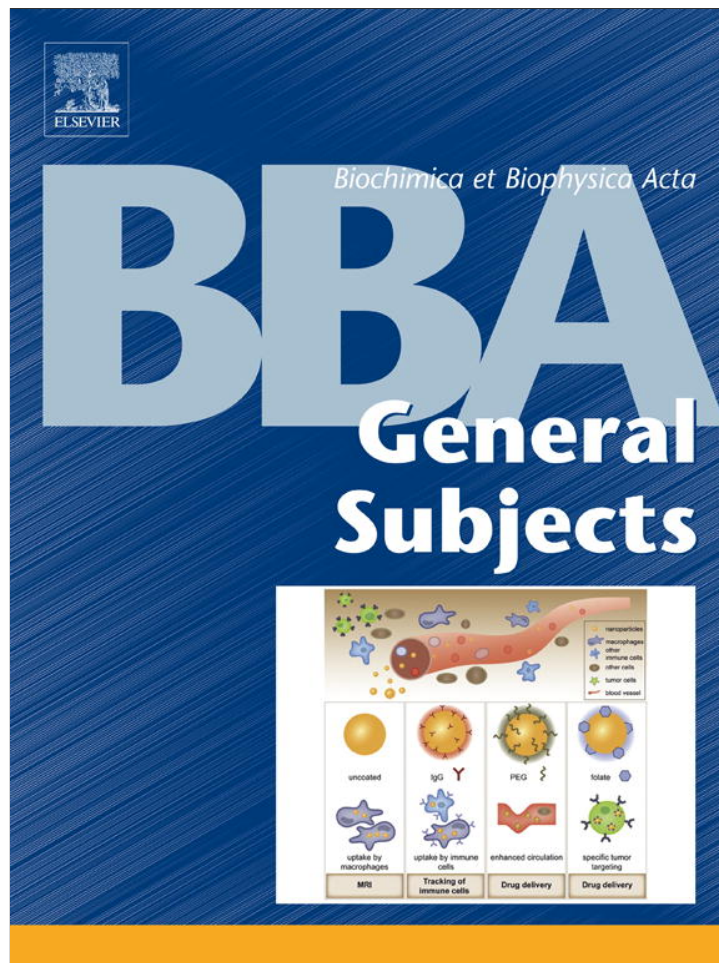


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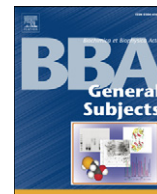
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## Cytotoxic effects of copper overload on human-derived lung and liver cells in culture

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### ABSTRACT

**Background:** Copper (Cu) is an essential trace metal used as a catalytic cofactor for many enzymes. However, it can have nocive effects when it participates in the Fenton reaction, producing reactive oxygen species (ROS). Excess Cu is present in the plasma of patients with diseases in which cell survival is crucial. In order to investigate the effect of Cu overload on the induction of cellular damage we chose two human cell lines derived from liver (HepG2) and lung (A-549) as representative cells exposed to exogenous (polluted air) and/or endogenous (systemic) Cu overload.

**Methods:** We studied ROS production using thiobarbituric acid reactive substances (TBARS) and fluorimetric measurements with dichlorofluorescein, cell viability by the trypan dye exclusion test, the methyltetrazolium (MTT) and lactate dehydrogenase leakage (LDH) assays, various cytotoxic indexes, and caspase-3 and calpain-dependent activation as the main signals involved in the apoptosis pathway.

**Results:** Cu overload induces cell death by a differential activation of calpains ( $m$ - and  $\mu$ -) and caspase-3, and modifies various proliferative indexes in a cell-type and concentration-dependent manner. The involvement of these two protease systems and the response of the two main Cu homeostatic proteins ceruloplasmin and metallothioneins are specific to each cell type. We demonstrated that Cu can trigger cell death by activation of specific protease systems and modify various proliferative indexes in a cell-type and concentration-dependent manner.

**General significance:** These findings contribute to understanding the diverse effects of Cu overload on the pathogenesis of human diseases like cancer, cirrhosis and degenerative disorders.

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### 1. Introduction

Copper (Cu) is an essential trace metal used as a catalytic cofactor for many enzymes [1–3] and is an important oligoelement in the food and water ingested by humans [3]. However, excess Cu is potentially hazardous to human health since it can participate in the Fenton reaction, producing radical species [4–6]. Many studies have reported that Cu overload leads to oxidative stress and subsequent oxidative damage to proteins, lipids and nucleic acids [7–10]. It is widely recognized that elevated levels of free radicals derived from oxygen (ROS) are related to the pathogenesis of various human diseases [11–23]. Cu-derived substances

are extensively used in a broad range of industries around the world, from smelting to the production of electrical and electronic goods [24], agrochemicals (pesticides and fungicides) [24–28] and Cu-based intrauterine devices (Cu-IUDs) [29]. Cu environmental pollution therefore comes as no surprise [24,27,30] and is already a matter of international concern [27]. More common than acute exposure [1,26,30], involuntary exposure to Cu overload under sub-clinical or sub-symptomatological conditions is very difficult to be detected [5,31]. It is a well-documented fact that farmers handling agrochemicals and women using Cu-IUDs are chronically exposed to Cu ions, resulting in elevated levels of Cu in their plasma [24–28].

Cross-sectional and case-control data have shown higher serum Cu levels in cancer patients [32–37] and Linder et al. [34] reported that tumor cells contain relatively high concentrations of Cu. However, very few studies have investigated the exact role of Cu in human cancer pathophysiology. High Cu intake is also associated with the development of childhood cirrhosis [38]. We and other authors have demonstrated that elevated concentrations of Cu in the plasma of patients with neurodegenerative disorders [23,39] and the putative clinical value of the Cu ion concentration in peripheral plasmas are useful tools for characterizing pathologies such as Alzheimer's, vascular dementia and Parkinson's disease [23].

**Abbreviations:** ATCC, American Type Culture Collection; CRP, ceruloplasmin; Cu, copper; Cu-IUD, copper-based intrauterine device; DCF-DA, dichlorofluorescein-diacetate; MDA, malondialdehyde; MEM, Eagle's minimum essential medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTs, metallothioneins; ROS, reactive oxygen species; SD, standard deviation; TBARS, thiobarbituric-reactive substances; TBA, thiobarbituric acid

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Our aim was therefore to study the role of Cu overload in the survival of human cells using an *in vitro* system. Since cultured cells are frequently used to investigate the effects of different noxae [40–42], we chose two human cell lines derived from liver (HepG2) and lung (A-549) as representative cells exposed to exogenous (polluted air) and/or endogenous (systemic overload) Cu in living organisms to study (i) the effect of different degrees of Cu overload in these two cell lines by determining various indexes of cellular proliferation and damage; (ii) the production of ROS as a possible causative factor of damage; and (iii) activation of the two main protease systems involved in programmed cell death (caspase-3 and calpains).

## 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. Monolayer cultures were grown in Eagle's minimum essential medium (MEM), with 15 mM HEPES, free of antibiotics, supplemented with 10% inactivated fetal calf serum (Natocor, Córdoba, Argentina) and microbiologically (pathogen-free) tested for cell culture.

### 2.3. Cell treatment

HepG2 and A-549 cell lines were seeded and grown to semi-confluence in disposable culture dishes (Falcon, CA, USA) in a humidified atmosphere with 5% CO<sub>2</sub> in air at 37 °C. When cultures reached the logarithmic phase of growth they were treated with fresh medium supplement with ultrafiltered (Millipore 0.22 µm, NY, USA) sterile PBS solutions of CuSO<sub>4</sub>, at different final concentrations (20–160 µM). Doses were on the basis of our assays demonstrating that concentrations around 80 µM Cu in the culture medium reduced viability by 50% with respect to control cultures. Other authors have used similar doses in HepG2 cells [43]. None of the treatments produced detectable changes in the pH of the culture medium. Control cultures were run simultaneously, supplemented with an equivalent aliquot of PBS. After 24-h treatment, some culture flasks were washed with cold sterile PBS (5 mL/three times), mechanically harvested and collected for centrifugation at 4 °C (10 min/2000 g). 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). The homogenates were used in the analysis of the markers listed below, except those for cell viability calculated with the values of total cellular protein (TCP), in which case the culture flasks were treated with Cu for 24 and 48 h and then processed as previously described. Three independent experiments were performed and each determination was repeated at least three times.

### 2.4. Copper concentration

Aliquots of washed cells were mineralized with a mixture of 4 mL of HNO<sub>3</sub> (c) and 1 mL HClO<sub>4</sub> (Aldrich or Sigma Chem. Co., Buenos Aires, Argentina) by heating at 120 °C for 60 min in a mineralization block [44]. The digests were cooled, diluted with ultrapure water (18 mΩ cm, Carlo Erba, Milan, Italy) and ultrafiltered using a 0.22 µm Millipore membrane (Milli-Q Purification System, Millipore, CA, USA). Ultrafiltered dissolutions were directly aspirated into the flame of a Perkin-Elmer 1100 B spectrophotometer equipped with a

Perkin-Elmer cathode lamp (Perkin-Elmer Corp., Norwalk, CT, USA) at a spectral width of 1 nm. Calibrations were performed with a standard solution of Cu(NO<sub>3</sub>)<sub>2</sub> in HNO<sub>3</sub> 0.5 N (Tritrisol, Merck Co., Darmstadt, Germany) and 18 Ω cm water ultrafiltered through a Millipore membrane. All measurements were performed in peak height mode (324.7 nm line). The intra-[(SD/ξ).100] and inter-[(ΔSD/Δξ).100] assay coefficients of variations were 15.5 and 6.0%, respectively. We routinely obtained a similar equation for the calibration curve ( $IR = 0.00055 + 0.04788 [Cu, \text{mg/L}]$ ) and the statistical analyses demonstrated a correlation coefficient always comprise between 0.956 and 0.991). In addition, we explored the so-called matrix effects that may modify the slopes of the standard regressions. In spiked samples the obtained values varying from 0.00041 to 0.00062 resulted very similar to those of copper standard solutions. So, matrix effect was considered not significant or negligible. The mean for recovery and RSD for spiked samples were 99.7% and 3.3%, respectively. Detection limit was 0.09 mg/L. Concerning accuracy of the method; we explored the influence of time after dilution, temperature of acid digestion, and concentration of HNO<sub>3</sub>/HClO<sub>4</sub> following the suggestions of Terrés-Martos et al. [45]. Also our results with biological samples (homogenates) were checked against a Seronorm™ Trace Elements Serum (Sero Labs, Billingstad, Norway) with no significant differences between the obtained and the declared (certified) concentrations. The concentrations of copper in test media were routinely checked by atomic absorption methodology and the results obtained were not significantly different to those of nominal concentrations. Based on previous experiments using similar copper concentrations, and on the results of measurements of total, free, and unbound copper performed in serum samples of rat and human patients made in our laboratory, the availability of copper in the test media seemed to be almost complete (the amount of bound copper to proteins was negligible in comparison to that used for the experiments) [23,28,29].

### 2.5. Copper homeostatic proteins

#### 2.5.1. Ceruloplasmin (CRP)

Samples were analyzed by conversion of p-phenylenediamine into a blue-colored product [46] measured at 550 nm at 37 °C in a buffer of glacial acetic/sodium acetate (50 mM, pH 5.5) directly into flat-bottomed plates, using a Microplate Reader SpectraMax M2/M2e model from Molecular Devices Analytical Technologies (Sunnyvale, CA, USA), for 3 min. Intra- and inter-assay coefficients of variation were 8.3 and 4.4%, respectively. CRP concentrations were calculated by comparison with the reaction rate of pure human ceruloplasmin standard (Sigma Chem. Co., Buenos Aires, Argentina).

#### 2.5.2. Metallothioneins (MTs) determination

Appropriate aliquots of samples (100 to 150 µL) were added to an excess of Ag<sup>+</sup> (500 µL/20 µg Ag<sup>+</sup>/mL) and the mixture was incubated with 100 µL of red blood cell hemolysate (2% in a buffer of Tris/HCl 30 mM pH: 8). The samples were then heated (2 min at 100 °C) and the denatured proteins discarded by centrifugation (5 min at 15,000×g). MTs were quantified in the supernatant fraction previously acidified with HNO<sub>3</sub> using an atomic absorption spectrometer Avanta Ultra Z (GBC Scientific Equipment, Hampshire, IL, USA). For the calculations it was assumed that the stoichiometry of Ag<sup>+</sup>-thioinein was 17 g-at. Ag<sup>+</sup> per mole of MTs [47,48]. This method was previously studied in comparison with other available methodologies such as thiomolybdate or enzyme-linked immunosorbent assays. With the Ag<sup>+</sup>-saturation assay, recovery of purified Cu-MTs was 100 ± 8% within the range 0.012 to 1.805 µg of MTs. At higher concentrations the method tends to underestimate the real amount of MTs present in the sample. Reproducibility between assays, as calculated by different mean coefficient of variations did not exceed 8% with a limit of detection of 0.012 µg of MTs. Matrix effect (rat cytosol) as

studied by the above mentioned group was negligible. Other analytical parameters were essentially identical to those described in the above mentioned papers [47,48].

## 2.6. Determination of proliferative indexes

For the last 10 h of culture, the cells were treated with colchicine (Sigma Chem. Co., Buenos Aires, Argentina) at a final concentration of 0.1  $\mu\text{g}/\text{mL}$ . For light microscope studies the cells were seeded ( $5.0 \times 10^4$  cells/10 mL medium) on sterile glass slides placed in the plastic culture dishes. After the treatments, the cells were immediately fixed with methanol:acetic acid (3:1), air dried and thereafter stained with 5% Giemsa in Sorensen buffer (pH 6.80). As a parameter of mitotic arresting activity, the mitotic index (MI) was determined by scoring 1000 cells/slide. Normal and abnormal cell division stages were evaluated from at least 200 mitotic cells/slides (three slides per experiment). MI was expressed as a factor ( $f$ ) of the mean MI from treated cells (MI<sub>t</sub>) over the mean from control assays (MI<sub>c</sub>) ( $f = \text{MI}_t / \text{MI}_c$ ) [49], or as a relative mitotic index (RMI) calculated as MI<sub>t</sub>/MI<sub>c</sub>, where MI<sub>t</sub> was obtained from treated cultures and MI<sub>c</sub> from control assays. Abnormal mitotic figures were classified as initial C-metaphases and full F-metaphases according to Hadnagy et al. [50] and the C/F ratios were calculated. A minimum of 200 metaphase cells per sample was assessed to determine the percentage of cells which had undergone one (M1), two (M2), and three or more ( $M \geq 3$ ) mitoses. The proliferative rate index (PRI) was calculated for each experimental point according to the formula  $\text{PRI} = [\%M1 + 2 \cdot (\%M2) + 3 \cdot (\%M \geq 3)] / 100$ .

Other proliferative indexes were determined using the changes in total cellular protein (TCP). Appropriate aliquots were taken for determination of the TCP using the methodology of Lowry et al. [51]. The following parameters were calculated using the TCP data: relative increase in cellular mass (RICM) = [increase in TCP in treated cells (final – initial) / increase in TCP in control cells (final/initial)] · 100, and relative populations doublings (RPDs) = [number of populations doubling in treated cultures / number of populations doubling in control cultures] · 100, where population doubling (PD) =  $\log [( \text{post-treatment TCP} / \text{initial TCP} )] / \log 2$  [52].

## 2.7. Assessment of cellular viability

### 2.7.1. Lactate dehydrogenase activity (LDH)

Medium samples were collected and centrifuged (10 min at 10,000 g) and then ultrafiltered through Millipore membranes (0.22  $\mu\text{m}$ ) in order to completely remove cell debris. Appropriate aliquots were taken for determination of 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 triplicate and expressed as mUI LDH/mL of culture medium for A-549 and HepG2. In another series of culture flasks, attached cells were washed with PBS and treated with 100  $\mu\text{L}$  of 0.1% solution of trypan blue dye (in PBS, pH 7.40). After 1 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. [53]. At least four fields of one hundred cells per field were counted and the results were expressed as the percentage of viable cells.

### 2.7.2. MTT assay

The mitochondrial-dependent reduction of colorless 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue-colored formazan was performed as previously described [54]. In brief, cultured cells were treated with  $\text{CuSO}_4$  as indicated and then incubated for 30 min in MTT solution (500  $\mu\text{g}/\text{mL}$  medium). After washing with PBS, the intracellular formazan was dissolved in dimethyl sulfoxide and the absorbance determined at 595 nm.

## 2.8. Biomarkers of ROS production

### 2.8.1. Fluorescent detection of ROS production

Formation of intracellular reactive oxygen species, specifically hydrogen peroxide, was measured spectrophotometrically using dichlorofluorescein-diacetate (DCF-DA), as described by Osseni et al. [55]. DCF-DA readily diffuses through the cell membrane and is hydrolyzed by intracellular esterases to nonfluorescent 2',7'-dichlorofluorescein. It is then rapidly oxidized to highly fluorescent 2',7'-DCFH-DA in the presence of reactive oxygen species. The fluorescence intensity is proportional to the amount of intracellular reactive oxygen species formed.

### 2.8.2. Thiobarbituric acid-reactive substances (TBARS)

The extent of lipid peroxidation in cellular homogenates was calculated by analyzing the levels of TBARS [56]. 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 tetrametoxipropene (TMP) solutions (TMP was purchased from Sigma Chem. Co., Buenos Aires, Argentina).

### 2.8.3. Total thiol content

Total thiol concentration was determined by the dithionitrobenzoate (DTNB) method [57]. An aliquot of sample containing approx. 100  $\mu\text{g}$  protein was mixed with sodium phosphate buffer 80 mM (pH 8.0), EDTA 2 mM and 250  $\mu\text{M}$  (final concentration) of DTNB. The mixture was incubated for 10 min at 30 °C and the optical density was recorded at 415 nm in a two-beam spectrophotometer (Cintra-20, Sydney, Australia). GSH was utilized as the calibration standard. Results were expressed as nanomoles of thiol groups/mg TCP.

## 2.9. Apoptosis biomarkers

### 2.9.1. Caspase-3 activity (commercial kit from Sigma Chem. Co, USA)

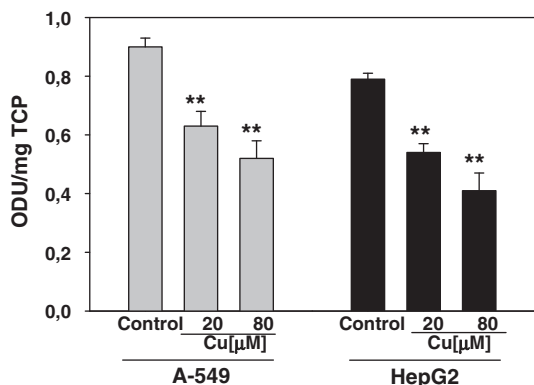
Caspase-3 activity was measured by a colorimetric assay kit (CASP-3-C), based on the hydrolysis of the synthetic peptide substrate acetyl-Asp-Glu-Val-Asp-p-nitroaniline (Ac-DEVD-pNA) by caspase-3. The resulting p-nitroaniline (p-NA) released was monitored at 405 nm. Three controls were used for each caspase-3 colorimetric assay: inhibitor-treated cell lysate (to measure the non-specific hydrolysis of the substrate), caspase-3 positive control (using commercial caspase-3, 5 mg/mL provided by the kit's manufacturer) and a blank of boiled (inactive) cellular lysate. A calibration curve using a standard solution of p-nitroaniline (p-NA) was run in parallel to calculate the activity of caspase-3 expressed as  $\mu\text{mol}$  of p-NA released/min mL of sample (activity =  $\text{OD} \times \text{dilution factor} / \epsilon$  (10.5 mM)  $\times$  time  $\times$  vol). All reagents used were provided with the commercial kit.

### 2.9.2. Milli (m) and micro ( $\mu$ ) calpains

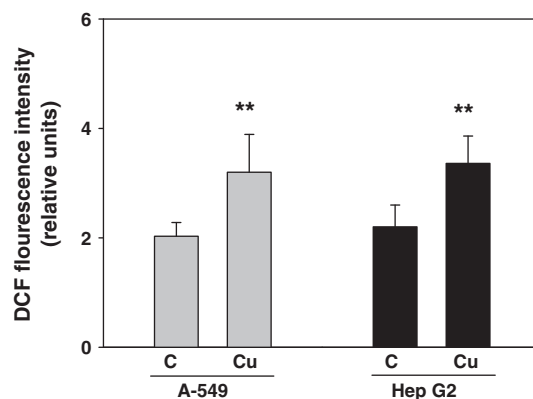
The assay involves the hydrolysis of whole ultra-pure casein (Sigma Chem. Co.) by calpain activity [58] and the subsequent detection of trichloroacetic acid (TCA)-soluble peptide fragments at 280 nm. The level of calcium in the medium was regulated (5 mM or 500  $\mu\text{M}$  of  $\text{CaCl}_2$  for m- or  $\mu$ -calpain, respectively) for the determination of calpain subtypes. Calculation was performed on the basis that a unit of calpain is the amount of enzyme that produces a change of absorbance of 0.01 at 280 nm and the results were expressed as units/min mg of TCP.

## 2.10. Statistical analysis

All values represent the mean of at least 3 independent experiments and each experimental point is expressed as mean  $\pm$



**Fig. 1.** MTT assay (formazan production) estimated by means of the optical density units (ODU) per milligram of total cellular proteins (TCP) in HepG2 (black bars) and A-549 (gray bars). Results are expressed as the mean of 3 independent experiments assayed in triplicate  $\pm$  standard deviation (SD). Significant differences compared to control assays are indicated with asterisks (\*\*,  $p \leq 0.01$ ).



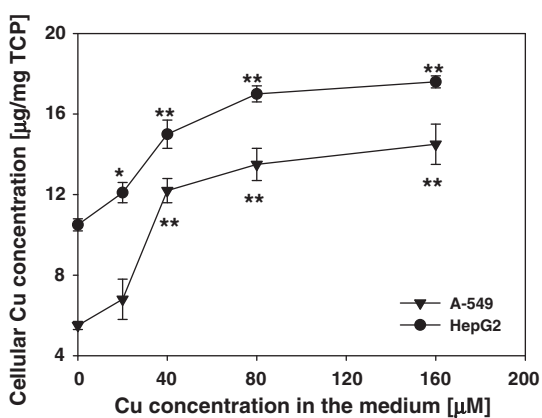
**Fig. 3.** ROS production assessment in HepG2 and A-549 treated with zero (control) or 20  $\mu\text{M}$  Cu concentration using DCF-DA (dichlorofluorescein-diacetate) test. Results are expressed as the mean of 3 independent experiments assayed in triplicate  $\pm$  standard deviation (SD). Significant differences compared to control assays are indicated with asterisks (\*\*,  $p \leq 0.01$ ).

standard deviation (SD). Data were analyzed by Student's *t*-test or ANOVA plus Tukey's test with the aid of Systat (version 15.0 for Windows) from SPSS Science (Chicago, IL). The 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 asterisks or letters, as appropriate. Levels of significance were tested at  $p \leq 0.05$  (significant) and  $p \leq 0.01$  (very significant).

### 3. Results

We clearly observed that exposure of A-549 and HepG2 to Cu overload significantly increased the concentration of this metal inside the cells in a dose-dependent manner (Fig. 2), with a concomitant increase in ROS production – more specifically in hydrogen peroxide (Fig. 3). This effect was also observed by indirect parameters of ROS production, such as increased lipid peroxidation measured as TBARS and a significant decrease in total thiol content (Table 1).

Since Kim et al. [59] have reported that the underlying mechanism of most chemicals that determine cytotoxic effects is impossible to be determined without a battery of assays, we used the trypan blue exclusion test and the LDH and MTT methods. A dose-dependent



**Fig. 2.** Copper concentration in HepG2 (black circles) and A-549 (triangles) cell lines ( $\mu\text{g}$  Cu/mg TCP) vs copper concentration in extracellular medium (20–160  $\mu\text{M}$ ). Results are expressed as the mean of 3 independent experiments assayed in triplicate  $\pm$  standard deviation (SD). Significant differences compared to control assays are indicated with asterisks (\*,  $p \leq 0.05$  and \*\*,  $p \leq 0.01$ ).

decrease in the production of formazan was observed (Fig. 1) in both the HepG2 and A-549 cells exposed to Cu overload. The increase in cell death was also confirmed by the trypan blue exclusion test (Fig. 4) and the elevated levels of LDH (Fig. 5). The observed decrease in cellular viability followed a similar pattern in both types of cells and the damage caused was aggravated by increments in Cu concentration.

To study the involvement of programmed cell death in the damage observed, we determined calpain ( $\mu$ - and *m*-) and caspase-3 activities. Figs. 6 and 7 show the increased activities of caspase-3 and the two calpain isoforms, respectively. The activity of caspase-3 increased very significantly at 80  $\mu\text{M}$  Cu concentration in both cells; however, at 80  $\mu\text{M}$  concentration this enzyme was more active in the liver- than in the lung-derived cells (Fig. 6). Calpains showed a very different behavior. Above 40  $\mu\text{M}$  Cu concentration a significant increase in both calcium-dependent calpain subtypes was observed. Concentrations as low as 20  $\mu\text{M}$  significantly increased calpain activity only in Hep G2 cells (Fig. 7).

We also observed significant decreases in the value of different proliferative parameters indicating a reduction in cell division rates and/or alterations in cell cycle progression as assessed by the mitotic index (MI), proliferative rate indexes (PRI), initial/full metaphases ratios (C/F), mitotic factors (*f*), relative increase in cellular mass (RICM) and relative population doubling (RPD) (Table 2).

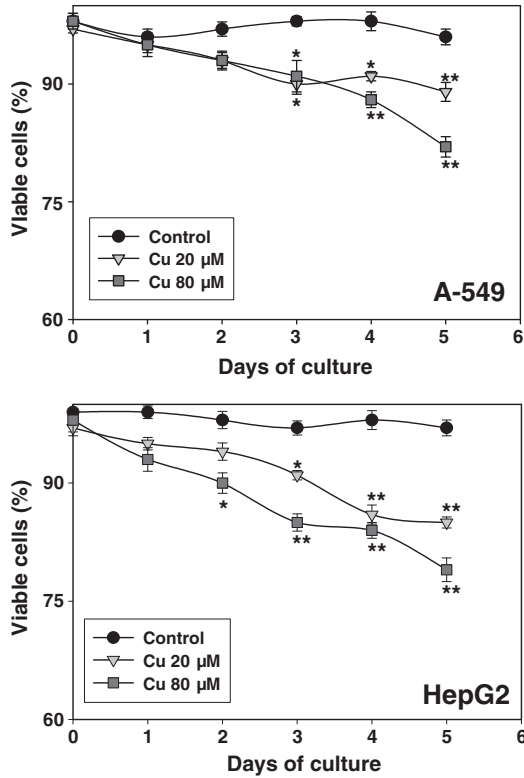
Considering the elevated levels of Cu inside the lung and liver cells, we also analyzed the concentration of two main proteins

**Table 1**

Thiobarbituric acid reactive substances and thiol content for control and Cu-treated HepG2 and A-549 cells in culture.

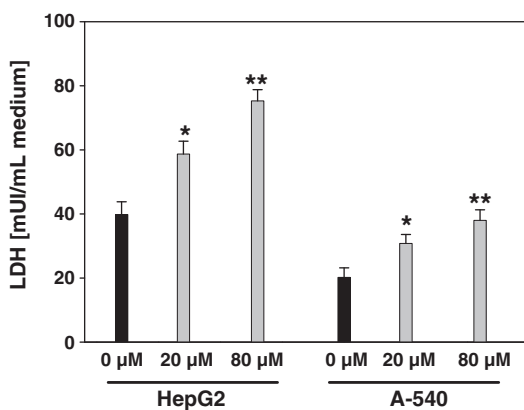
Culture	Treatments		
	Cu concentration [ $\mu\text{M}$ ]		
	0	20	80
<i>HepG2</i> cells			
TBARS	0.31 $\pm$ 0.05a	0.97 $\pm$ 0.1b	1.88 $\pm$ 0.11c
Total thiol	204 $\pm$ 15.1a	150.7 $\pm$ 12.8b	107.4 $\pm$ 4.5c
<i>A-549</i> cells			
TBARS	0.23 $\pm$ 0.03a	0.57 $\pm$ 0.1b	1.40 $\pm$ 0.2c
Total thiol	100.4 $\pm$ 6.6a	72.1 $\pm$ 4.5b	39.6 $\pm$ 3.7c

Results were expressed as the mean  $\pm$  standard deviation of three independent experiments (each experimental point assayed in triplicate). Samples were processed as indicated in the Materials and methods section. Significant differences between results were denoted with letters (results with different letters are statistically significant at the level  $p \leq 0.05$ ).

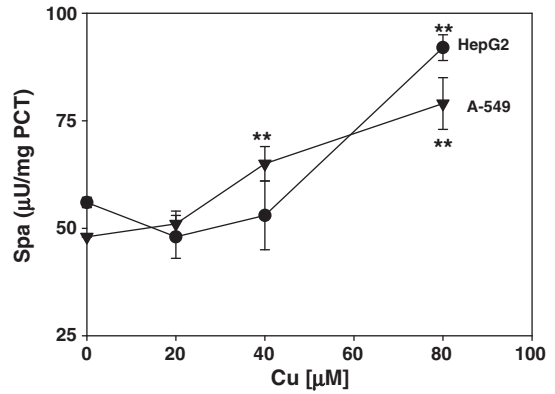


**Fig. 4.** Percentage of cellular viability in A-549 (upper panel) and HepG2 (lower panel) treated with zero (control), 20 or 80  $\mu\text{M}$  Cu concentration for 1 to 5 days. Results are expressed as the mean of 3 independent experiments assayed in triplicate  $\pm$  standard deviation (SD). Significant differences compared to control assays are indicated with asterisks (\*,  $p \leq 0.05$  and \*\*,  $p \leq 0.01$ ).

associated with Cu homeostasis, metallothioneins (MTs) and ceruloplasmin (CRP) (Fig. 8). An increase in CRP was observed; it did not correlate linearly with the levels of Cu ions, though the increase was significant at concentrations as low as 20  $\mu\text{M}$  and very significant at higher concentrations for both cell lines (Fig. 8-A). In the case of MTs levels there was an almost linear correlation with the amount of endocellular Cu in both cell lines, mainly in A-549 cells (Fig. 8-B). The increase was very significant at 80  $\mu\text{M}$ ,



**Fig. 5.** Lactate-dehydrogenase (LDH) leakage measured in ultrafiltered culture medium from HepG2 or A-549 human culture cells treated with Cu (gray bars), compared to control flasks treated only with PBS (black bars). LDH activity results are expressed as milliunits per mL of medium (mUI/mL) and are the mean of 3 independent experiments (each point assayed in triplicate)  $\pm$  standard deviation (SD). Significant differences compared to control assays are indicated with asterisks (\*,  $p \leq 0.05$  and \*\*,  $p \leq 0.01$ ).

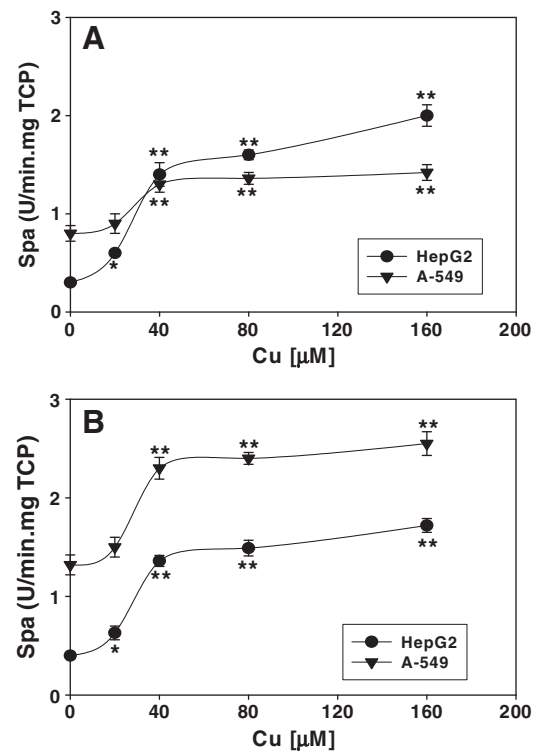


**Fig. 6.** Caspase-3 activity ( $\mu\text{U}/\text{mg}$  TCP) in HepG2 (black circles) and A-549 (triangles) cell lines treated with zero (control), or 20 to 160  $\mu\text{M}$  Cu concentration. Results are expressed as mean of 3 independent experiments assayed in triplicate  $\pm$  standard deviation (SD). Significant differences compared to control assays are indicated with asterisks (\*,  $p \leq 0.05$  and \*\*,  $p \leq 0.01$ ).

whereas below this value the level of significance depended on the cell type.

#### 4. Discussion

Our results demonstrated that exposure of lung (A-549) and liver (HepG2) human-derived cells to concentrations of Cu ions below 80  $\mu\text{M}$  produced a concomitant increase in the intracellular concentration of this metal. At higher levels, Cu input reaches a plateau probably because the ions have a specific homeostatic system that regulates metabolism of the metal in all eukaryotic cells [60,61]. Cu uptake also provokes an increase in hydrogen peroxide production,



**Fig. 7.** Mili (m-; upper panel) and micro ( $\mu$ -; lower panel) calpain activities in HepG2 (black circles) and A-549 (triangles) cell lines treated with zero (control), or 20 to 160  $\mu\text{M}$  Cu concentrations. Results (U/min mg TCP) are expressed as the mean of 3 independent experiments assayed in triplicate  $\pm$  standard deviation (SD). Significant differences compared to control assays are indicated with asterisks (\*,  $p \leq 0.05$  and \*\*,  $p \leq 0.01$ ).

**Table 2**  
Proliferative indexes for control and treated HepG2 and A-549 cell cultures after 24 h treatment with Cu.

Cell culture	Treatments		
	Cu concentration [ $\mu\text{M}$ ]		
	0	20	80
<i>Hep G2 cells</i>			
MI (o/oo)	37.1 $\pm$ 3.0a	27.0 $\pm$ 3.2b	11.2 $\pm$ 2.8c
PRI	1.99 $\pm$ 0.01a	1.71 $\pm$ 0.11b	1.58 $\pm$ 0.04c
C/F	1.25 $\pm$ 0.06a	0.58 $\pm$ 0.11b	0.34 $\pm$ 0.03c
f	–	0.72 $\pm$ 0.03a	0.29 $\pm$ 0.02b
RICM (o/o)	–	74.3 $\pm$ 3.5a	62.5 $\pm$ 2.4b
RPDs (o/o)	–	72.0 $\pm$ 4.1a	43.2 $\pm$ 2.8b
<i>A-549 cells</i>			
MI (o/oo)	39.2 $\pm$ 4.2a	32.0 $\pm$ 4.1a	17.0 $\pm$ 3.6b
PRI	2.01 $\pm$ 0.03a	1.73 $\pm$ 0.10b	1.65 $\pm$ 0.05c
C/F	1.75 $\pm$ 0.08a	0.55 $\pm$ 0.04b	0.39 $\pm$ 0.02c
f	–	0.82 $\pm$ 0.04a	0.44 $\pm$ 0.03b
RICM (o/o)	–	76.8 $\pm$ 4.0a	65.5 $\pm$ 4.5b
RPDs (o/o)	–	57.9 $\pm$ 3.7a	40.9 $\pm$ 3.1b

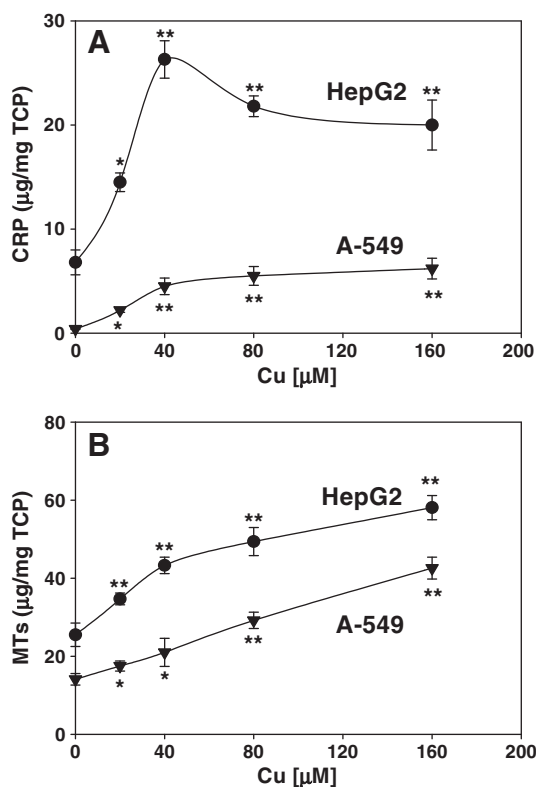
Results were expressed as the mean  $\pm$  standard deviation of three independent experiments (each experimental point assayed in triplicate). Mitotic indexes (MI), proliferative rate indexes (PRI), initial/full metaphases (C/F) ratios, index mitotic factor (f), relative increase in cellular mass (RICM), and relative population doubling (RPD) were calculated as described in the Materials and methods section. Significant differences between results were denoted with letters (results with different letters are statistically significant at the level  $p \leq 0.05$ ).

a reduction in the total thiol content and a significant rise in TBARS production. These findings are in agreement with previous results by Pourahmad and O'Brien [62]. Moreover, we observed a response of the Cu homeostatic system elicited by increased levels of the two main proteins involved in Cu buffering, CRP and MTs. CRP belongs to the  $\alpha_2$ -globulin fraction of human plasma and is considered to be

an acute or sub-acute phase reactant. The increase in CRP levels in both cell lines can be attributed to the oxidative stress condition produced by exposure to Cu, which triggers the synthesis of pro-inflammatory cytokines responsible for the activation of the gene that expresses CRP [63,64]. The dose-dependent increase in MTs observed in our work in response to Cu overload is also in agreement with the results of a previous paper [65]. In addition, other researchers have reported increased MTs in liver and lung cells following administration of ROS-producing agents [66–68]. HepG2 basal levels of MTs are above those found for A-549, probably because the liver is the main organ that synthesizes this family of proteins. Like CRP, the synthesis of MTs is induced by pro-inflammatory cytokines [68] and by transcription factors in response to metals (MRF-1), especially Cu [1].

The increased oxidative stress condition in response to Cu overload, a well-known causative factor of ROS-dependent damage of important biomolecules, is likely involved in the activation of the programmed cell death pathway (apoptosis). This may explain the progressive loss of viability in both cell types. It is widely known that calpains are activated in response to proapoptotic stimuli such as increased reactive oxygenated species [69]. Furthermore, caspase-3 is known to be the main effector protein common to the intrinsic and extrinsic pathways of apoptosis and that ROS overproduction is effectively involved in both pathways, indicating that both cell lines enter into apoptosis under Cu overload. However, calpains are also related to cell death by necrosis. This issue remains unresolved since there are studies demonstrating that both calpains are activated in the apoptotic and the necrotic pathways [70–72]. Though we observed a significant increase in calpain activity for HepG2 after exposure to 20  $\mu\text{M}$  of Cu, with even higher activity at higher concentrations of this metal, the increase in caspase-3 became significant only at the highest concentration of Cu assayed. This finding suggests that liver cells preferentially activate calpains, which are then relieved at higher overload stages by the activation of caspase-3. This explanation agrees with that of Nawaz et al. [73], who reported the calcium-dependent activation of  $\mu$ - and m-calpains in primary cultures of hepatocytes. Furthermore, van Raam et al. [71] demonstrated that the inactivation of calpains in granulocytes prevents the activation of caspase-3 by stabilizing XIAP, a target of calpains, and PKC- $\delta$ . Choi et al. [74] obtained similar results in stable pancreatic cells (MIN6N8a) and Ding et al. [75] demonstrated that calpains are the main proteases involved in apoptosis induced by microcystin-LR in hepatocytes.

As stated before, the matter of which protease system is mainly responsible for the activation of programmed cell death is still controversial, though the results reported here appear to indicate specificity for each type of cell. In recent years it has become increasingly clear that multiple mechanisms of cell death – as well as crosstalk between different pathways – contribute to determining cell survival or death by necrosis or apoptosis. The striking similarity between the substrates for caspases and calpains raises the possibility that both protease families contribute to structural dysregulation and functional loss of cells under oxidative stress conditions [76]. Moreover, caspases have been reported to up-regulate calpain activities through modification of calpastatin (an endogenous calpain inhibitor) by proteolytic cleavage [77]. On the contrary, several studies suggest that calpains could cleave and inactivate endogenous caspases such as caspase-3, -7, -8, and -9 [78]. Although there is multiple cross-talk between caspases and calpains, the exact signaling pathway linking the two protease families remains to be elucidated [79–83]. Our results suggest that activation of calpains prevents caspase activation, or vice-versa, depending on the level of Cu overload. The predominant activation of calpain proteases instead of caspases at higher Cu concentrations was reported by other authors in models of chronic injury [77,78]. Our own results strongly suggest a direct dependence of both proteolytic systems on the degree of Cu overload. Cu-induced oxidative



**Fig. 8.** Concentration of Cu homeostatic proteins, ceruloplasmin (CRP; panel A) and metallothioneins (MTs; panel B) ( $\mu\text{g/mg TCP}$ ) treated with zero (control), or 20 to 160  $\mu\text{M}$  Cu concentration. Results are expressed as mean of 3 independent experiments assayed in triplicate  $\pm$  standard deviation (SD). Significant differences compared to control assays are indicated with asterisks ( $p < 0.01$ ).

stress may also contribute to the apoptotic machinery in other ways. For example, ATP levels [84] and redox cellular status [85] appear to determine the specific pathway by which cells will die. It was further-observed that activation of caspase-3 is dependent on the maintenance of a thiol/redox status [85]. Experimental evidence also indicates that both types of calpains can be activated under the oxidative stress condition induced by Cu overload and that they could play a functional role in apoptotic death, as suggested in other biological systems [80,81,86]. Unlike liver cells, in A-549 the activities of caspase-3 and calpains increase in parallel with Cu concentration. Thus, we cannot assume – at least in the case of this cell line – that the involvement of these two protease systems is equivalent. Another indication of death by apoptosis could be the increased levels of LDH activity in the culture media of HepG2 and A-549 after Cu treatment. Although the extracellular increase in LDH activity has traditionally been considered as an indicator of death by necrosis, there are studies demonstrating an increase in LDH release in cells undergoing programmed cell death [58,87]. This marker should therefore be taken with caution, as indirect evidence.

In addition to the activation of programmed cell death after Cu exposure, we observed significant changes in many proliferative indexes in both cell lines. These findings may reflect a decrease in sister chromatid exchange and an increase in the doubling-time of cell division as a consequence of which Cu treatment produces significant decreases in the RCM and RPD. In agreement with these findings, other authors have demonstrated that exposure to Cu in a cell line derived from hamster ovary produced a delay in the progress of the S phase [88]. Also, Aston et al. [43] showed that HepG2 cells exposed to 64  $\mu$ M Cu lost their replicative capacity and underwent a significant decrease in cell viability.

Concerning the significance of the tested concentrations of copper and potential exposure cases, there are scarce data for humans because most of the available evidence (experimental or epidemiological) were obtained from animal models. However, the regulatory frameworks for copper chronic exposures in large human populations indicate that food, drinking water and copper-containing supplements are the main sources of human exposure [30]. Dietary reference intake for people from USA, United Kingdom, Europe and Australia varies from 0.16 to 0.98 EAR (Estimated Average Requirements)/RDA (Recommended Dietary Allowance) expressed in mg Cu/kg body weight with a great variation as a function of the age. PRI (Population Reference Intakes) were reported between 0.3 and 1.5 mg Cu/kg body weights [30,89]; however, these limits were largely surpassed in many circumstances such as ingestion of fish, bivalves, or contaminated drinking water [90]. In addition, copper ingestion and absorption is strongly influenced by many foods. For example, a negative correlation between copper levels in meals with DNA damage in a study with orange juices was demonstrated [91]. Also, other trace elements modify significantly the bioavailability of copper (especially zinc) [92]. The usual concentration of copper in human plasma (as determined by us and other groups) is between the range 0.3 to 2.1 mg/L for intakes of 1.4 to 2.0 mg copper/day [90]. Considering the available data, we can say that our experimental conditions resemble those Cu levels commonly found as a consequence of involuntary exposure through air, food and water pollution [88], in professionals engaged in agrochemical activities [22,28], or female users of Cu-based intrauterine devices [29,90,93].

## 5. Conclusion

We concluded that the effect of Cu exposure on human cell survival depends not only on the degree of overload but also on the cell type. The production of ROS appears to be involved in a differential response of the two main protease systems – caspase-3 and calpains – for programmed cell death. Furthermore, the behavioral response of Cu-homeostatic proteins to the same degree of Cu exposure is

different. These findings are the starting point for more in-depth studies aimed at elucidating the diverse effects of Cu overload in the pathogenesis of a variety of human diseases like cancer, cirrhosis, atherosclerosis and neurodegenerative diseases [90].

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