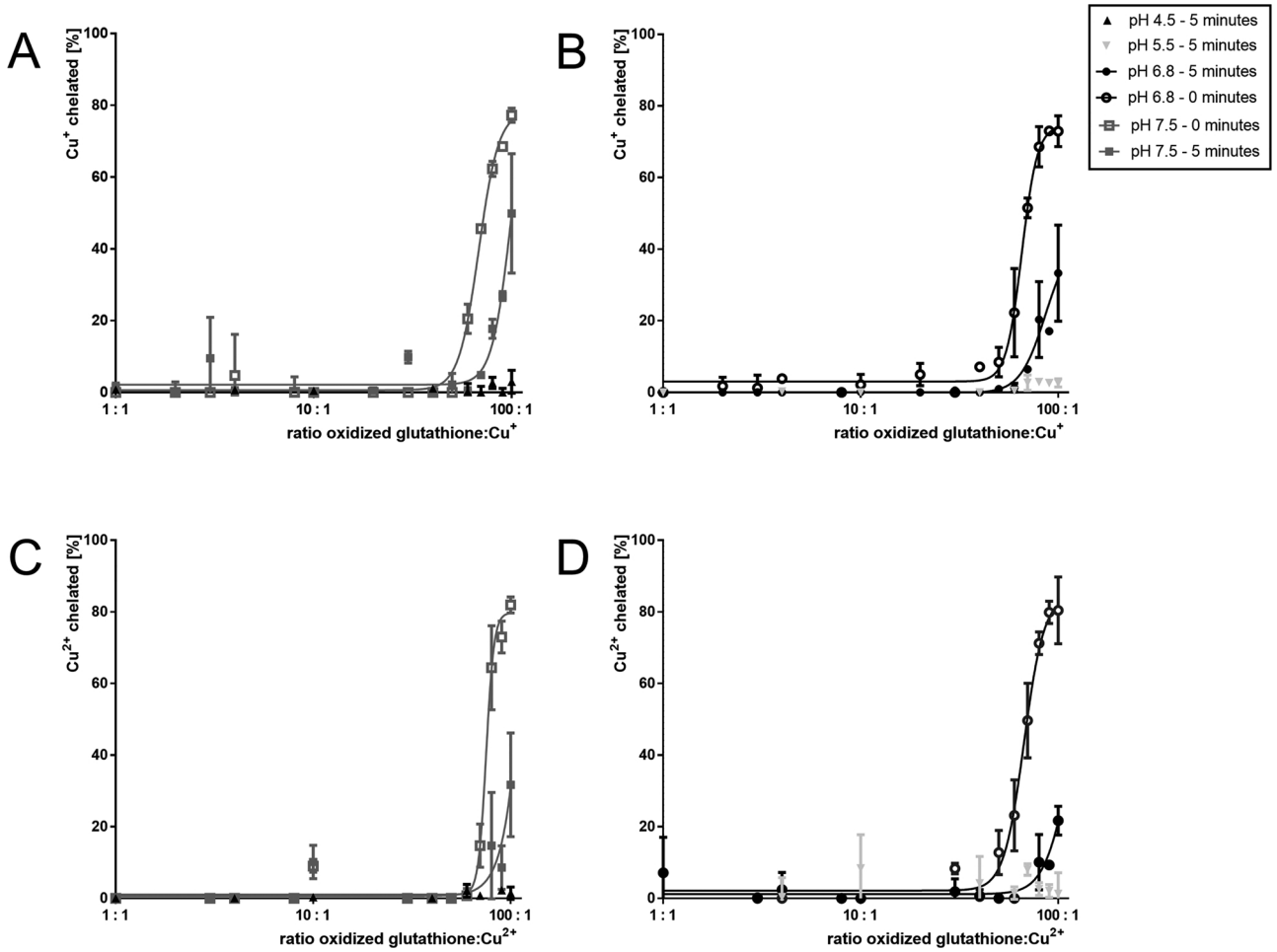

Fig. 3. Copper chelation by oxidized glutathione measured by BCS method. **A, B**: cuprous ions; **C, D**: cupric ions. The data are from experiments where oxidized L-glutathione was dissolved in water.

Table 1
Summarized stoichiometry of cupric complexes of reduced and oxidized L-glutathione under all tested pH conditions. Arrows refer to change in stoichiometry at higher ratios of glutathione to copper.

compound	The Job´s method	complementary method
L-glutathione oxidized Cu^{2+}		
pH 4.5	1:1	
pH 5.5		
pH 6.8		
pH 7.5		
L-glutathione oxidized Cu^{+}		
pH 4.5	2:1	2:1
pH 5.5	1:1	1:1 → 2:1
pH 6.8	1:1	1:1
pH 7.5	1:1	1:1
L-glutathione reduced Cu^{2+}		
pH 4.5	1:1	1:1 → 3:1
pH 5.5	1:1	1:1 → 3:1
pH 6.8	1:1	1:1
pH 7.5	1:1	1:1
L-glutathione reduced Cu^{+}		
pH 4.5	2:3	2:1
pH 5.5	2:3	2:3 → 2:1
pH 6.8	2:3	2:3
pH 7.5	2:3	2:3

Fig. S6. Additional experiments analysed the stoichiometry of the formed complexes by the two methods (see Fig. S7 for illustrative case). This is summarised in Table 1.

To confirm our hypothesis, cupric ion reduction was tested in the last step. With the exception of GSSG, all compounds dissolved in water reduced Cu^{2+} ions. In all pH conditions, vitamin C was clearly the least potent reducer while trolox was the most active. GSH and HA were not less effective than trolox at pH 4.5 and 5.5 from the statistical point of view, but were significantly less potent under higher pH conditions. With the exception of pH 6.8, GSH and HA were equally potent (Fig. 4). GSSG did not reduce cupric ions at all and even attenuated the spontaneous reduction of cupric ions at pH 7.5 and 6.8 (Fig. 5). Again with the exception of HA, there were no or minor differences between the results with compounds dissolved in DMSO and water (Fig. S8)

4. Discussion

There is logically a paucity of data on metal chelation by HA, trolox and vitamin C. In particular, the chemical structures of HA and trolox do not include any chelation sites. Isolated carboxy and hydroxy groups are not sufficient functionalities for metal chelation [19,20]. Vitamin C has a potential chelation site (ortho-dihydroxygroup) but the addition of copper ions resulted in the disappearance of its UV absorption band (Fig. S5B) suggesting too the disappearance of a conjugated system likely due to oxidation of the hydroxyls mentioned. We conclude that none of these compounds, chelate copper ions and served in our study as model compounds. Notwithstanding the inability of these compounds to chelate metals, all these compounds showed strong (HA, vitamin C) or weak (trolox) “chelation” effect using the hematoxylin method (Fig. 2). Hence, such results are clearly false positives and this method should be always used in combination with the BCS method to

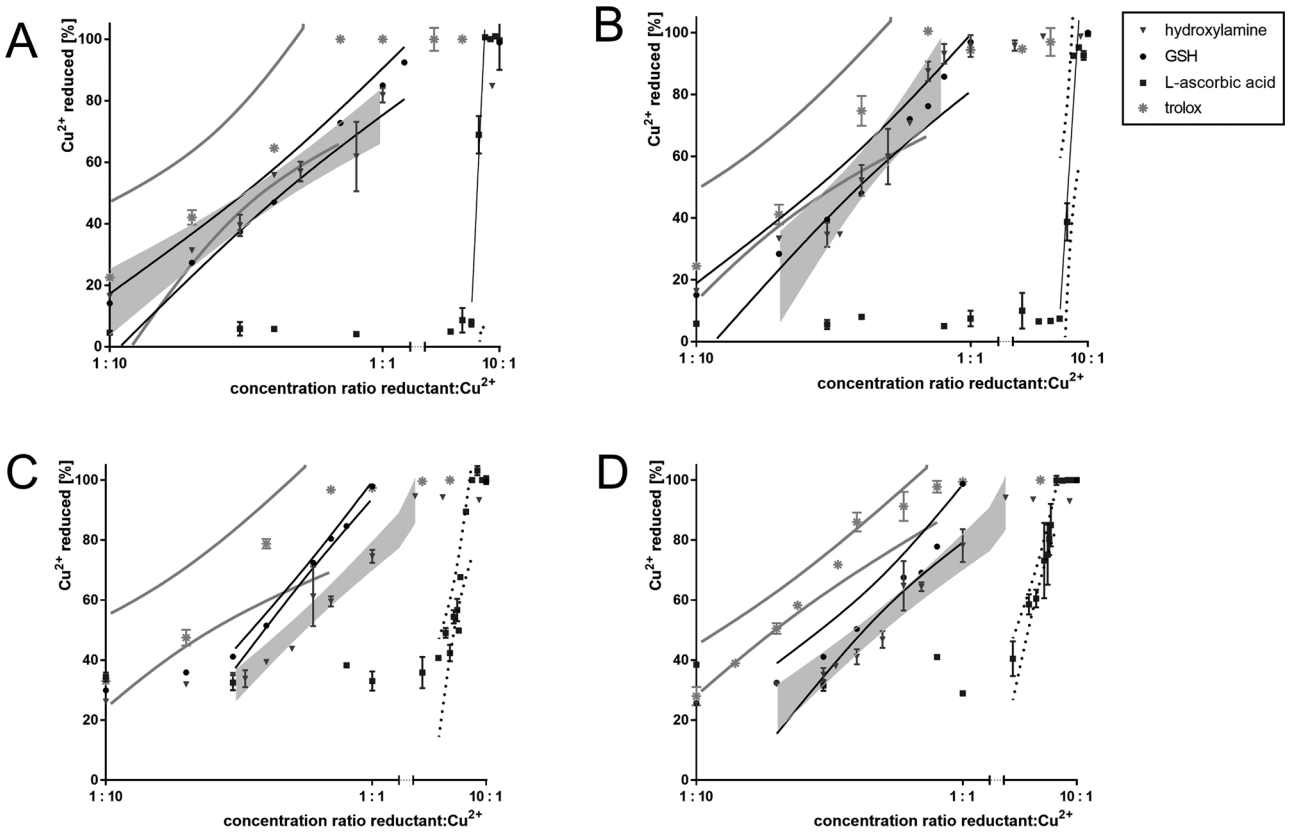

Fig. 4. Cupric ion reduction by hydroxylamine, reduced L-glutathione, vitamin C and trolox. **A**: pH 4.5, **B**: pH 5.5, **C**: pH 6.8 and **D**: pH 7.5. Data shows 95% confidence intervals of reduction lines. The compounds were dissolved in water. For easier comparison, the confidence intervals for GSH are shown as black lines, for trolox as gray lines and for hydroxylamine as light gray areas.

obtain a real assessment of copper chelation when testing compounds with reducing properties (antioxidants). The selection of the solvent (DMSO or water) generally played no important role (Figs. S2 and S8).

For a more complete overview, we included endogenous and hence pathophysiologically relevant GSH and GSSG as well. The first is a strong antioxidant compound with chelation properties while the second had no direct antioxidant (reducing) properties as can be demonstrated at least with cupric ions (Fig. 5). From the literature, it is apparent that interaction of GSH with the physiological trace metals, iron and copper, is the subject of lively discussion and interest.

However, the definite composition including the stoichiometry of these complexes remains unclear, as there are a number of factors implicated, including pH, environment and ratio of GSH to metal [3,21]. Moreover, at least in the case of iron, mixed complexes with oxidized and reduced metals have been documented, likely due to partial reduction of ferric ions [22]. Indeed, in this study, the stoichiometry was largely dependent on the valence of copper, pH and excess glutathione (Table 1). The changing stoichiometry of GSH-metal complexes has also been indirectly suggested, since GSH progressively promotes DNA damage in GSH:Cu^{2+} ratios up to 1:1 while the effect is reversed from a ratio of 3:1 [21,23]. This is relevant to tumour cells which often overexpress GSH to protect themselves from oxidative stress. When GSH levels are reduced, tumour cells are more susceptible to anticancer drugs [12,13].

There are also articles describing metal chelation by GSSG [3,22]. These data are quite surprising since GSSG was considered simply the oxidized form of GSH with no direct positive biological effect. There is also discussion about which atoms chelate the metals. Hamed and Silver concluded that sulphurs of GSSG were not involved in iron chelation and suggested that the metal binding was mediated by carboxyl and amide groups [22]. Similarly, sulphur does not seem to be involved in copper chelation [3]. The present study was not targeted to ascertain the functional groups involved. At least, the reduction of GSSG to GSH with subsequent chelation of copper ions by GSH can be excluded based on our data (e.g. if GSH for example chelated one Cu^{2+} atom, GSSH would chelate two copper atoms but this was not observed, Table 1). Similarly, the iron complexes for GSH and GSSG are different [22]. In the present study, we clearly showed that GSSG chelates both cupric and cuprous ions but has no potential to reduce cupric ions. This is also apparent from reduction experiments where GSSG decreased the degree of cupric reduction apparently due to its chelation potential (Fig. 5). In contrast, GSH forms unstable complexes with cuprous and cupric ions which is likely the base for its physiological role in the intracellular copper shuttle. In brief, GSH is a strong reducing agent with intracellular concentration in units of mM and hence it seems to form intracellular complexes predominantly with Cu^{+}. Such complexes are instable and hence can transfer the copper for further intracellular utilisation [14]. The dominant reducing effect was clearly shown in this study too, where its cupric reducing effect (Fig. 4) prevailed over the chelation effect. For this reason, only the GSSG chelation effect observed in this study in the hematoxylin method was a true outcome. If there is no copper reduction, e.g. the tested compound is not a direct antioxidant, the combination of positive hematoxylin assay with negative BCS reduction assay might be sufficient to confirm the cupric chelation but a BCS chelation assay is recommended.

The secondary outcome showing that GSSG is a copper chelator, which does not enable copper reduction in contrast to GSH, may have important biological impact in trace element pathophysiology. Hence, oxidation of GSH to GSSG seems to be another important step in cell protection since GSSG can chelate free metals. Its importance can be documented by the fact, that although GSH is a potent Cu^{2+} reductant, it's behaviour toward oxidative damage is complicated. Although not

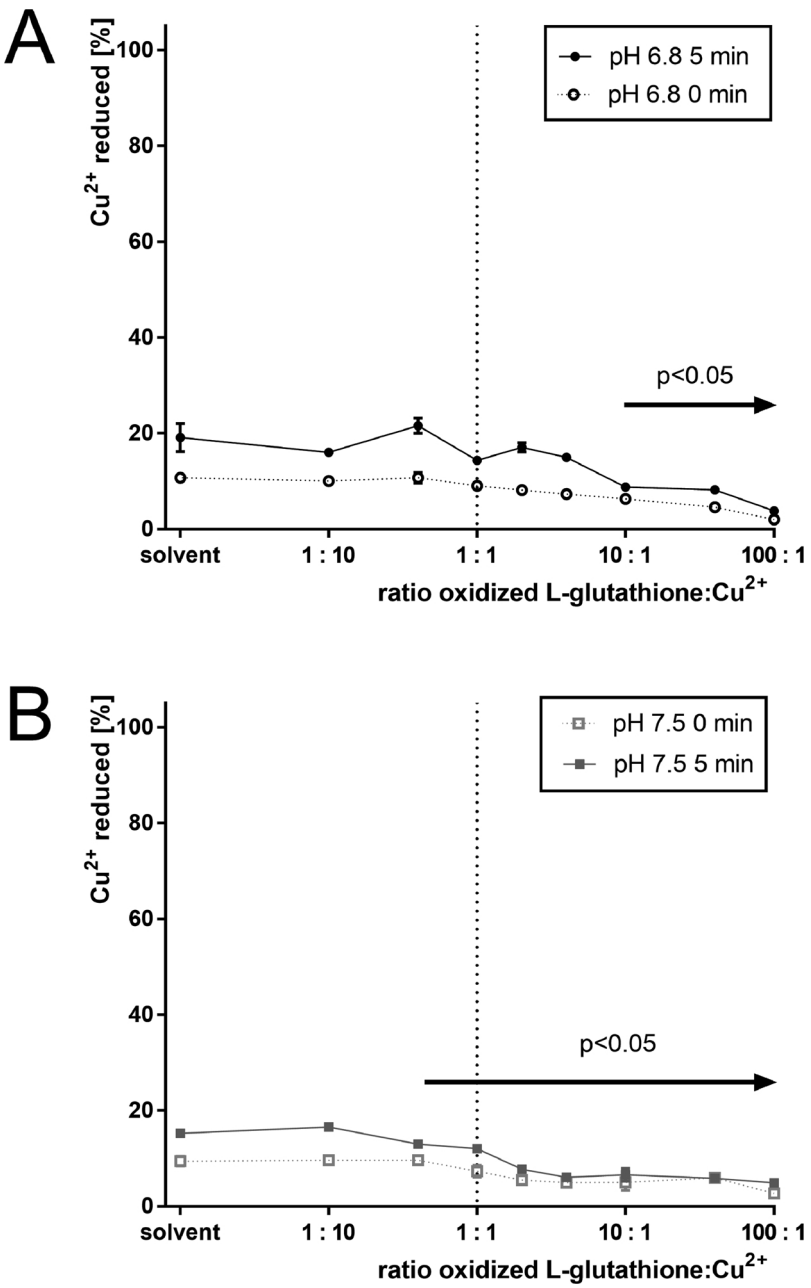

Fig. 5. The attenuation of cupric reduction by oxidized L-glutathione (GSSG) at pH 6.8 (**A**) and pH 7.5 (**B**). Under lower pH conditions, there was no reduction either in the control (solvent) or by GSSG (data not shown). GSSG was dissolved in water. Arrows show statistical significance *vs.* solvent (spontaneous reduction).

observed in all studies [24], it seems that low ratios (GSH to Cu ions) enable metal based oxidative damage, while in greater excess of GSH over Cu ions, protective antioxidant effects are observed [21,23,25]. This appears to correspond to our data suggesting that copper chelation is not involved and copper reduction may be responsible for the phenomenon in low ratios. In higher ratios, direct ROS, in particular OH· scavenging can mediate the antioxidant effects. Interestingly, based on our data, GSSG, which is formed by oxidation of GSH, can chelate both Cu^{+} and Cu^{2+} ions and might therefore contribute the final antioxidant effect.

This "by-product" of the study will need a further study, in particular analysis of the behaviour of GSH/GSSH toward the Fe/Cu-based Fenton reaction. It would also be of interest in the future to test N-acetylcysteine, which is used pharmacologically in the place of GSH due to the low bioavailability of the latter.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jtemb.2018.10.022.

References

[1] M. Riha, J. Karlickova, T. Filipsky, K. Macakova, R. Hrdina, P. Mladenka, Novel method for rapid copper chelation assessment confirmed low affinity of D-penicillamine for copper in comparison with trientine and 8-hydroxyquinolines, J.

Inorg. Biochem. 123 (2013) 80–87.
[2] I.F. Benzie, J.J. Strain, The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay, Anal. Biochem. 239 (1) (1996) 70–76.
[3] M.E. Aliaga, C. Lopez-Alarcon, R. Bridi, H. Speisky, Redox-implications associated with the formation of complexes between copper ions and reduced or oxidized glutathione, J. Inorg. Biochem. 154 (2016) 78–88.
[4] O. Zitka, S. Skalickova, J. Gumulec, M. Masarik, V. Adam, J. Hubalek, L. Trnkova, J. Kruseova, T. Eckschlager, R. Kizek, Redox status expressed as GSH:GSSG ratio as a marker for oxidative stress in paediatric tumour patients, Oncol. Lett. 4 (6) (2012) 1247–1253.
[5] A.A. Ensafi, H. Karimi-Maleh, S. Mallakpour, A new strategy for the selective determination of glutathione in the presence of nicotinamide adenine dinucleotide (NADH) using a novel modified carbon nanotube paste electrode, Colloids Surf. B Biointerfaces 104 (2013) 186–193.
[6] V.J. Forrest, Y.H. Kang, D.E. McClain, D.H. Robinson, N. Ramakrishnan, Oxidative stress-induced apoptosis prevented by Trolox, Free Radic. Biol. Med. 16 (6) (1994) 675–684.
[7] T.L. Duarte, J. Lunec, Review: when is an antioxidant not an antioxidant? A review of novel actions and reactions of vitamin C, Free Radic. Res. 39 (7) (2005) 671–686.
[8] J. Marczewska, J.H. Koziorowska, E.L. Anuszewska, Influence of ascorbic acid on cytotoxic activity of copper and iron ions in vitro, Acta Pol. Pharm. 57 (6) (2000) 415–418.
[9] M.L. Circu, T.Y. Aw, Glutathione and modulation of cell apoptosis, Biochim. Biophys. Acta 1823 (10) (2012) 1767–1777.
[10] A. Glasauer, N.S. Chandel, Targeting antioxidants for cancer therapy, Biochem. Pharmacol. 92 (1) (2014) 90–101.
[11] K. Malarz, A. Mrozek-Wilczkiewicz, M. Serda, M. Rejmund, J. Polanski, R. Musiol, The role of oxidative stress in activity of anticancer thiosemicarbazones, Oncotarget 9 (25) (2018) 17689–17710.
[12] D. Trachootham, J. Alexandre, P. Huang, Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat. Rev. Drug Discov. 8 (7) (2009) 579–591.
[13] N. Traverso, R. Ricciarelli, M. Nitti, B. Marengo, A.L. Furfaro, M.A. Pronzato, U.M. Marinari, C. Domenicotti, Role of glutathione in cancer progression and chemoresistance, Oxid. Med. Cell. Longev. (2013) (2013) 972913.
[14] P. Delangle, E. Mintz, Chelation therapy in Wilson's disease: from D-penicillamine to the design of selective bioinspired intracellular Cu(I) chelators, Dalton Trans. 41 (21) (2012) 6359–6370.
[15] J.H. Freedman, M.R. Ciriolo, J. Peisach, The role of glutathione in copper metabolism and toxicity, J. Biol. Chem. 264 (10) (1989) 5598–5605.
[16] T. Homma, J. Fujii, Application of glutathione as anti-oxidative and anti-aging drugs, Curr. Drug Metab. 16 (7) (2015) 560–571.
[17] T. Filipsky, M. Riha, R. Hrdina, K. Vavrova, P. Mladenka, Mathematical calculations of iron complex stoichiometry by direct UV-Vis spectrophotometry, Bioorg. Chem. 49 (2013) 1–8.
[18] J. P, Recherches sur la formation des complexes mineraux en solution, et sur leur stabilité, Ann. Chim. (1928) 113–134.
[19] M.C. Catapano, J. Karlickova, V. Tvrdy, S. Sharma, A.K. Prasad, L. Saso, A.K. Chhillar, J. Kunes, M. Pour, V.S. Parmar, P. Mladenka, Mono and dihydroxy coumarin derivatives: copper chelation and reduction ability, J. Trace Elem. Med. Biol. 46 (2018) 88–95.
[20] P. Mladenka, K. Macakova, T. Filipsky, L. Zatloukalova, L. Jahodar, P. Bovicelli, I.P. Silvestri, R. Hrdina, L. Saso, In vitro analysis of iron chelating activity of flavonoids, J. Inorg. Biochem. 105 (5) (2011) 693–701.
[21] N. Spear, S.D. Aust, Hydroxylation of deoxyguanosine in DNA by copper and thiols, Arch. Biochem. Biophys. 317 (1) (1995) 142–148.
[22] M.Y. Hamed, J. Silver, Studies on the reactions of ferric iron with glutathione and some related thiols. 2. Complex-formation in the Ph range 3 to 7, Inorgan. Chim. Acta-Bioinorg. Chem. 80 (3) (1983) 115–122.
[23] C.J. Reed, K.T. Douglas, Chemical cleavage of plasmid DNA by glutathione in the presence of Cu(II) ions. The Cu(II)-thiol system for DNA strand scission, Biochem. J. 275 (Pt 3) (1991) 601–608.
[24] P.M. Hanna, R.P. Mason, Direct evidence for inhibition of free radical formation from Cu(I) and hydrogen peroxide by glutathione and other potential ligands using the EPR spin-trapping technique, Arch. Biochem. Biophys. 295 (1) (1992) 205–213.
[25] N. Spear, S.D. Aust, Effects of glutathione on Fenton reagent-dependent radical production and DNA oxidation, Arch. Biochem. Biophys. 324 (1) (1995) 111–116.