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Copper Enhances Antitumor Activity of Water Soluble Garlic Extract Components on a Human Hepatoma Cell Line

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Abstract: Water garlic extract combined with Copper (GE-Cu) shows specific anti-proliferative and antitumoral activity in human hepatocarcinoma (HepG2) cancer cell line, while it is not active on differentiated or primary isolated cells, normal human dermal fibroblasts (HDF) and human epidermal keratinocytes (HEK). Since other transition metals do not affect cell viability, the observed antineoplastic property, was found specific for copper (Cu), and atomic absorption analyses demonstrate an accumulation of this metal inside the cell nuclei. GE-Cu treatment of HepG2a cancer cells, induces cell differentiation, growth arrest and programmed cell death. Furthermore, GE-Cu produces a continuous and prolonged flux of oxidative radicals, which down-regulates nuclear antioxidant defenses and repair systems, thus generating direct double-strand breaks to DNA. Indeed, it was observed that in GE-Cu-treated HepG2 cancer cells, histone deacetylase (HDAC) is inhibited, and histone H2AX is early phospho-activated. DNA double strand break downstream, likely responsible for the observed cell death in HepG2 cell, was evidenced by an early over expression of p53 and p21. JNK/c-Jun and p-38 phosphorylative cascade are activated as response to oxidative stress also. The resulting apoptosis appears to be caspases-independent because, under our experimental conditions, nuclear translocation of the apoptosis inducing factor (AIF) is operative. Our data suggest that GE-Cu possess an active specific antitumor capability, which could provide valuable new tools in cancer prevention and in supporting traditional chemotherapy.

Keywords: Garlic, Copper, p-Jun, HDAC, Apoptosis, HepG2

1. Introduction

In a previous paper [1] we reported that water-soluble garlic extracts (GE), supplemented with copper (Cu), exerts an enhanced anti-proliferative and a caspase-independent apoptotic activity in a human hepatocarcinoma HepG2 cancer cell line. The bioavailability of garlic compounds depends on several factors, first of all the method of processing the bulbs to obtain the garlic extract. The oil-soluble organosulfur compounds, including allicin, ajoene, and DADS are not found in the urine or blood after garlic or garlic oil consumption. Likely, they are not considered as active phytocompounds *per se*, [2] and reasonably cannot reach target organs directly *via* circulation. Water-soluble garlic preparations contain stable and odorless phytocompounds and do not show toxic effects in the liver [3]. The anticarcinogenic and antioxidant activity of water garlic extracts may be related to the elevated content in organosulfur and selenium compounds [4, 5]. However, the specific phytocompounds present in GE, which are involved

in molecular and cellular events underlying specific anticancer properties are unknown as yet [6, 7]. Phytochemicals have generated interest because of their pharmacological effects such as vasoprotective and anti-inflammatory actions [8-10]. Many of these effects are related to their antioxidant properties and to the synergistic effects with other phytochemicals [11]. Interestingly phytochemicals can interact with metal ions, especially Cu, leading to formation of chelate Cu-phytochemical compounds, acting in promotion of free-radical reactions, tumor cell cytotoxicity and neuroprotective actions [12-17]. Several recent studies focused on the synergistic activity of Cu and sulfur compound present in garlic, where the phytochemical complexes formed, show enhancement of the fungicidal activity. [18, 19].

Cu is a trace element essential for different physiological functions in growth and development and is required for the catalysis of several cuproenzymes [20], but Cu becomes toxic when not properly bound and at high concentration. Under these conditions its redox reactivity may lead to cell damage through oxidative modification of proteins, lipids and nucleic acids, adversely affecting their structure and function (21).

In this work, a patented water based garlic extract combined with Cu (GE-Cu) was used. This GE-Cu formulation has shown induction of apoptotic death in HepG2 hepatoma cells, evidencing specific antitumor activity in an *in vitro* experimental system, while it does not affect the viability of HDF normal human fibroblasts or primary isolated HEK. These findings give support to the use of ethno pharmacological solution of GE-Cu in anticancer treatment, in preventing tumorigenesis or in combination with the current chemotherapies.

2. Experimental Protocol

2.1. Cell Cultures

Human hepatocarcinoma HepG2 cells were purchased from the European Collection of Cell Cultures (Promochem, UK), grown in glutamine-containing RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 1% sodium pyruvate, 1% non-essential amino acids, 10% fetal calf serum (FCS), and incubated at 37°C in an atmosphere of 5% CO_2 in air. Cells were routinely trypsinized, and plated at a density of 4×10^4 cells/cm^2.

Normal human keratinocytes (HEK) are plated on a murine fibroblast (3T3-J2) plated "feeder layer" in the flask of 75 cm^2. The 3T3-J2 cells were lethally irradiated (6000 RAD). The first change of medium was performed on the third day after plating. Before reaching the confluence, the soil should be changed every two days. The cells are supplied already plated from IDI-Pharmaceutical (Italy). Thanks to the presence of the "feeder layer", keratinocytes do not undergo differentiation and mimic very well the behavior of the skin.

3T3-J2 cells were cultured in Dulbecco's-Vogt Eagle's medium containing FCS (10%), glutamine (4 mM), and

penicillin-streptomycin (50 IU-50 µg/mL). NHK were cultured as previously described [22]. Briefly, skin biopsy samples were minced and trypsinized (0.05% trypsin/0.01% EDTA) at 37°C for 3 h. Cells were collected every 30 min, plated $(2.5\times10^4/cm^2)$ on lethally irradiated 3T3-J2 cells $(2.4\times10^4/cm^2)$, and cultured in 5% CO_2 and humidified atmosphere in keratinocytes growth medium: DMEM and Ham's F12 media (2:1 mixture) containing 10% FCS, insulin (5 µg/mL), adenine (0.18 mmol/L), hydrocortisone (0.4 µg/mL), cholera toxin (0.1 nmol/L), triiodothyronine (2 nmol/L), epidermal growth factor (10 ng/mL), glutamine (4 mmol/L), and penicillin-streptomycin (50 IU-50 µg/mL). Subconfluent primary cultures plated at a cell density of $5\times10^3/cm^2$ were serially propagated. Efficiency of colony formation (CFE) for keratinocytes was determined by plating 100-1000 cells, fixing colonies with 3.7% formaldehyde 9-14 days later, and staining them with 1% rhodamine B [22].

Human Dermal Fibroblasts (PH10605A-HDF), derived from the dermis of normal human neonatal foreskin or adult skin, were purchased from Genlantis (San Diego, CA. USA). HDF were plated at a density of $4\times10^4/cm^2$ and maintained in humidified 5% CO_2 in air [23]. Cultures were fed twice weekly with Fibroblast Growth Medium, PM116500 (Genlantis). Cells were collected by centrifugation and washed extensively with cold phosphate-buffered saline (PBS; 10 mmol/L phosphate buffer, 2.7 mmol/L KCl, and 137 mmol/L NaCl, pH 74). The cells were trypsinized routinely.

2.2. Water Garlic Extracts

Fresh garlic (*Allium sativum)* bulbs, collected at the end of July, were kindly provided by local garlic producers. Further steps on preparation of water garlic extraction are described in patent n° RM2008A00008 (registered 2008-14-02). Briefly, 5 g of the powdered garlic sample was extracted with 100 mL of ultra-filtered water at 80°C for 20 min in a water bath shaker (Shaking Bath 5B-16-Techne, Ltd., UK). After cooling, the extract was centrifuged at 5,000 x*g* for 10 min and filtered by a Millipore filter with a 0.2 µm cellulose membrane, under vacuum at 23°C. The filtrate was stored at 4°C until use within 24 hr.

2.3. Cell Treatments

Water garlic extracts were maintained at room temperature just before use and added to the cell medium at concentrations of 0.1%, 0.5% and 1% (v/v) together with a water solutions of $CuSO_4$ (final concentration on the cell medium ranging from 50 µM to 1mM). It was chosen the optimal concentration of GE-Cu (0.1% GE plus 100 µM Cu), proved sufficiently cytostatic and less toxic effect to the cells (Figure 1).

Cu (II) sulfate anhydrous ($CuSO_4$), ferrous sulfate ($FeSO_4$ *x* $7H_2O$), zinc sulfate ($ZnSO_4$ x $7H_2O$) (MERCK, Darmstadt, Germany) were dissolved in water, sterilized by filtration on a 0.2 µm cellulose membrane just before use, and utilized at different concentrations.

N-Ethylmaleimide (NEM), was from Sigma. Retinoic acid (20 µmol/L) was used to induce differentiation of HepG2 [24]; before treatments, HepG2 cells were pre-incubated with retinoic acid every two days over a period of two weeks.

2.4. Evaluation of Oxidative Damage

Detection of intracellular reactive oxygen species (ROS) was performed by incubation of the cells with 50 µM 2'-7'-dihydrodichlorofluorescein diacetate (DCF-DA) (Molecular Probes, Eugene, USA) dissolved in dimethyl sulfoxide for 30 min at 37°C prior the treatments [25]. Treatment with 100 µM *tert*-butyl hydroperoxide was used as a positive control.

Carbonylated proteins were detected using the Oxyblot Kit (Intergen, NY, USA). Briefly, 20 µg of proteins were reacted with 2,4-dinitrophenylhydrazine (DNP) for 15 min at 25°C. Samples were resolved on 12% SDS-polyacrylamide gels and DNP-derivatized proteins were identified by immunoblotting using an anti-DNP antibody.

2.5. Analysis of Cell Viability and Apoptosis

Cell viability was evaluated in a hemocytometer chamber under a phase contrast optical microscope, by their capacity to exclude Trypan blue (0.2%). Adherent apoptotic cells were detected with a fluorescence microscope directly on chamber slides, by analyzing the nuclear fragmentation after staining with Hoechst 33342 (Calbiochem-Novabiochem) dye as previously described [26]. Early apoptosis was also detected with a FACScalibur instrument [Becton Dickinson, San Josè, CA) by analyzing phosphatidylserine externalization after staining with the impermeant dye Annexin V-FITC (Bender MedSystems Vienna, Austria). Alternatively, adherent (after trypsinization) and detached cells were combined, washed in PBS and stained with 50 µg/mL propidium iodide for the cytofluorimetrical evaluation of late apoptotic cells. The percentages of apoptotic cells were determined according to Nicoletti et al. [27] by calculating the peak area of hypo diploid nuclei. Data from 10,000 cells were collected for each data file. Cell cycle analyses were performed with WinMDI 2.8 software.

2.6. Preparation of Cell Lysates, Subcellular Fractionation and Western Blot Analyses

Cell pellet was re-suspended in a lysis buffer containing 10 mmol/L Tris-HCl, pH 7.4, 5 mmol/L EDTA, 150 mmol/L NaCl, 0.5% IGEPAL CA-630, and protease inhibitors (Sigma). After 30 min incubation in a ice-bath, cells were disrupted by 10 s sonication. Lysates were then centrifuged at $14,000 \times g$ for 15 min at 4°C and supernatants were removed and stored at –80°C. 20 µg (for p53 and p21) or 50 µg (for the phospho isoforms of JNK and c-Jun) of proteins were loaded on 10% polyacrylamide gel and transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA).

The fractionation of HepG2 cells was obtained by cell lysis in NP-40 lysis buffer (NLB), (0.5% Nonidet P-40, 0.5 mol/L sucrose, 15 mmol/L Tris, pH 7.5, 60 mmol/L KCl, 0.25 mmol/L EDTA, 0.125 mmol/L EGTA, 0.5 mmol/L spermine, 0.125 mmol/L spermidine, 50 mmol/L NaF, 1 mmol/L Na_3VO_4 (sodium *o*-vanadate), 1 mmol/L dithiothreitol at 4°C (28). Briefly, cells pellet were re-suspended in NLB, incubated on ice for 30 min, and then centrifuged at 450 *x g* for 10 min at 4°C. The supernatant represents the cytosolic fraction and the pellet (nucleus fraction) was washed with NLB and centrifuged at 600 *x g* for 5 min. After centrifugation the nuclei were re-suspended in lysis buffer containing 50 mmol/L Tris/HCl, pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, protease inhibitor cocktail, 40 mmol/L NaF, 1 mmol/L Na_3VO_4.

Polyclonal anti-c-Jun (1-200), anti-JNK (1:200), anti-p21 (1:5000) or monoclonal anti-phospho-activated c-Jun and JNK isoforms (1:200), polyclonal anti Nrf2 (1:500); polyclonal anti-AIF (1:2500) (Chemicon International, Billerica, MA, USA); monoclonal anti-H2A.X (1:1000) (Upstate Cell Signaling Solutions, Billerica, MA, USA); monoclonal anti-Hsp90 (1.10000) (BD Transduction Laboratories, San Jose, CA, USA); monoclonal anti-catalase (1:2500), monoclonal anti-p53 (1:5000), monoclonal anti-Lamins A/C (1:100) (UCS Diagnostic, Italy); were used as primary antibodies. The specific protein complex formed upon appropriate secondary antibody (Bio-Rad) treatment (1:10000) was identified using a Fluorochem Imaging system (Alpha Innotech – Analitica De Mori, Milano, Italy) after incubation with ChemiGlow chemiluminescence substrate (Alpha Innotech). Proteins were determined according to the method of Lowry et al. [29].

2.7. Cu Determinations and ESR Spectra

Cytosolic and nuclear fractions were diluted 1:2 with 65% HNO_3. After at least 1 week at room temperature, Cu concentration was measured by atomic absorption spectrometry using an AAnalyst 300 Perkin-Elmer instrument, equipped with a graphite furnace with platform (HGA-800) and an AS-72 auto sampler.

ESR spectral were recorded with a Bruker ESP 300 spectrometer.

2.8. HDAC Assay

Boc-Lys (Ac) (MAL, N-(4-methyl-7-coumarinyl)-N-α-(*tert*-butyloxy-carbonyl)-N-Ω-acetyllysinamide (AMC) from Alexis, NY, USA, was used as substrate for a microplate HDAC assay. Cellular extracts of 2×10^5 cells re-suspended in 200 µl PBS buffer, were obtained by sonication (three times for 3s on ice with 30s breaks between sonication steps). HDAC assay was performed taking aliquots corresponding to 20 µg of proteins. The HDAC reaction was started adding 5 µl of substrate solution (500 µM final concentration) to 45µl of sample solution in buffer (50 mmol/L Tris-HCl, pH 8.0, 137 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L $MgCl_2$) followed by 60 min of incubation at 37°C. The reaction was stopped by addition of 50 µl stop solution (10 mg/mL trypsin from porcine pancreas, Sigma, in 100 mM ammonium bicarbonate, pH 8.0, buffer and 2 µmol/L tricostain A. After 20 min incubation at 30°C, the release of AMC was

monitored by measuring the fluorescence at 460 nm (λ_{ex} = 360 nm). The AMC signals were recorded against a blank with buffer and substrate but without the enzyme. All experiments were carried out in triplicate.
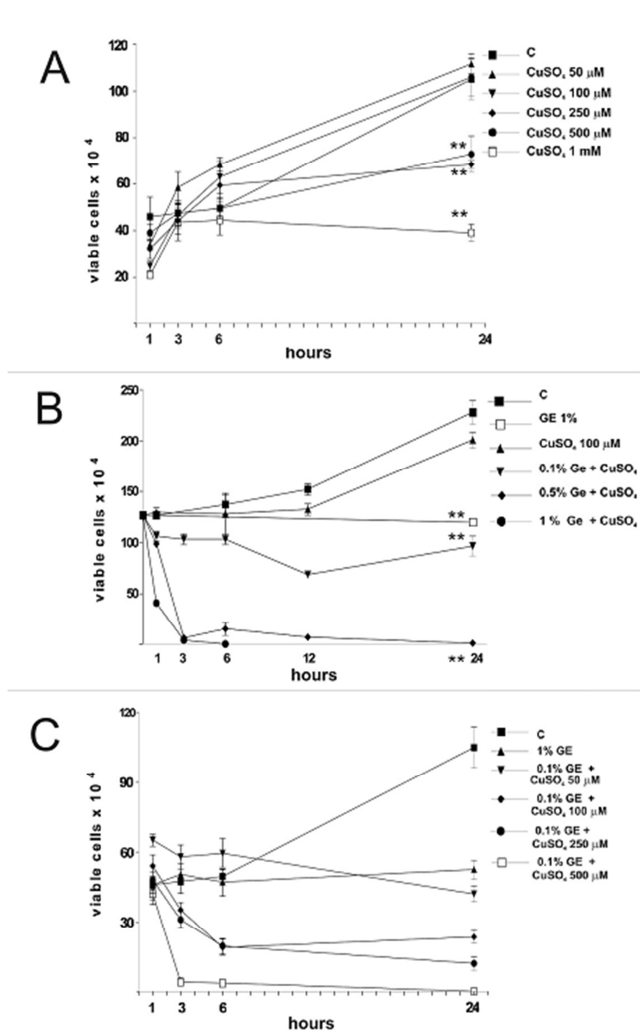
Figure 1. *Effects of GE-Cu treatments on HepG2 hepatoma cells growth. HepG2 cells were incubated with Cu at different concentrations (A); with 0.1, 0.5 and 1% GE (B) and with different composition of GE-Cu (C). Viable cells were evaluated by direct counts after Trypan blue staining under a phase contrast optical microscope. Data are expressed as means ± S.D. **p<0.001 (n = 6).*

2.9. Fluorescence Microscopy Analyses

Cells were plated on chamber slides at $6 \times 10^4/cm^2$. To evaluate mitochondrial integrity, cells were stained with 50 nmol/L of the mitochondrial transmembrane potential-sensitive probe MitoTracker Red (Invitrogen-Molecular Probes), washed, then fixed with 4% paraformaldehyde and permeabilized. Afterwards, they were washed exhaustively with PBS, blocked with PBS containing 10% FCS, and incubated with Monoclonal anti-Ser139-phosphorylated histone H2AX antibody, and further probed with an Alexa Fluor-488 goat anti-mouse secondary antibody (1:1000) (Invitrogen-Molecular Probes). To determine the shape of

nuclei, cells were also incubated with Hoechst 33342 (1:1000), and visualized by fluorescence microscopy. Images of cells were digitized with a Delta Vision Restoration Microscopy System (Applied Precision Inc., Issaquah, WA) equipped with an Olympus IX70 fluorescence microscope. All images were captured under constant exposure time, gain, and offset and subjected to a de-convolution algorithm to remove signal originating beyond each focal plane.

2.10. Statistic

All experiments were carried out at least 4 times (n = 4) unless otherwise indicated. The results are presented as means ± S.D. Statistical evaluation was conducted using ANOVA, followed by the post hoc Student-Newman-Keuls. Differences were considered to be significant at p < 0.05.

3. Results

3.1. GE-Cu Inhibits Proliferation and Induces Cell Death in HepG2 Hepatoma Cells

In this study we focused on the effects of GE supplemented with copper (GE-Cu) on the viability of HepG2 hepatoma cells. Cells were treated for different times with $CuSO_4$ ranging from 50 µmol up to 1 mmol, harvested and counted by Trypan Blue exclusion. Fig. 1A shows that 50 and 100 µmol $CuSO_4$ did not affect cell viability. By contrast, higher concentrations induced a dose-dependent decreased in the total number of viable cells. We used different volumes of GE and different concentrations of Cu to select optimum experimental conditions. Fig. 1B shows that 1% GE was cytostatic per se, in agreement with our previous reported data (1). However, the addition of 100 µmol $CuSO_4$ to increasing concentrations of GE (from 0.1 to 1%) evidenced a time and dose-dependent decrease of cell viability. We performed a second set of experiment by keeping constant GE concentrations (0.1% v/v) and increasing $CuSO_4$amounts. Results depicted in Fig. 1C show that, except for 50 µmol, GE-Cu solutions containing 100-250-500 µmol/L $CuSO_4$ yielded a significant decrement of viable cells, also in this case, in a time and dose-dependent manner. Altogether, these results pointed out that, although the highest dose of GE did not show cytotoxicity, the addition of $CuSO_4$ at doses higher than 100 µmol determined a dramatic decrease of cell viability. Along this line, Data show that a direct relationship between decrease of viable cells and increase of dead ones occurred upon treatment with 0.1% GE along with 100 µmol/L $CuSO_4$. This was also the condition which allowed performing experiments at longer times (24 and 48 h).

### 3.2. GE and Cu Treatments Do Not Affect the Viability of Normal and Differentiated Cells

Anti-tumor therapies should not compromise cell viability of primary or differentiated cells or, at least, their efficacy should be much selective towards cancer cells. In this context, we induced differentiation of HepG2 cells by 15

days treatment with 20 µmol retinoic acid. Then, according to our preliminary experiments, we treated HepG2 cells with increasing amount of GE, ranging from 0.1 to 1% v/v, supplemented with 100 µmol/L CuSO$_4$. At different times of treatment, cells were stained with propidium iodide and analyzed by cytofluorimetric analyses for the extent of apoptosis. Differentiated HepG2 were completely resistant to Ge-Cu-mediated apoptosis, even at the highest concentrations used, suggesting that GE and Cu treatments do not affect cell viability of differentiated HepG2 cells (data not shown). Moreover, experiments performed with 1% GE supplemented with 100 µmol/L CuSO$_4$ on FDH and NHK cells evidenced no sign of toxicity (Fig. 2).
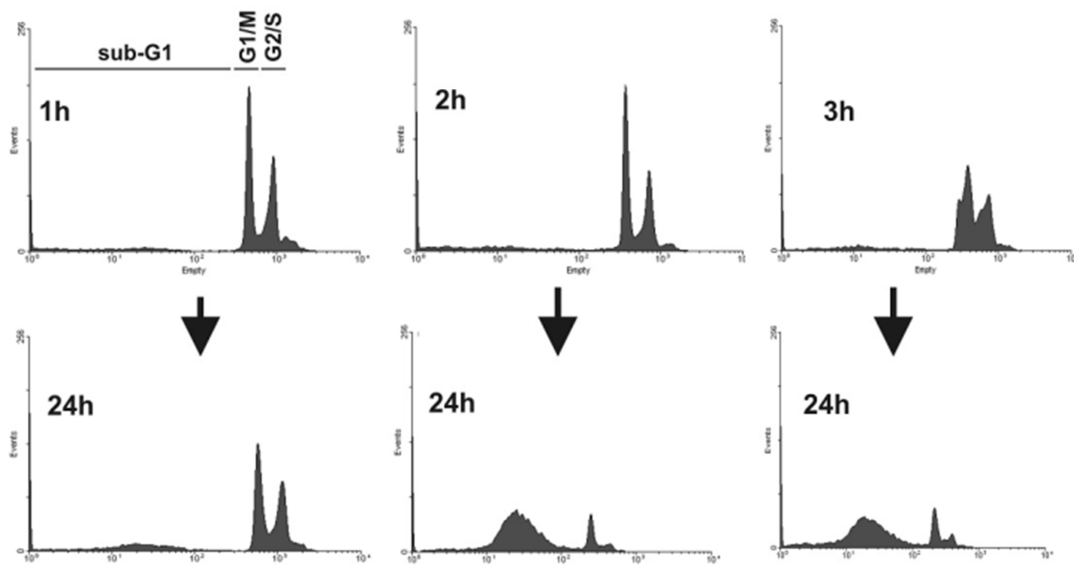
3.3. Cytotoxic Effect of GE-Cu Is Rapidly Activated and Irreversible

In order to assess when cell death was induced, we treated HepG2 cells for 1-3 h with 1% GE supplemented with 100 µmol/L CuSO$_4$, then cells were washed and replaced with fresh medium without GE-Cu. After 24 h of recovery, cells were stained with propidium iodide and analyzed for apoptosis. Fig. 3 shows that cells were rescued from death as long as the medium was removed within the first hour of treatment. Conversely, longer times of incubations with GE-Cu brought on irreversible programmed cell death with

values of sub-G1 (apoptotic) cell population close to 70%, independent on the time of incubation.
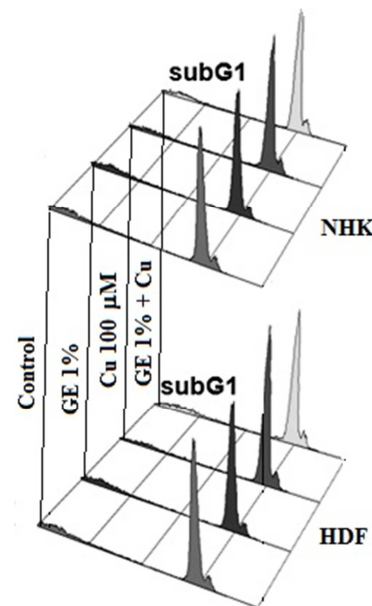
Figure 2. *Cu treatments do not induce apoptosis in normal human cells: NHK and HDF cells were incubated with 100µM Cu plus 1% GE. Percentages of sub-G$_1$ (apoptotic cells) cells was evaluated by a FACScalibur instrument.*

Figure 3. *Cytotoxic effects of GE-Cu treatments are rapidly activated and irreversible.*

HepG2 cells were treated with 1% GE and Cu 100 µmol/L. After 1, 2 or 3 h, the media was removed and substituted with fresh media till 24 h. After this time, cells were washed and stained with propidium iodide. Percentages of sub-G$_1$ (apoptotic cells) cells was evaluated by a FACScalibur instrument and are expressed as means ± S.D. **p<0.001 (n = 6).
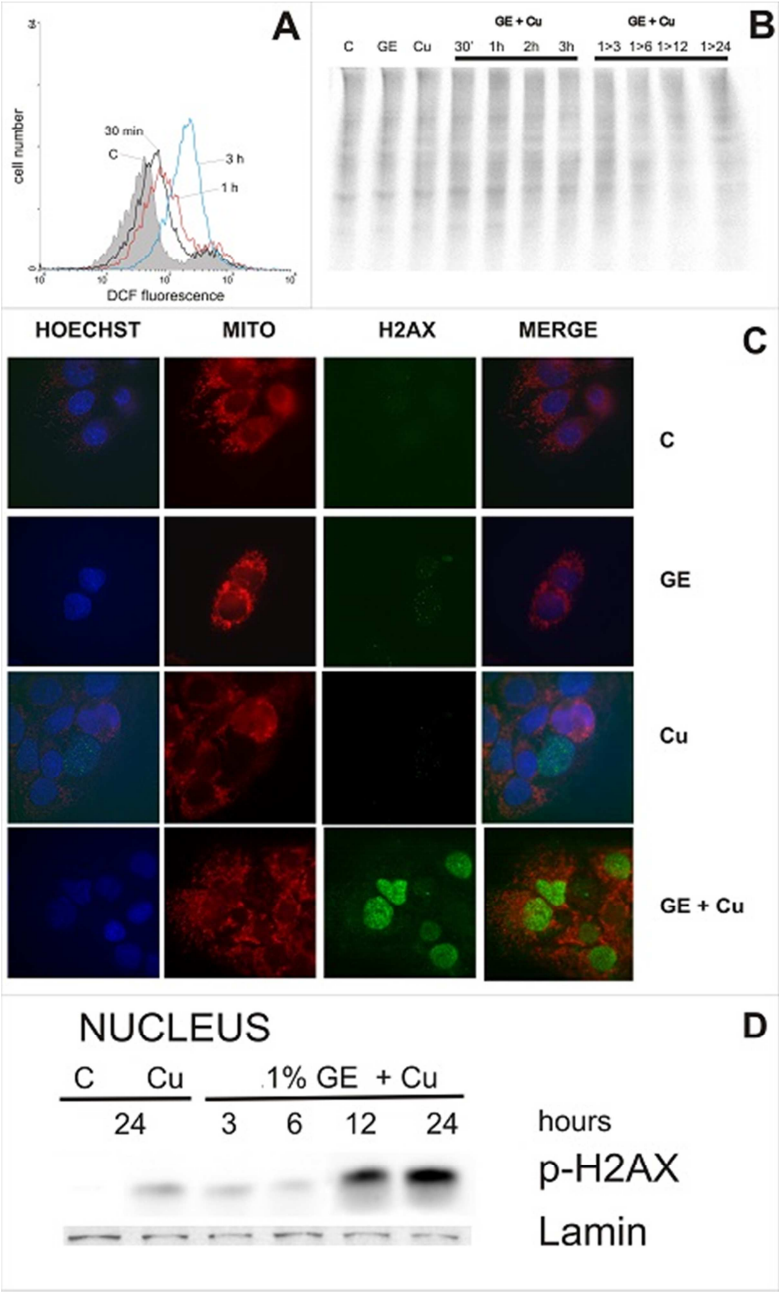
3.4. GE-Cu Induces ROS Production and Oxidative Damage to DNA

Results obtained so far strongly indicated that a burst event should occur in order to entirely induce cell death in the first hour of treatment with GE-Cu. Due to the presence of Cu, we wonder whether reactive oxygen species (ROS) could be

produced by GE-Cu. To this aim, we treated HepG2 cells with 1% GE supplemented with 100 µmol/L CuSO$_4$ for different times and analyzed intracellular ROS concentration upon staining with (DCF-DA). Fig. 4A shows a representative cytofluorimetric panel indicating that ROS increased as early as 30 min of treatment with GE-Cu, reaching a 10-fold increase in DCF fluorescence at 3 h of

treatment with respect to control cells. Next we evaluated the possible occurrence of oxidative damage to proteins and DNA. Fig. 4B shows Western blot analyses of carbonylated proteins. No change in the extent of protein carbonylation was detected neither during the early 3 h of treatment nor during different times of recovery up to 24 h. DNA damage was evidenced by fluorescence microscopy analyses of the phospho-activation of the double strand break-sensitive histone H2AX. Fig. 4C shows that cells treated for 1 h with 1% GE supplemented with 100 μmol/L CuSO$_4$ displayed a massive activation of histone H2AX, indicating that GE-Cu treatment induced damage to DNA. Concomitantly, cells

were stained with the mitochondrial transmembrane potential-sensitive probe MitoTracker Red. Figure 4C shows that mitochondria of GE-Cu-treated cells appeared as single less fluorescent spots, compared to the network present in control cells or in cells treated with 1% GE or 100 μmol/L CuSO$_4$. Fluorescence microscopy results were confirmed by Western blot analyses of phospho-active H2AX, within nuclear compartment of HepG2 cells, treated with GE-Cu for different times. Fig. 4D shows that histone H2AX was rapidly activated to reach maximum level at 12 and 24 h of treatment.


Figure 4. *GE-Cu treatments induce ROS production and oxidative damage to DNA: (A) HepG2 cells were treated with 1% GE and Cu 100 μmol/L up to 3 h, incubated with 50 μM DCF-DA at 37°C. Figure show one experiment representative of three that gave similar results. (B) Carbonylated proteins of HepG2 cells treated with 1% GE and Cu 100 μmol/L. Immunoblots are from one experiment representative of three that gave similar results. (C) HepG2 cells were grown on chamber-slides and treated for 1 h with 1% GE and Cu 100 μmol/L. (D) HepG2 cells were treated with 0.1% GE and Cu 100 μmol/L for 3, 6, 12 or 24 h. for detection of p-H2AX in the nucleus.*

3.5. GE-Cu Modulates DNA Damage-Related Proteins

To confirm whether DNA double strand break downstream was responsible for cell death observed by GE-Cu treatment, we analyzed the expression level of p53, which normally responds to DNA damage and activates cell cycle arrest and apoptosis. Fig. 5A shows Western blot analyses of p53 upon treatment with 1% GE supplemented with 100 µmol/L CuSO$_4$, indicating that p53 immunoreactive band rapidly increased already at 30 min, to return to control levels as soon as after 2 h of treatment. Western blots after 1 or 2 h of recovery from GE-Cu showed that p53 remained over expressed only when the treatment was performed for 1 h, while additional time of incubation with GE-Cu resulted in lack of p53 activation. To corroborate these data we also analyzed the expression level of the p53 downstream factor: the cyclin dependent kinase inhibitor p21. Fig. 5A shows the same trend of induction as previously observed for p53. Next we evaluated whether HDAC was also affected by GE-Cu treatment, since it has been suggested that its inhibition results in a decrease of DNA repair machinery efficiency.
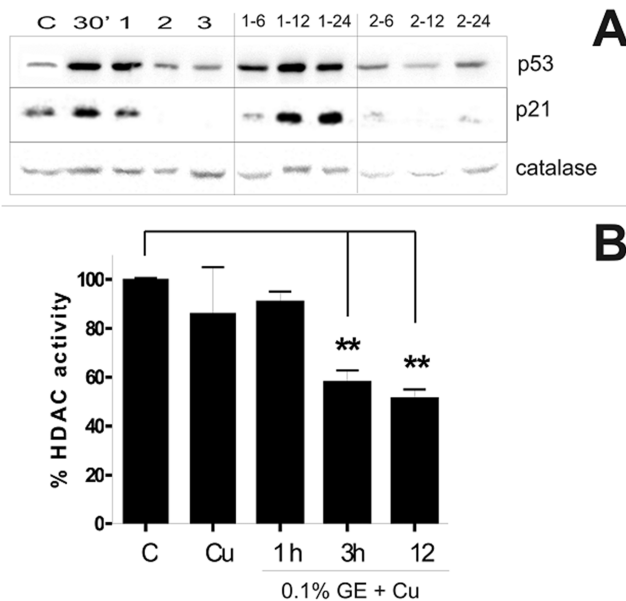
Figure 5. GE-Cu treatments modulates DNA damage-related proteins, p53 and p21.(A) HepG2 cells were treated with 1% GE and Cu 100 µmol/L for 30 min, 1, 2 or 3 h. After 1h or 2 h of treatment, cell media was removed and substituted with fresh media and cells growth on for 6, 12 and 24 h for detection of p53 and p21 protein levels. (B) HepG2 cells were treated with 0.1% GE and Cu 100 µmol/L for 1, 3 and 12 h. for detection of HDAC activity. Data are expressed as means ±S.D. **p<0.001 (n = 4).

Cell were then treated with 0.1% v/v of GE supplemented with 100 µmol/L CuSO$_4$ to allow extending the experimental time up to 12 h. Fig. 5B shows that HDAC activity decreased significantly already at 3 h to reach a minimum at 12 h (about 50% decrease).
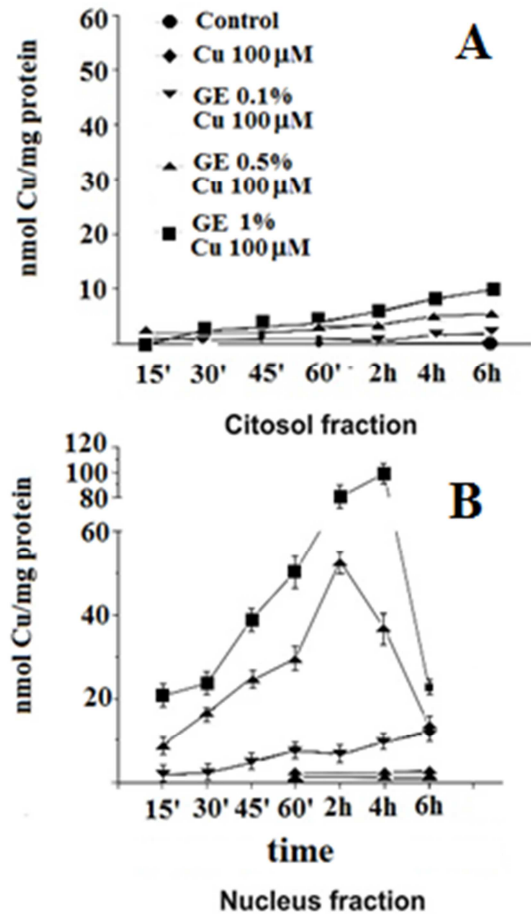
Figure 6. Cu accumulates specifically within nuclear compartment. HepG2 cells were treated with 0.1, 0.5 or 1% GE and Cu 100 µmol/L from 15 minutes to 6 hours. HepG2 cells were fractionated into cytosolic and nuclear fractions. Cu concentration was measured by Atomic Absorption Spectrometry and expressed as nmol Cu/mg total proteins. Cu concentration evaluated after GE-Cu treatment in cytosolic (A) and nucleus (B) fractions. Data are expressed as means ±S.D. **p<0.001 (n = 4).

3.6. Cu Accumulates Specifically Within Nuclear Compartment upon GE-Cu Treatment

The result obtained indicated that GE-Cu could cause nuclear-specific oxidative damage. We therefore purified cytosolic and nuclear fractions of HepG2 cells treated with different concentrations of GE supplemented with 100 µmol/L CuSO$_4$ and analyzed Cu levels by atomic absorption spectroscopy. Figure 6A shows that only minor differences could be detected in the cytosol, with Cu concentration remaining very low (under 10 nmol/mg protein) in each experimental condition. Conversely, a dose-dependent increase of Cu content was measured in purified nuclear fractions, with 1% GE reaching values close to 100 nmol/mg prot. It is worth noting that the decrease of nuclear Cu observed after 2-4 h treatment with 0.5 and 1% GE was a consequence of cell death. Instead, treatment with 0.1% GE supplemented with 100 µmol/L CuSO$_4$ showed a time-dependent continuous increase of nuclear Cu up to 6 h (Fig. 6B). To evaluate the specificity of Cu in inducing toxicity of

GE-Cu, we used other transition metals, Fe and Zn, and analyzed cell viability by Trypan blue exclusion upon treatment with their sulfate salts alone or together with 1% GE. No toxic effect could be ascribed to Fe or Zn (data not shown), suggesting that only Cu in combination with GE was able to accumulate inside the nucleus and caused toxic effects. Because GE is rich in sulfur-containing molecules, it is very likely a role for sulfur in coordinating Cu.

Thiols and perthiols are good ligands for transition metals; in addition, disulfides and polysulfides are capable to form metal complexes too, in a way resembling multi-dentate ligands. Since, all these sulfur-containing molecules are present in GE, to study the formation of the GE-Cu complex, in our experimental conditions and to assess the role of thiols, we used the EPR spectroscopy. While GE alone did not give any signal, 1% GE supplemented with 100 µmol/L $CuSO_4$ showed a peculiar EPR stabile spectrum both in PBS or in cell medium. Moreover, treatment with NEM, a sulfur-alkylating agent, did not cause any change in the CE-Cu spectrum, indicating that reasonably disulfides or polysulfides-containing molecules concurred in coordinating Cu in GE-Cu (data not shown).

3.7. GE-Cu Induced Cell Death is Caspase-Independent and Associated with p38 MAPK/c-Jun-Dependent Pathway

To dissect the mechanisms underlying GE-Cu-induced apoptosis, we performed Western blot analyses of JNK and p38, the pro-apoptotic members of MAPK super-family. Cells were treated with 0.1% GE supplemented with 100 µmol/L $CuSO_4$ and the levels of the phosphorylated active forms p-JNK and p-p38 were evaluated at different times. Fig. 7A shows that no significant change in phospho JNK was detectable upon GE-Cu treatment; by contrast, p38 rapidly activated to remained phosphorylated up to 6-12 h. The same wave of activation was visible by following the phosphorylated levels of the downstream transcription factor c-Jun, which remained sustained and significantly higher than control up to 6 h, suggesting a cause-effect relationship between p38 and c-Jun (Fig. 7A). To establish the dependence of GE-Cu-induced apoptosis upon caspase activation, we treated HepG2 cells with 0.1% GE supplemented with 100 µmol/L $CuSO_4$ and analyzed the caspase activation by Western blot analyses. No activation was evidenced even at high doses of GE (data not shown), however, Western blot analyses of apoptosis inducing factor (AIF) in the nuclear compartment indicated that it translocated within the nuclei as soon as after 3-6 h of treatment with GE-Cu (Fig. 7B), suggesting the existence of caspase-independent apoptosis occurring under our experimental conditions.

4. Discussion

Several studies suggest that polyphenols might inhibit free radical formation and the propagation of free radical reactions through the chelation of transition-metal ions,

particularly those of iron and Cu [41, 42]. Furthermore, many studies have demonstrated the antioxidant effects of quercetin in lipid systems in the presence of a range of pro-oxidants
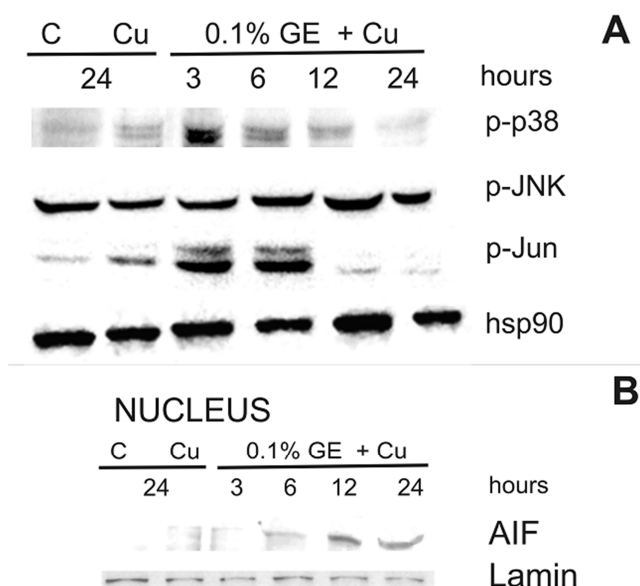
Figure 7. *GE-Cu treatments induce phospho-activation of p38 and p-Jun signaling cascade and apoptosis is AIF mediated.(A) Western blots for detection of the phospho-activated isoforms of JNK (p-JNK), Jun (p-Jun) and p38 (p-p38). (B) HepG2 cells were treated with 0.1% GE and Cu 100 µmol/L for 3, 6, 12 or 24 h. 50 µg of proteins of total nucleus lysates were loaded onto each lane for detection of AIF. Immunoblots shown are from one experiment representative of four that gave similar results.*

[43, 44]. In transition metal-free lipid systems, the phenolics act as antioxidants by virtue of their ability to act as hydrogen donors, leading to the formation of aryloxyl radicals; compounds containing the *o*-dihydroxycatecholate structure are the most effective. In the presence of transition metal ions, however, it is not clear to what relative extents radical scavenging or metal chelation might contribute to the antioxidative effects. Thiols and perthiols are excellent ligands for transition metals; in addition, disulfides and polysulfides are capable to form metal complexes too, in a way resembling multi-dentate ligands [45].

Oxidative stress represents a condition in which a persistent and uncontrolled generation of ROS occurs in the cells. The resulting redox imbalance in favor of pro-oxidants species gives rise to a series of irreversible damages to biological macromolecules with consequent cell death. The cellular equipment of enzymatic and not enzymatic anti-oxidant molecules is exploited by cells to contrast and remove the deleterious effect of ROS. A key role in the antioxidant defense is carried out by the several molecules introduced with the diet, which directly participate to counteract oxidative damages providing a protection to the whole organism.

The role of foods and dietary phytochemicals in prevention and treatments of cancer has been extensively discussed [30-33]. The potential chemo-preventive and anti-tumoral effects of garlic are of particular interest, since garlic contains numerous pharmacologically active substances, including

sulfur and seleno-compounds, which have been shown to inhibit cancer cell growth and induce cell death [34, 35]. Indeed, at high concentrations, phytochemicals present in garlic extract, may act as pro-oxidants showing a pro-apoptotic activity [36-38].

Results obtained in this work show that high concentrations of Cu (up to 1 mmol/L) or of GE (1%) produce cytostatic effects with arrest in cell proliferation.

On the contrary, the combined GE-Cu treatment causes cell death, through a synergistic action of the garlic extract with the Cu, in undifferentiated HepG2 cells. The cytotoxic specificity of GE-Cu, was confirmed by the lack of damaging effects, when other transitional metals, such as iron and zinc, were added.

Since GE is rich in mercaptans and disulfide molecules, which have been long known to chelate transition metals, it was assumed that the formation of a stable complex could occur also in our experimental conditions. ESR spectroscopy data, show the presence of a stable complex in GE-Cu solution. This ESR signal did not change, even after the addition of NEM, a sulphydryl alkylating agent, suggesting that disulfides, more likely than sulphydryls, play a pivotal role in binding Cu.

Cu enters the cell only through specific transporters and in the cytosol, rapidly binds to intracellular thiols to avoid ROS production by Fenton's reaction. However, recently, it has been demonstrated that a mixture of allicin and Cu, shows antimycotic effects, since it can freely diffuse across the yeast cell membrane, causing a strong efflux of potassium ions and inducing the death [18]. Our results revealed very low levels of Cu in the cytosolic fraction, but a specific and drastic increase of this metal inside the nucleus. These data suggested that one of the main targets of the damage, caused by GE-Cu treatments, could be the DNA. This was confirmed by the early phospho-activation of the histone H2AX and the decrease of HDAC activity, which are the result of DNA double strand breaks. Moreover, fluorescent microscopy analyses provided further evidence for a significant alteration of the mitochondrial function, indicating that in the presence of GE, Cu is vehicled within cellular organelles and induces selective damages. This is also corroborated by the absence of protein carbonylation in the cytosol, because Cu reasonably accumulates in the nucleus and is not able to catalyze oxidative reactions in the cytosolic space.

Since nuclear translocation of the apoptosis inducing factor (AIF) is operative is confirmed a caspase-independent type of apoptosis.

Recently, it has been shown that selenium, which is found to be particularly abundant in garlic, is able to activate the phosphorylation of p38, but not that of JNK [39]. Results obtained upon treatment with GE-Cu, are in agreement with these observations, since the exclusive activation of p38, was found, without change in the phosphorylation state of JNK. Since this wave of activation seems to involve c-Jun as well, work is in progress, in our laboratory, in order to identify a possible link between p38 and c-Jun. Moreover, p53 and p21 are activated early, in response to GE-Cu, especially when Cu is used in combination with 0.1% GE. These results are in line with those from groups which demonstrated that selenium is able to damage DNA with the consequent phosphorylation of histone H2AX in prostate cancer cells. These events were associated with cell death due to p53 [40], thus resembling the sequence of events obtained in our experimental system.

A fundamental requirement of chemotherapeutics is that they should be directed towards transformed cells without affecting vitality of differentiated cells. GE-Cu had no effect on either differentiated cells, or primary fibroblast and keratinocytes, indicating possible roles of GE-Cu in anticancer treatment.. This result provides additional support for consideration the possibility that GE-Cu, could be applied in chemotherapeutic treatments.

Conflict of Interest

The authors have no potential conflicts of interest.

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