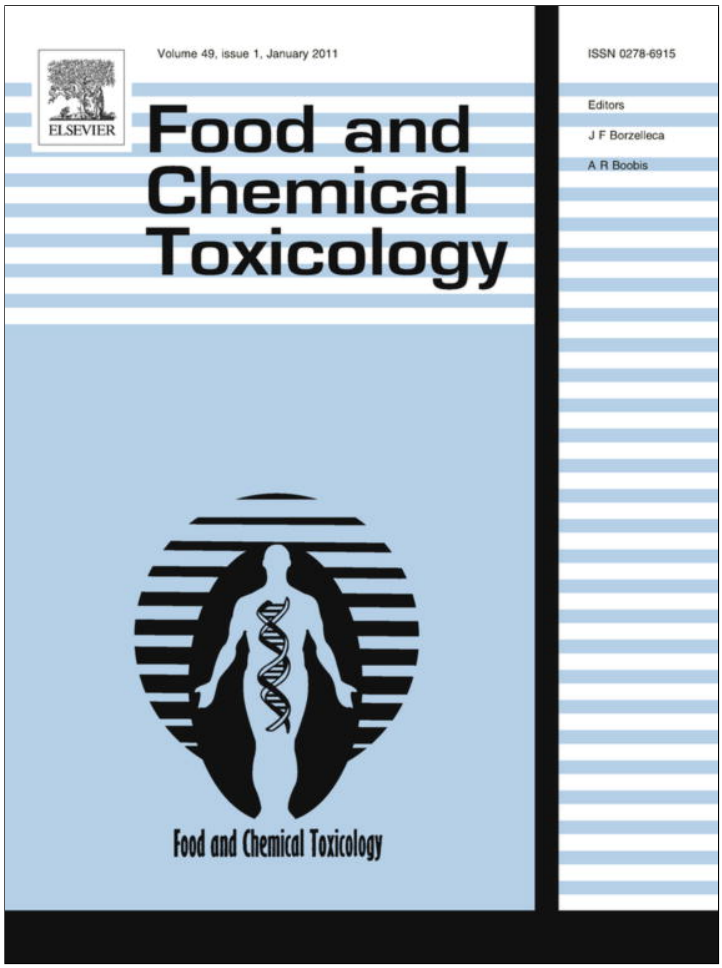

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Natural polyphenols may ameliorate damage induced by copper overload

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ABSTRACT

The effect of the simultaneous exposure to transition metals and natural antioxidants frequently present in food is a question that needs further investigation. We aimed to explore the possible use of the natural polyphenols caffeic acid (CA), resveratrol (RES) and curcumin (CUR) to prevent damages induced by copper-overload on cellular molecules in HepG2 and A-549 human cells in culture. Exposure to 100 μM/24 h copper (Cu) caused extensive pro-oxidative damage evidenced by increased TBARS, protein carbonyls and nitrite productions in both cell types. Damage was aggravated by simultaneous incubation with 100 μM of CA or RES, and it was also reflected in a decrease on cellular viability explored by trypan blue dye exclusion test and LDH leakage. Co-incubation with CUR produced opposite effects demonstrating a protective action which restored the level of biomarkers and cellular viability almost to control values. Thus, while CA and RES might aggravate the oxidative/nitrative damage of Cu, CUR should be considered as a putative protective agent. These results could stimulate further research on the possible use of natural polyphenols as neutralizing substances against the transition metal over-exposure in specific populations such as professional agrochemical sprayers and women using Cu-intrauterine devices.

1. Introduction

Copper (Cu) is an essential trace metal used as a catalytic cofactor for many enzymes including cytochrome oxidase, superoxide dismutase and tyrosinase (Tapiero et al., 2003; Arredondo and Núñez, 2005; Fraga, 2005) and is an important oligoelement in the food and water ingested by humans (Fraga, 2005). Nevertheless, excess Cu is potentially hazardous to human health since it can participate in the Fenton reaction, producing radical species (Halliwell and Guteridge, 1999; Uriu-Adams and Keen, 2005; Kozlowski et al., 2009). Many studies have reported that Cu overload leads to overproduction of ROS and the subsequent establishment of an oxidative stress condition responsible for the oxidative damage of proteins, lipids and nucleic acids in cells (Hussain et al., 2000; Dalle-Donne et al., 2003; Valko et al., 2006; Gupte and Mumper, 2009). It is widely recognized that elevated levels of ROS are related to the pathogenesis of various diseases like asthma, cirrhosis, neurodegenerative diseases and atherogenic disorders as a result of damage to important biomolecules such as lipids, proteins and DNA (Banerjee et al., 1999; Green-Ruiz and Páez-Osuna, 2001; Prakasam et al., 2001; Ranjbar et al., 2002; Gaetke and Chow, 2003; Ranjbar et al., 2005; Hernández et al., 2006; Jia and Misra, 2007; Lima et al., 2006 Lukaszewicsz-Hussain, 2008; Muniz et al., 2008; Astiz et al., 2009; Arnal et al., 2010a).

Cu-derived substances are used in a wide range of industries from smelting to electrical and electronic supplies (Newhook et al., 2003), the production of agrochemicals (pesticides and fungicides) (Campbell, 2001; Calderón et al., 2003; Newhook et al., 2003; Riedel, 2008; Arnal et al., 2011) and in copper-based intrauterine devices (CuIUDs) (Arnal et al., 2010b) all widely used around the world (Sivin, 2007). Cu environmental pollution therefore comes as no surprise (Newhook et al., 2003; Riedel, 2008; Cockell et al., 2008). The increasing concentrations of this metal in the air, water and food is becoming a matter of international concern (Riedel, 2008) in particular because of the involuntary exposure to Cu overload under sub-clinical or sub-symptomatological conditions which are obviously very difficult to detect (Uriu-Adams and Keen, 2005). Cu toxicity as a result of acute ingestion is rare; it is the much more common chronic exposure that causes a problem, precisely because it goes largely unnoticed (Calderón et al., 2003; Tapiero et al., 2003; Cockell et al., 2008). Fortunately, mammalian cells have a highly effective system for

Abbreviations: ATCC, American type culture collection; CA, caffeic acid; Cu, copper, CuIUD; copper intrauterine device, CUR, curcumin; LDH, lactate dehydrogenase, MDA, malondialdehyde; MEM, Eagle's minimum essential medium; [NOx], nitrite + nitrate; PCs, protein carbonyls; RES, resveratrol; ROS, reactive oxygen species; SD, standard deviation.

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regulating Cu homeostasis through a complex mechanism involving different kinds of specialized proteins (Puig and Thiele, 2002; Bertinato and Lábbé, 2004; Prohaska and Gybina, 2004; Balamurugan and Schaffner, 2006). However, Cu overload cannot always be neutralized by this system, leading to a pro-oxidative condition (Calderón et al., 2003; Bertinato and Lábbé, 2004; Uriu-Adams and Keen, 2005). It is widely known that many human pathophysiological conditions are associated with a specific failure of the Cu-homeostatic machinery (Uriu-Adams and Keen, 2005).

It is a well-documented fact that farmers handling agrochemicals and women using Cu-IUDs are chronically exposed to Cu ions. We and other authors have reported an enhancement of lipid peroxidation, protein oxidation and many other adverse effects in these two groups (Banerjee et al., 1999; Green-Ruiz and Páez-Osuna, 2001; Prakasam et al., 2001; Ranjbar et al., 2002; Gaetke and Chow, 2003; Ranjbar et al., 2005; Suzuki et al., 2004; Hernández et al., 2006; Jia and Misra, 2007; Lima et al., 2006; Lukaszewicz-Hussain, 2008; Muniz et al., 2008; Astiz et al., 2009; Arnal et al., 2010a,b, 2011).

Natural plant products have been used for both culinary and medicinal purposes for centuries, many of them being secondary metabolites produced as a defense against disease or infection. Curcumin (diferuloil-methane) is a polyphenol derived from the perennial herb *Curcuma longa*, commonly called "turmeric". It is used as a spice in curries and masalas, and because of its intense yellow color, as coloring for cheese, butter, and pastries (Goel et al., 2008). This substance has been used for hundreds of years in different parts of the world as an active ingredient in therapeutic preparations (Goel et al., 2008). Resveratrol (*trans*-3,5,4′-trihydroxiestilbeno) is also a polyphenolic compound derived from plants and is used as an antioxidant in the treatment human illnesses such as gonorrhea and hyperlipidemia (Saiko et al., 2008). This compound was first extracted in 1940 from the roots of the *Veratrum grandiflorum* plant, commonly known as "hellebore", and has more recently been found in many other plant species (vines, groundnuts and tropical fruits such as mango, among others). Saiko et al. (2008) reported that despite having a diet relatively rich in saturated fats, people in France suffer almost 40% fewer heart attacks than people from the rest of Europe. It is believed that there is a correlation between red wine consumption and the incidence of cardiovascular disease. Research suggests that resveratrol may be one of the active ingredients in red wine that protect people from cardiovascular risk. There are other types of polyphenols in plants belonging to the hydroxycinnamic acid family, whose main representative compound is caffeic acid (3,4-hydroxycinnamic acid). Coffee is the main source of this compound, though it is also found in apples, pears, strawberries, artichokes and eggplants (Clifford, 1999). This compound showed antioxidant activity *in vitro* and multiple pharmacological properties including anti-inflammatory and antitumorigenic effects (Clifford, 1999).

Transition metals and polyphenols are frequently present simultaneously in food and the nature of their interaction in terms of final biological activity presents an intriguing interrogative. There are many studies indicating that polyphenols behave as antioxidants both *in vivo* and *in vitro* (Young et al., 2000; Ahn et al., 2002; Cai et al., 2002; Eybl et al., 2006; Wei et al., 2006; Gladine et al., 2007) but also a number of studies showing that polyphenols may act as pro-oxidants *in vitro*, especially in the presence of transition metals (Zheng et al., 2006; Bhat et al., 2007; Chen et al., 2008; Wang et al., 2008). Many researchers agree that various polyphenols such as curcuminoids, flavonoids and stilbenes break the double helix of DNA in the presence or absence of Cu (Jacobi et al., 1998; Yoshino et al., 2004; Azmi et al., 2006; Bhat et al., 2007; Wang et al., 2008). This topic remains a matter of controversy and calls for further investigation.

With this in mind our aim was to investigate (i) the possible use of polyphenols to prevent Cu overload-induced damage in cellular lipids and proteins tested in two human culture cells (HepG2 and A-549) and (ii) the differential response of CUR, CA and RES with and without co-supplementation with Cu in terms of their protective action relative to cell viability.

2. Materials and methods

2.1. Chemicals

All chemicals used were of analytical grade and obtained from Sigma Chem. Co. (Buenos Aires, Argentina or USA), Merck (Darmstadt, Germany) or Carlo Erba (Milan, Italy).

2.2. Cell culture

Human liver (HepG2) and lung (A-549) cell lines from ATCC (American Type Culture Collection) were used. These human lung- and liver-derived cell culture lines were chosen as representative cells exposed to exogenous (polluted air) and/or endogenous (systemic overload) Cu in living organisms. Both culture systems have frequently been used as an appropriate experimental approach for testing the effect of transition metals in general and Cu-induced damage in particular (Aston et al., 2000; Fatur et al., 2002; Fotakis and Timbrell, 2006). Monolayer cultures were grown in Eagle's minimum essential medium (MEM). We used streptomycin and penicillin during stock culture conditions but no antibiotics were added during the experiments. This decision was taken in order to define more clearly incubation medium and to avoid possible interferences with the addition of the polyphenols assayed. MEM was supplemented with 10% inactivated fetal calf serum (Natocor, Córdoba, Argentina) and microbiologically (pathogen-free) tested for cell culture.

2.3. Cell treatment and homogenization

HepG2 and A-549 cell lines were seeded and grown to semi-confluency in disposable culture flasks (Falcon, Ca. USA). Forty-eight hours after seeding, cultures were treated with fresh medium supplement with ultrafiltered (Millipore 0.22 μm, NY, USA) sterile solutions of CuSO4, resveratrol (RES), curcumin (CUR) or caffeic acid (CA) alone or in combination (Cu, RES, CUR, CA, RES/Cu, CUR/Cu or CA/Cu) (final concentration 100 μM each). CuSO4 was dissolved in PBS. Polyphenols were dissolved in dimethylsulfoxide (DMSO, Sigma Chem. Co. Buenos Aires) and immediately diluted into the culture medium as suggested by Lima et al. (2006) (final concentration of DMS was 0.5% V/V). Control cultures supplemented with an equivalent aliquot of DMSO were run in parallel; however, there were no statistical differences in the parameters assayed between cells treated with or without the addition of DMSO. After 24-h treatment, cells were harvested, washed with cold sterile PBS (5 mL/three times), centrifuged at 4 °C (10 min at 1500g) and the pellet was manually homogenized on ice with 4 mL of PBS using a stainless-steel hand homogenizer (Khonte, IL, USA) to complete lysis (10 strokes, controlled by optical microscopic observation).

2.4. Estimation of cellular viability

Parallel cultures were analyzed for cellular viability. Medium samples were collected and centrifuged (10 min at 1500g) and then ultrafiltered through Millipore membranes (0.22 μm) in order to completely remove cell debris. Appropriate aliquots were taken in order to determine the lactate dehydrogenase activity (LDH) by a kinetic UV method using the commercial kit Optima-LDH-P UV/AA from Wiener Laboratories (Rosario, Argentina). Results were determined in quadruplicate and expressed as the percentage change compared to control flasks (4.5 ± 0.2 and 7.9 ± 0.4 IU LDH/mL of culture medium for A-549 and HepG2, respectively). In another series of culture flasks, attached cells were washed with PBS and treated with 100 μL of 0.1% solution of trypan blue dye (in PBS, pH 7.40). After one min incubation at room temperature they were examined under optical microscopy to determine the percentage of viable cells according to the method described by Jauregui et al. (1981). At least four fields of one hundred cells per field were counted and the results were expressed as the percentage change compared to control flasks (98 ± 1% viability on average for both types of culture cells).

2.5. Biomarkers of damage

2.5.1. Nitrate and nitrite ([NOx])

Total nitrite plus nitrate formation ([NOx]), as the end metabolic products of nitric oxide (NO) and peroxinitrite ($ONOO^-$), were measured in cellular homogenates using the method of Griess in samples previously deproteinized and reduced with vanadium chlorhydrate (Miranda et al., 2001). Briefly, appropriate aliquots of samples were treated with 4 volumes of a methanol:chloroform mixture (3:1) at 4 °C.

After centrifugation at 10.000*g* for 10 min, they were reduced by an excess of VCl3 in 1 M HCl. Samples were combined with the Griess detection reactive (*N*-1-(naphthyl) ethylenediamine (1 mM) plus sulfamide (20 mM) in 5% HCl. The formation of the chromophore from the diazotization of sulfamide by nitrite at low pH, followed by the coupling with the bicyclic amine, was detected at 405 nm after incubating the mixtures at 37 °C for 30 min. Quantification was performed after calibration with standard solutions of sodium nitrate from Merck Co. (Darmstadt, Germany).

2.5.2. Protein carbonyls (PCs)

The method of Reznick and Packer (1994) was used. Aliquots of cellular homogenates were incubated with dinitro-phenylhydrazine in HCl 2 N at 37 °C in the dark for 30 min. The corresponding hydrazone-derivatives present in the proteins were revealed after addition of excess NaOH and measured at 505 nm. The concentration of PCs was calculated from a calibration curve prepared with a stock solution of sodium pyruvate (Sigma Chem. Co., Argentina).

2.5.3. Thiobarbituric acid-reactive substances (TBARs)

The extent of lipid peroxidation in homogenates was calculated by analyzing the levels of TBARs (Yagi, 1976). An aliquot of cellular homogenates (50–100 μL) was reacted with 200 μL of SDS (8.10%, W/V) and 1.5 mL of acetic acid 10% (V/V) (pH 3, 5). Subsequently, 1.5 mL of thiobarbituric acid (TBA 0.8%) and 600 μL of water were incorporated and the mixture was heated in sealed tubes at 95 °C for 60 min. Under these conditions TBARs (mainly malondialdehyde (MDA) generated by lipid peroxidation) reacted with TBA to yield TBA-MDA adducts which were quantified at 532 nm. The concentration of the chromophore was calculated from a calibration curve prepared with fresh tetrametoxipropane (TMP) solutions (TMP was purchased from Sigma Chem. Co., Buenos Aires, Argentina).

2.5.4. Total glutathione (G) content and its reduced (GSH) and oxidized (GSSG) forms

Total glutathione was determined by the glutathione reductase/dithio-nitrobenzoic (DTNB) method (Anderson and Meister, 1980) that can measure both GSH and GSSG. An aliquot of sample was mixed with sodium phosphate buffer 140 mM with EDTA 6.3 mM (pH 7.5). The mixture was supplemented with NADPH (0.25 mg/mL) and DTNB (6 mM) and incubated for 10 min at 30 °C. Five IU/reaction tube of GSH-reductase (Sigma Chem. Co.) was then added and the optical density was recorded for 2 min at 412 nm in a two-beam spectrophotometer (Cintra-20, Sydney, Australia). To calculate the ratio GSH/GSSG samples were re-analyzed after derivatization with divinyl-pyridine (3 mM final concentration).

2.6. Cellular copper and total polyphenol determinations

After incubations, cell monolayers were washed, harvested and homogenized as described previously. Aliquots (400 μL) of cellular homogenates were subjected to mineralization and copper content was determined by atomic absorption spectrophotometry as described in detail by Arnal et al. (2010b). Results were expressed as μmoles of copper/mg total cellular protein (TCP). Appropriate aliquots (200–400 μL) of the same homogenates were assayed spectrophotometrically at 715 nm by total phenolic compounds using the Folin–Ciocalteu method described by Ali et al. (2006). Calibration curves were performed for each phenol drug (CA, RES and CUR). Results were expressed as μmoles of the corresponding phenolic compound/mg TCP.

2.7. Statistical analysis

All values represent the mean of at least 4 independent determinations indicated as mean ± standard deviation (SD). Data were analyzed by Student́s *t*-test or ANOVA plus Tukey test with the aid of Systat (version 15.0 for Windows) from SPSS Science (Chicago, IL). Results were also plotted and analyzed using Sigma Scientific Graphing Software (version 11.0) from Sigma Chem. Co. (St. Louis, MO) and/or GB-STAT. Professional Statistics Program (version 6.0) from Dynamic Microsystems Inc. (Silver Springs). The statistical significance of differences was indicated by * ($p < 0.01$) in figures or with superscript letters in the Table 1 (values within the same row with different superscript letters were statistically significant at $p < 0.05$ or less).

3. Results

Copper was effectively taken up by the cells as demonstrated by the cellular concentration of the metal within the HepG2 or A-549 cells (0.31 ± 0.03 and 0.27 ± 0.04 μmoles Cu/mg TCP, respectively). Co-treatments (polyphenols plus Cu) did not modify significantly the intracellular concentration of Cu that was incorporated by the cells. Determination of total polyphenols in washed cells indicated that the presence of Cu in the culture medium did not alter the internal concentration reached for each drug (0.17 ± 0.04; 0.19 ± 0.03 and 0.15 ± 0.04 μmoles of CA, RES or CUR (respectively)/mg TCP in HepG2 cells; and 0.16 ± 0.03; 0.21 ± 0.02 and 0.17 ± 0.03 μmoles of CA, RES or CUR (respectively)/mg TCP in A-549 cells).

Treatments with caffeic acid or resveratrol produced no significant changes on the parameters studied. They seem to have a neutral effect. Fig. 1 shows basal levels of protein carbonyls (PCs) as markers of protein damage in both cell lines (higher in A-549 than in HepG2). Clearly, the treatment with Cu induced a significant increase in the formation of PCs. Supplementation with natural polyphenols in the absence of Cu did not cause significant changes in the baseline production of PCs. However, co-treatment with Cu and caffeic acid (CA) or resveratrol (RES) significantly increased the production of PCs to levels even higher than those observed in the group supplemented with Cu alone. In contrast to the co-addition of CA or RES, co-treatment with the polyphenol curcumin (CUR) gave rise to a marked decrease in the generation of PCs. TBAR determinations showed a pattern similar to that observed for PCs. The addition of Cu ions alone significantly increased TBAR production and co-supplementation with RES or CA further increased their formation (Fig. 2). Interestingly, supplementation with Cu/RES had a deleterious effect which was stronger in HepG2 (Fig. 2A) than in A-549 (Fig. 2B). Again, co-treatment with Cu/CUR reduced TBARs to levels comparable to those measured in control

Table 1
Total glutathione, reduced (GSH), oxidized (GSSG) forms, and the GSH/GSSG ratio in HepG2 and A-540 cells treated with copper and combinations of copper plus polyphenols.

	Additions							
	−Cu				+Cu			
	None	+CA	+RES	+CUR	None	+CA	*+RES*	*+CUR*
HepG2								
Total	758 ± 29^{a}	1976 ± 84^{b}	1811 ± 71^{b}	1327 ± 69^{c}	1773 ± 59^{d}	3165 ± 112^{e}	3084 ± 134^{e}	3249 ± 109^{e}
GSH	674 ± 22^{a}	1341 ± 51^{b}	1457 ± 63^{b}	1215 ± 70^{b}	1462 ± 44^{b}	2492 ± 115^{c}	2433 ± 153^{c}	2845 ± 138^{d}
GSSG	84 ± 5^{a}	268 ± 14^{b}	354 ± 18^{c}	112 ± 5^{d}	311 ± 12^{e}	673 ± 25^{f}	651 ± 31^{f}	404 ± 21^{g}
GSH/GSSG	8.0 ± 0.5^{a}	4.9 ± 0.2^{b}	4.1 ± 0.3^{b}	10.8 ± 1.1^{a}	4.7 ± 0.3^{b}	3.7 ± 0.2^{c}	3.7 ± 0.3^{c}	7.0 ± 0.6^{a}
A-540								
Total	318 ± 11^{a}	324 ± 15^{a}	295 ± 17^{a}	298 ± 21^{a}	322 ± 14^{a}	345 ± 23^{a}	351 ± 25^{a}	348 ± 20^{a}
GSH	289 ± 9^{a}	296 ± 12^{a}	269 ± 13^{a}	279 ± 14^{a}	266 ± 15^{a}	281 ± 10^{a}	282 ± 8^{a}	315 ± 16^{a}
GSSG	29 ± 2^{a}	28 ± 3^{a}	26 ± 1^{a}	19 ± 2^{b}	56 ± 2^{c}	64 ± 3^{d}	69 ± 5^{d}	33 ± 3^{a}
GSH/GSSG	9.9 ± 1.2^{a}	10.5 ± 2.0^{a}	10.3 ± 0.9^{a}	14.6 ± 0.8^{b}	4.7 ± 0.1^{c}	4.3 ± 0.2^{c}	4.1 ± 0.3^{c}	9.5 ± 0.7^{a}

Results were expressed (nmoles/ mg TCP) as the mean ± standard deviation of three independent experiments each assayed in triplicate. Cells were treated with copper or combinations of cooper and the corresponding polyphenols (100 μl final concentrations) as described in the experimental protocol. Cell cultures with no additions were run in parallel as control assays. Total glutathione and the sub-fractions (GSH and GSSG) were measured as described in Section 2. Results with different superscript letters were statistically different between them ($p < 0.05$ or less).

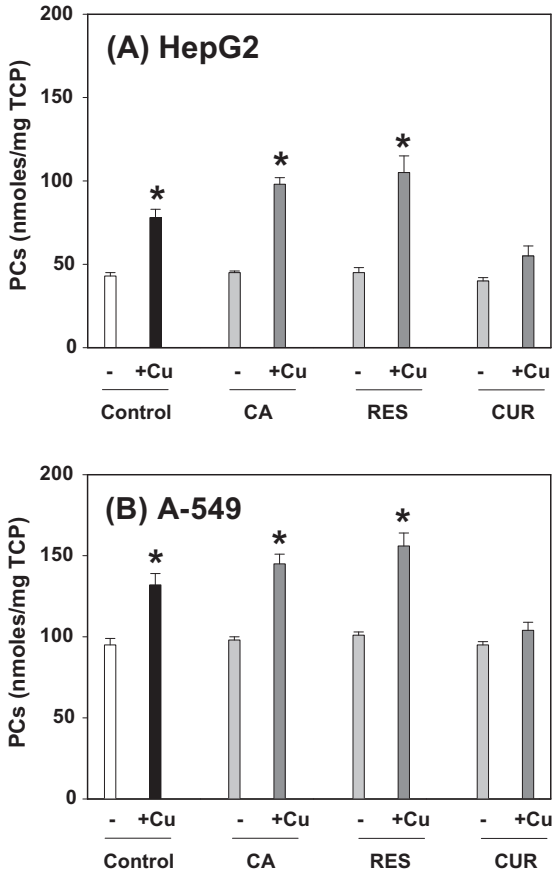

Fig. 1. Protein carbonyl concentration (PCs) in HepG2 (A) or A-549 (B) human culture cells treated with copper (Cu) alone (black bar), polyphenols (grey bars), or combined supplementation with Cu/polyphenols (dark grey bars) compared to control flasks (white bar). Forty-eight hours after seeding (semiconfluency), cultures were treated for 24 h with fresh medium supplement with Cu, resveratrol (RES), caffeic acid (CA) or curcumin (CUR) (alone or in combination) each at a final concentration of 100 μM. Results are expressed as nmoles/mg total cellular protein (TCP) and are the mean of 4 independent determinations assayed in duplicate ± standard deviation (SD). Significant differences compared to control assays are indicated with an asterisk ($p < 0.01$).

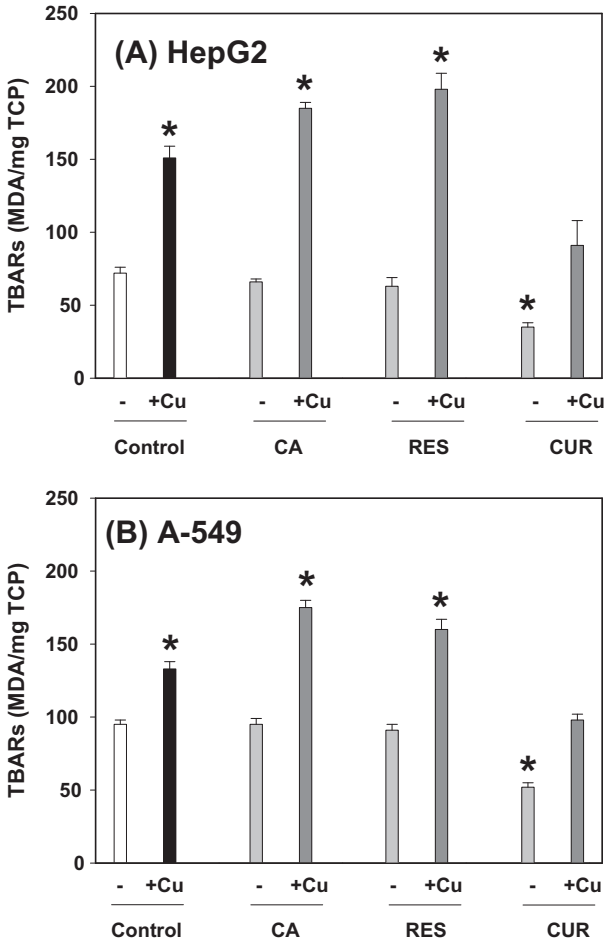

Fig. 2. Thiobarbituric acid-reactive substances (TBARs) in HepG2 (A) or A-549 (B) human culture cells. Cell treatments were as described in Fig. 1. Results are expressed as nmoles/mg total cellular protein (TCP) and are the mean of 4 independent determinations assayed in duplicate ± standard deviation (SD). Significant differences compared to control assays are indicated with an asterisk ($p < 0.01$).

flasks. In addition, CUR alone suppressed TBARs concentration below control levels.

Fig. 3 shows the results of the determination of [NOx] (used as biomarker of nitrative stress) in both cell lines. The findings are similar to above. It is clear that the adverse effects of the combined treatments Cu/CA and Cu/RES are much more noticeable in HepG2 (Fig. 3A) than in A-549 (Fig. 3B). It is interesting to note that the addition of CUR to A-549 significantly decreased the levels of [NOx] to even lower values than those obtained with control incubations (Fig. 3B). Another differential response of this biomarker was observed for HepG2 cultures where supplementation with Cu/CUR failed to restore the values of [NOx] (Fig. 3A). Interestingly, curcumin has an antioxidant effect only in the TBARs and [NOx] measurements in the absence of Cu ions.

The effect of co-exposure to Cu and polyphenols was also investigated for the main endogenous water soluble antioxidant glutathione (Fig. 4). Cells from liver showed a significant increase in glutathione levels after exposure to Cu, even when they had been co-supplemented with any of the three antioxidants studied (Fig. 4A). Unlike in the case of HepG2 cells, A-549 did not show any significant changes in the levels of this antioxidant (Fig 4B). To differentiate the contribution of the reduced (GSH) and oxidized (GSSG) forms of glutathione we determine these compounds separately (Table 1). Copper treatment increased significantly the level of GSSG and produced a substantial reduction of the GSH/GSSG ratio. Addition of CA or RES not only did not restore the physiological proportions of GSH *vs* GSSG but also produced a detrimental effect. On the contrary, CUR added to cells -with or without Cu- elevated the GSH proportion and completely restores the values of the GSH/GSSG ratio (Table 1). The behavior of the cells is obviously different since only HepG2 cells have the capacity to biosynthesize the GSH. However, in A-540 cells the oxidative stress induced by Cu alone (or associated with CA or RES) increased the proportion of GSSG that is completely normalized by treatment with CUR. Moreover, CUR treatment in A-540 cells not exposed to Cu produced accumulation of GSH over control values.

Cell viability was found to be significantly affected by the addition of Cu. Treatment of both cell lines with Cu/CA or Cu/RES also gave rise to significantly reduced viability as measured by the trypan blue dye exclusion test (Fig. 5). However, co-supplementation with Cu/CUR restored cell viability values to those observed in control incubation flasks. In agreement with these results, we observed increased LDH activity in both cell lines when they were supplemented with Cu, Cu/CA or Cu/RES, but not in the case of co-incubation with Cu/CUR (Fig. 6). The addition of any of the antioxidants alone had no significant impact on cell viability or LDH activity. Co-supplementation with Cu/CUR restored the values of LDH activity to those obtained for control flasks in both cell lines.

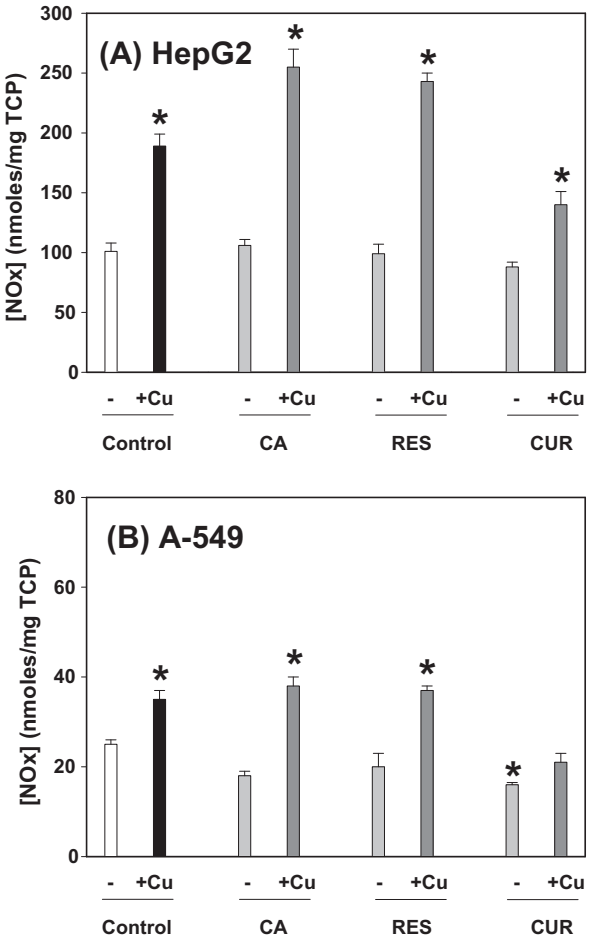

Fig. 3. Nitrate plus nitrite ([NOx]) concentrations in HepG2 (A) or A-549 (B) human culture cells. Cell treatments were as described in Fig. 1. Results are expressed as nmoles/mg total cellular protein (TCP) and are the mean of 4 independent determinations assayed in duplicate ± standard deviation (SD). Significant differences compared to control assays are indicated with an asterisk ($p < 0.01$).

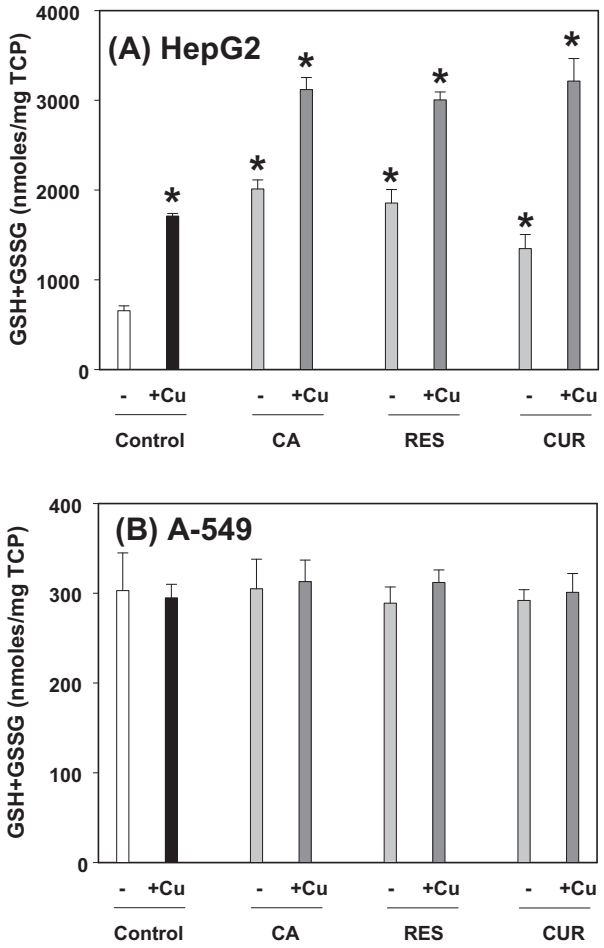

Fig. 4. Reduced (GSH) plus oxidized (GSSG) glutathione concentrations in HepG2 (A) or A-549 (B) human culture cells. Cell treatments were as described in Fig. 1. Results are expressed as nmoles/mg total cellular protein (TCP) and are the mean of 4 independent determinations assayed in duplicate ± standard deviation (SD). Significant differences compared to control assays are indicated with an asterisk ($p < 0.01$).

4. Discussion

Our findings show that supplementation of cells with natural polyphenols such as resveratrol (RES) or caffeic acid (CA) produces no changes in the basal levels of the stress biomarkers of pro-oxidative damage (TBARs, PCs and [NOx]). The co-addition of these substances with Cu produced a deleterious effect at least equivalent to that observed for the transition metal-overload itself. Our results agree with the published findings of other researchers who reported that addition of CA could increase TBAR production in isolated lymphocytes (Bhat et al., 2007). Apparently, RES treatment triggers Cu mobilization from endogenous stores, causing lipid damage and increasing levels of [NOx] in human peripheral lymphocytes (Azmi et al., 2006). Other authors have demonstrated that, at least in the case of RES, the biological effects of the polyphenol/Cu association are critically dependent on both cell type and incubation conditions (Saiko et al., 2008).

Contrary to the effects displayed by CA or RES, curcumin (CUR) decreased TBARs to below control values in both A-549 and HepG2 cell lines. These results are in agreement with those reported by Naik et al. (2004). The addition of CUR alone also decreased [NOx] in A-549 cells to below control values, demonstrating a protective effect against the nitrative damage induced by the overproduction of peroxinitrite. In relation to this finding, some researchers have reported that CUR efficiently reduces the activation of iNOS in primary cultures of rat macrophages, leading us to postulate that the decrease in [NOx] in A-549 may be the result of a similar inhibitory mechanism (Goel et al., 2008).

After co-supplementation in both cell lines with Cu/RES or Cu/CA, we observed increases in TBAR, PCs and [NOx] formation even up to concentrations higher than those found in the treatments with Cu alone. Wang et al. (2008) reported that CA in the presence of Cu ions leads to increased ROS production, particularly of hydroxyl and superoxide ions. They also suggested that CA could be a good reducing agent of Cu^{++}, increasing its capacity to induce damage through the generation of Cu^{+}. In agreement with the results of Azmi et al. (2006) in relation to the RES effect, we observed no significant increase in the formation of biomarkers of oxidative damage. However, co-supplementation of RES with Cu produced additional increases in the production of TBARs, PCs and [NOx] to levels even higher than those obtained by supplementing with Cu alone. Further experiments will be necessary to elucidate the mechanism(s) by which the association between the natural polyphenol RES and Cu ions triggers such pro-oxidative synergism.

The addition of CUR in combination with Cu totally inhibited the formation of PCs and TBARs in both A-549 and HepG2 cells. In HepG2 cells, supplementing with Cu/CUR led to a significant increase in [NOx] production but not to the level observed with Cu treatment alone. This effect was not observed for A-549 cells. This difference lead us to speculate the existence of a specific response in each cell line that probably involves iNOS regulation. This issue

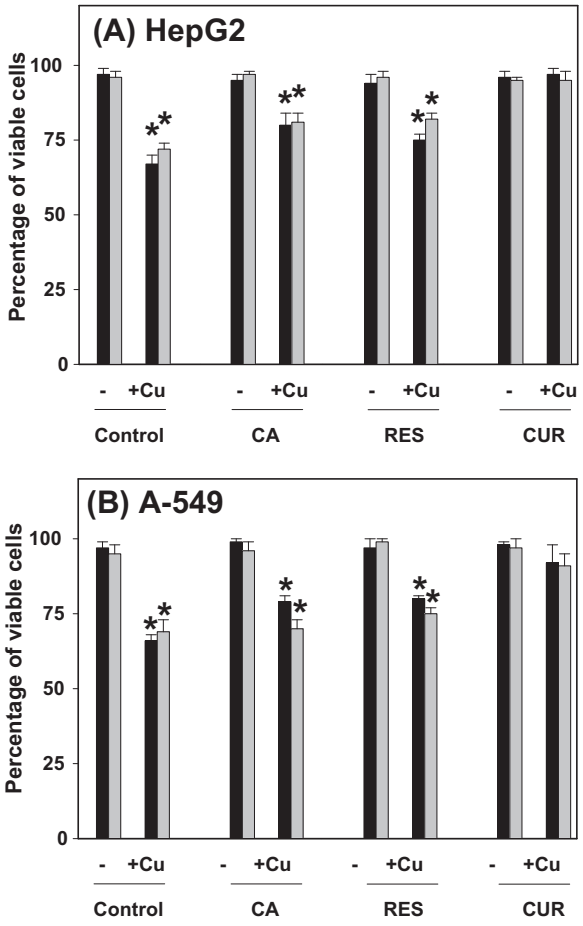

Fig. 5. Cellular viability estimated by trypan blue dye exclusion test in HepG2 (A) or A- 549 (B) human culture cells treated with copper Cu (alone or in combination with polyphenols; grey bars) compared to their respective controls without Cu addition (black bars). Cell treatments were as described in Fig. 1. Results are expressed as percentages of living (viable) cells and are the mean of 4 independent determinations assayed in duplicate ± standard deviation (SD). Significant differences compared to control assays are indicated with an asterisk ($p < 0.01$).

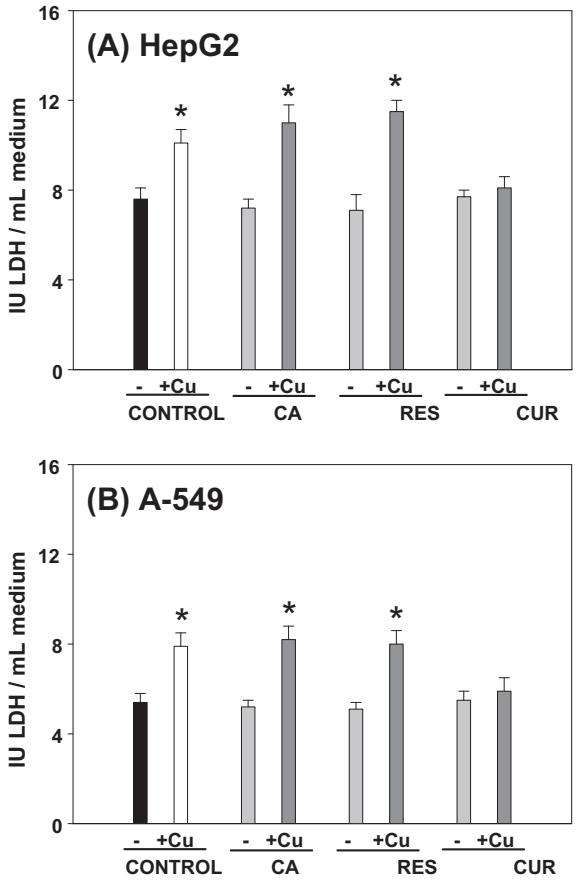

Fig. 6. Lactate-dehydrogenase leakage measured in ultrafiltered culture medium from HepG2 (A) or A-549 (B) human culture cells treated with copper (Cu) alone (black bar), polyphenols (grey bars), or a combination of Cu/polyphenols (dark grey bars) compared to control flasks (white bar). Cell treatments were as described in Fig. 1. LDH activity was measured at each experimental point and then expressed as IU/mL of medium. Results are the mean of 4 independent determinations assayed in duplicate ± standard deviation (SD). Significant differences compared to control assays are indicated with an asterisk ($p < 0.01$).

deserves further research for its elucidation. It is well known that nitric oxide plays a central physiological role in lungs, and its biosynthesis is regulated differently in this tissue with respect to liver.

Daniel et al. (2004) suggested that the antioxidant properties of turmeric (a curcumin-enriched preparation obtained from *Curcuma longa*) against the toxic effects of Cd are due to the binding between the two substances. Thus, the formation of a complex might reduce the effective concentration of free metal and in turn its toxic effect(s). Based on this finding we speculated that CUR displays a similar effect by reducing or neutralizing the effective concentration of Cu ions. Although the cellular concentration of copper in CUR-treated cells was indistinguishable to those measured in the other experimental groups, the real availability of copper ions inside the cells could be interfered by the presence of the CUR and the subsequent formation of [CUR-Cu] complexes. However, this hypothesis deserves further investigation.

Although there were no significant changes in the concentration of the oxidative damage biomarkers after treatment with polyphenols alone, we did observe a higher concentration of total glutathione in HepG2 cells. Moreover, this increase also occurred after co-supplementation with Cu and polyphenols and was even more marked than that observed after the addition of Cu alone. These effects are probably a consequence of the pro-oxidative insult in conjunction with the induction of GSH biosynthesis in liver cells, which is likely to occur in this tissue but highly improbable in lungs (Jaeschke, 1990).

Liver is the primary organ responsible for the biosynthesis and exportation of this water soluble antioxidant, and it has an active mechanism for over-expressing the key enzyme that controls the biosynthetic pathway (Dringen, 2000).

Consistent with the results obtained in A-549 and HepG2 in relation to the production of TBARs, PCs and [NOx], we also found no significant changes in terms of cell viability in the presence of polyphenols alone. After treatment with Cu alone or in association with RES or CA, however, the percentage of viable cells markedly decreased. These results are in agreement with data published by Wang et al. (2008) who showed that in the presence of Cu, CA was able to break the double helix of DNA, producing significant increases in the formation of ROS. Similarly, Zheng et al. (2006) reported that RES can act *cooperatively* with Cu to cause structural damage to the DNA double helix. Results reported by other researchers suggest that resveratrol inhibits entry to the S phase of the cell cycle by preventing the onset of G2/M (Notas et al., 2006; Colin et al., 2008). For this reason, one might have expected a lower number of cells (or rate of mitosis) under the influence of this polyphenol, but not necessarily lower viability of already divided cells. In fact, we did not observe a decrease in any of the viability markers determined under RES treatment. Supplementation with Cu/CUR clearly showed protective effects in both cell lines. The latter result is consistent with data previously published

by Naik et al. (2004) using primary cultures of rat hepatocytes treated simultaneously with other ROS-producing agents and CUR.

5. Conclusions

Among the polyphenols assayed in this study, curcumin supplementation -but not RES or CA- was efficient in the protection of transformed hepatocytes HepG2 and A-549 lung cells against adverse effects of Cu ions. It is hoped that these findings will encourage more in-depth research into the use of natural polyphenols as nutritional complements to protect people chronically exposed to Cu overload, such as specialists handling agrochemical sprayers, Cu-IUD users (Banerjee et al., 1999; Green-Ruiz and Páez-Osuna, 2001; Prakasam et al., 2001; Ranjbar et al., 2002; Gaetke and Chow, 2003; Ranjbar et al., 2005; Suzuki et al., 2004; Hernández et al., 2006; Jia and Misra, 2007; Lima et al., 2006; Lukaszewicz-Hussain, 2008; Muniz et al., 2008; Astiz et al., 2009; Arnal et al., 2010b; Arnal et al., 2011) and persons genetically conditioned (Mercer, 2001; González et al., 2008) to exposure to the Cu in food or even tap water (Spitalny et al., 1984; Buchanan et al., 1999; Lagos et al., 1999; Sparks and Schereurs, 2003; Rech Franke et al., 2006; Becaria et al.,2006). More research in this field will shed light on the real biological effect(s) of the extensive list of natural polyphenols in food and their putative use as protecting/therapeutic agents against exposure to Cu and other transition metals with pro-oxidant activities.

Conflict of Interest

The author declares no directly or indirectly conflict of interest.

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