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Polyphenol Effects on CuO-Nanoparticle-Mediated DNA Damage, Reactive Oxygen Species Generation, and Fibroblast Cell Death

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Abstract

The ability of ten polyphenolic antioxidants to prevent CuO nanoparticle (^{NP}CuO) and H_2O_2-mediated DNA damage and cytotoxicity was investigated. Five of the polyphenols (MEPCA, PREGA, MEGA, ECG, and EGCG) prevent ^{NP}CuO/H_2O_2-mediated DNA damage (IC_{50} values of 7.5–800 μM), three have no effect (PCA, VA, and EC), and two (GA and EGC) result in increased DNA damage. Most polyphenols had similar antioxidant/prooxidant activity in the presence of ^{NP}CuO or free copper ions. Electron paramagnetic resonance (EPR) spectroscopy of reactive oxygen species (ROS) generated by ^{NP}CuO/H_2O_2 in the presence of representative polyphenols correlate with results of DNA damage studies: in the presence of ^{NP}CuO/H_2O_2, MEPCA prevents ROS formation, VA has no effect on ROS levels, and EGC increases ROS levels. EPR results with CuO nanoparticles washed to remove dissolved copper in solution (^{W}CuO) in the presence of H_2O_2/ascorbate suggest that MEPCA prevents ROS formation on the nanoparticle surface in addition to preventing ROS formation from dissolved copper. In mouse fibroblast (L929) cells, combining ^{NP}CuO with H_2O_2 results in significantly greater cytotoxicity than observed for either component alone. After 3 h incubation with MEPCA or MEGA, the viability loss in L929 cells induced by ^{NP}CuO/H_2O_2 challenge was significantly rescued at physiologically relevant polyphenol levels (1 μM). These studies show that polyphenols can protect DNA and inhibit cytotoxicity generated by ^{NP}CuO under oxidative stress conditions.

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Transparency document

The transparency document associated with this article can be found online.

Conflicts of interest

The authors have no conflicts of interest to declare.

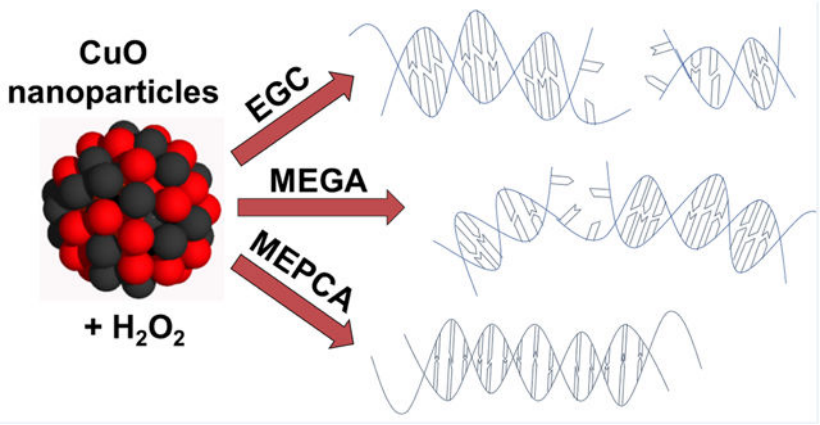
Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A: Supplementary Material

All data presented in this work, including DNA gel electrophoresis data and IC_{50} plots, cell viability data and EC_{50} plots, data from dynamic light scattering measurements, and electron paramagnetic resonance spectra are provided in the associated Supplementary Material.

Graphical Abstract



Keywords

polyphenol; CuO nanoparticles; DNA damage; reactive oxygen species; cell death prevention; prooxidant

1. Introduction

Copper oxide nanoparticles (^{NP}CuO) are widely used in consumer products such as cosmetics (Borkow, 2014), electronics (Son et al., 2009), sensors (X. Zhang et al., 2008), wood preservation (Evans et al., 2008), antifouling paints (Detty et al., 2014), and antibacterial textiles (Ren et al., 2009; Suresh et al., 2013). The physicochemical properties of these nanoparticles raise concerns about risks to human health (Karlsson et al., 2008a). However, the evidence is limited for acute toxicity from nanoparticles at realistic doses, especially at low concentrations that may cause oxidative stress and adverse long-term health effects. Toxicity from nanoparticles is generally different than from the constituent ions due to differences in nanoparticle uptake/pharmacokinetics as well as surface dependent biochemical properties (Angelé-Martínez et al., 2017; Klaine et al., 2020). Therefore, considerable efforts have been placed on identifying the potential toxicity of nanoparticles to cells and organisms (Bondarenko et al., 2013; Karlsson et al., 2008a; Karlsson et al., 2009; Oberdorster et al., 2005; Rim et al., 2013; Stone et al., 2007).

To reduce the toxic effects of ^{NP}CuO, one can directly modify the nanoparticle surface by coating it with an inert shell, by adding ligands to the ^{NP}CuO surface, or by altering methods of synthesis (Barua et al., 2013; Jo et al., 2012; Kanninen et al., 2008; C. C. Li and Chang, 2004; Studer et al., 2010). Since ^{NP}CuO toxicity is due to effects of reactive oxygen species (ROS) generated both from dissolved copper from the nanoparticles and to ROS generated directly on the nanoparticle surface (Aruoja et al., 2009; Bondarenko et al., 2013; Heinlaan et al., 2008; Isani et al., 2013; Jo et al., 2012; Karlsson et al., 2008a; Karlsson et al., 2009; Karlsson et al., 2008b; Kasemets et al., 2009; Midander et al., 2009; Misra et al., 2012; Mortimer et al., 2010; Nel et al., 2006; Shi et al., 2012), another possible strategy to prevent ^{NP}CuO toxicity is to use radical scavenging antioxidants to

prevent the ROS damage, as reported in similar ROS-generating systems. Sulforaphane, an isothiocyanate with anticancer properties (Juge et al., 2007; Y. Zhang et al., 1994) (Figure 1A) found primarily in green vegetables (Juge et al., 2007; Liang et al., 2007; Liang et al., 2006), reduces ROS generation and increases cell viability in mouse embryonic fibroblast (BALB 3T3) cells exposed to ^{NP}CuO (Akthar et al., 2012). Treatment of ^{NP}CuO-exposed cells (HEp-2; 5 h) with resveratrol (100 μM) resulted an 80% reduction in 8-isoprostane levels, a marker for oxidative stress, compared to cells without resveratrol treatment (Fahmy and Cormier, 2009). More recently, rats treated with curcumin (200 mg/kg; Figure 1A) and ^{NP}CuO (250 mg/kg) showed improved renal toxicity markers, including lower creatinine and blood urea-nitrogen levels, compared to rats treated with only ^{NP}CuO (250 mg/kg) (Elkhateeb et al., 2020). Ameliorating ^{NP}CuO toxicity is potentially important since ^{NP}CuO and copper nanoparticles are being examined as breast cancer treatments (Kamble et al., 2016; Mariadoss et al., 2020), and dietary polyphenols could thus affect both the therapeutic response in tumors and side-effects in normal tissue.

Polyphenols, such as those shown in Figure 1B, are the most common dietary antioxidants, with an average daily intake around 1 g/day in humans (Scalbert and Williamson, 2000). Their antioxidant properties can protect DNA, lipids, and proteins from oxidative damage in cells, rodents, and humans (Asensi et al., 2011; Babich et al., 2011; Blokhina et al., 2003; Haslam, 1996). Epicatechin (EC) protects plasmid DNA from γ-irradiation (Nair and Salvi, 2008), and propyl gallate (PREGA), ellagic, gallic, and tannic acids protect human lymphocytes from DNA damage and lipid peroxidation induced by food mutagens or hydrogen peroxide (C. H. Chen et al., 2007). In general, the antioxidant and radical scavenging ability of gallols (polyphenolic compounds with three adjacent OH groups on an aromatic ring; e.g., gallic acid) is greater than analogous catechols (with two adjacent OH groups; e.g., protocatechuic acid) (Perron et al., 2011; Reis et al., 2010; H. C. Wang and Brumaghim, 2011). Other polyphenol structural modifications also correlate with antioxidant activity; for example, the ROS scavenging activity of protocatechuic acid (PCA) and its esters increases as the length of the alkylated ester chain increases (methyl, ethyl, and propyl) (Reis et al., 2010). Polyphenol antioxidants can scavenge ROS produced in normal cellular processes and protect cells from oxidative damage, so they are widely studied for their ability to prevent development of diseases such as cancer and heart disease (Dall'Asta et al., 2015; Ding et al., 2013; Oak et al., 2018; Z. Wang et al., 2012).

Herein, we systematically investigate ten polyphenols for their effects on ^{NP}CuO-mediated DNA damage. For representative polyphenols, activity observed in the DNA gel electrophoresis experiments were also correlated with ROS levels as determined using electron paramagnetic (EPR) spectroscopy. The most effective polyphenols for preventing ^{NP}CuO-mediated DNA damage, MEPCA and MEGA, were then examined for their ability to prevent ^{NP}CuO/H_2O_2-induced oxidative damage in mouse fibroblast (L929) cells. The ability of polyphenols to modulate ^{NP}CuO toxicity is an important aspect of these dietary antioxidants as incorporation of ^{NP}CuO into consumer materials increases and the medicinal effects of ^{NP}CuO are explored.

2. Materials and Methods

2.1 General

Water was purified using a Barnstead NANOpure DIamond Life Science water deionization system (Barnstead International). 3-(*N*-Morpholino)propanesulfonic acid (MOPS, Alfa Aesar), $CuSO_4$ (Acros Organics), L-ascorbic acid (30% v/v in water, Alfa Aesar), Chelex 100 resin (Sigma-Aldrich), hydrogen peroxide (H_2O_2, Alfa Aesar), methyl 3,4-dihydroxybenzoate (MEPCA, Alfa Aesar), methyl 3,4,5-trihydroxybenzoate (MEGA, Alfa Aesar), *n*-propyl gallate (PREGA, Acros), gallic acid (GA, TCI America), protocatechuic acid (PCA, Frontier Scientific), (−)-epigallotechin (EGC, Alfa Aesar), (−)-epcatechin (EC, MP Biomedicals), (−)-epigallotechin-3-gallate (EGCG, Alfa Aesar), (−)-epicatechin-3-gallate (ECG, Frontier Scientific), vanillic acid (VA, Frontier Scientific), and copper(II) oxide (CuO) nanoparticles (50% weight, Alfa Aesar) were used as received. Cu^{2+}, polyphenol, and H_2O_2 solutions were prepared prior to each experiment and used immediately. Microcentrifuge tubes were rinsed in 1 M HCl, rinsed in deionized water, and dried prior to use. Buffered solutions were treated with Chelex resin (2 g / 80 mL buffer) for 24 h prior to use.

Mouse fibroblast (L929) cells were purchased from the American Type Culture Collection (ATCC). Dulbecco's modified Eagle's medium (DMEM), Eagle's minimum essential medium (EMEM), glutamine, and fetal bovine serum (FBS) were purchased from Corning. A CellTiter 96® Aqueous One Solution Cell Proliferation MTS assay kit was purchased from Promega.

2.2 Preparation of CuO nanoparticle suspensions and copper solutions

In addition to commercial CuO nanoparticle suspensions (^{NP}CuO), washed CuO nanoparticles (^{w}CuO), and CuO nanoparticle leachate after removal of dissolved Cu ions (^{l}CuO) were used in this study. ^{w}CuO were prepared by separating ^{NP}CuO from the suspensions using a bench-top centrifuge at 14,000 rpm (30,074 g RCF) for 45 min, removing the supernatant via pipette, and then re-dispersing the ^{NP}CuO pellet in deionized water. This centrifugation and re-dispersion process was repeated 6 times. Removal of dissolved copper from the supernatant of ^{NP}CuO suspensions to yield ^{l}CuO solutions were performed as previously described (Angelé-Martínez et al., 2017). Dissolved copper solutions were prepared using $CuCl_2$. The concentrations of Cu^{2+} solution used for the cell studies were based on the measured dissolved copper concentration from the ^{NP}CuO supernatant prior to copper removal (Angelé-Martínez et al., 2017). All ^{NP}CuO, ^{w}CuO, ^{l}CuO, and copper suspensions/solutions were freshly prepared prior to each experiment and used immediately.

2.3 Transfection, amplification, and purification of plasmid DNA

Plasmid DNA (pBSSK) was purified from *E. coli* strain DH1 using a PerfectPrep Spin kit (Fisher). The plasmid DNA was dialyzed at 4 °C against EDTA (1 mM) and NaCl (50 mM) for 24 h and then against NaCl (130 mM) for 24 h to remove metal ions from the DNA. For all experiments, the absorbance ratios for DNA solutions were $A_{250}/A_{260} \leq 0.95$ and $A_{260}/A_{280} \geq 1.8$.

2.4 DNA damage assays

To evaluate the activity of polyphenols samples for the DNA damage assays, reactions were prepared by adding polyphenol (0.50 – 800 μM) to samples containing ^{NP}CuO (500 μM), NaCl (130 mM), MOPS (pH 7, 10 mM), and ethanol (10 mM, as a radical scavenger) in 10 μL of total volume. This mixture was allowed to stand at room temperature for 5 min and plasmid DNA (0.1 pM in 130 mM NaCl) was added. After 5 min, H_2O_2 (50 μM) was added to initiate DNA damage. After 150 min, the reaction was stopped by adding EDTA (200 mM, 0.5 μL) and loading dye (2 μL) to give a final volume of 12.5 μL.

Gel electrophoresis was run on a 1% agarose gel in TAE buffer for 60 min at 140 V and 255 mA to separate the nicked and supercoiled forms of the plasmid DNA. Gels were then stained for 5 min using ethidium bromide and washed for an additional 10 min with deionized water before imaging under UV light. The intensities of the damaged and undamaged DNA gel bands were quantified using UVIproMW software (Jencons Scientific Inc.). Ethidium stains supercoiled DNA less efficiently than nicked DNA, so supercoiled DNA band intensities were multiplied by 1.24 prior to comparison (Hertzberg and Dervan, 1982). Intensities of the nicked and supercoiled bands were normalized for each lane so that % nicked + % supercoiled = 100%. Sample gel images (Figures S1–S10) and tables of gel data (Tables S1–S10) are provided in the Supplementary Material.

2.5 Electron paramagnetic resonance (EPR) studies

EPR spectra were measured on a Bruker EMX spectrometer using a quartz flat cell at room temperature using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a standard (g = 2.0036 (Mani et al., 2004)) centered at 3500 with a sweep width of 100 G. The modulation amplitude was 0.50–1.00 G, time and conversion constants were 81.92 s, and microwave power and frequency were 20.02 mW and 9.752 GHz, respectively. Samples (500 μL total volume) were prepared in MOPS buffer (10 mM, pH 7), containing ^{NP}CuO (300 μM), ascorbate (375 μM), H_2O_2 (22.5 mM), polyphenol (600 μM), and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) as a spin trap, and measured in less than 5 minutes. In all cases, H_2O_2 was added last to initiate the reaction. Complete EPR data for ^{NP}CuO generation of ROS with ascorbic acid and H_2O_2 are provided in Figures S12 and S13.

2.6 Dynamic light scattering (DLS) size measurements of ^{NP}CuO and ^{w}CuO

DLS measurements were performed with a Brookhaven NanoBrook Omni particle sizer and zeta potential analyzer system at 640 nm and 589 nm wavelengths. After loading the sample in the cuvette, the sample was left to sit for 5–10 min to allow any turbulence to dissipate before spectral acquisition. A detection angle of 90° was chosen for all size measurements. EMEM was used for particle characterization studies. Unless otherwise stated, EMEM suspensions were supplemented with 10% FBS, L-glutamine (2 mM), non-essential amino acids, streptomycin (100 μg/mL), and penicillin G (100 UI/mL). Aliquots were taken from ^{NP}CuO or ^{w}CuO stock suspensions and mixed with 3 mL of either EMEM or sterile deionized water. The solutions were mixed well and incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air for 24 h. The test suspensions were mixed gently prior to DLS measurements. DLS measurements of ^{NP}CuO and ^{w}CuO in water and EMEM are presented in Table S11 and Figure S14 in the Supplementary Material.

2.7 Cell culturing procedures

Mouse fibroblast cells (L929) were cultured in 75 cm^2 tissue-culture flasks in presence of EMEM at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. EMEM was supplemented with 10% FBS, L-glutamine (2 mM), non-essential amino acids, streptomycin (100 μg/mL), and penicillin G (100 UI/mL). At 95% confluence, cells were harvested using 0.25% trypsin and were seeded at a concentration of 1.5×10^4 cells/well in 96-well plates. Cells were allowed to attach the surface for 24 h prior to treatment. The appropriate dilutions of ^{w}CuO and ^{NP}CuO suspensions were then sonicated using a sonicator bath at room temperature for 10 min to avoid nanoparticle aggregation prior to administration. Cells not exposed to nanoparticles served as controls in each experiment. Selection of final concentrations of 15.625–625 μM dose range for both ^{w}CuO and ^{NP}CuO nanoparticles was based on a previous report on copper oxide nanoparticle interactions with lung epithelial cells (Z. Wang et al., 2012). When cells reached sub-confluence, they were pretreated for 24 h with or without H_2O_2 and the indicated concentrations of ^{w}CuO, ^{NP}CuO, ^{l}CuO, or copper solution. Following treatment, the culture supernatant was removed, and cells were washed three times with phosphate-buffered saline (PBS) before determining cell viability.

To study the effects of MEPCA and MEGA addition, 1.5×10^4 cells/well were incubated with various concentrations (1–400 μM) of MEPCA or MEGA at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air for 3 h. Next, a mixture of ^{NP}CuO (153.5 μM) and H_2O_2 (80 μM) was added. After 24 h incubation, the culture supernatant was removed, and cells were washed three times with phosphate buffered saline (PBS) before determining cell viability. Cell viability results with ^{NP}CuO/H_2O_2, with and without polyphenol addition, are provided in Figures S15–S22 and Tables S12–S19 in the Supplementary Material.

2.8 Cell viability measurements

After L929 cell treatment with ^{w}CuO, ^{NP}CuO, ^{l}CuO, or copper solutions with or without H_2O_2, MEPCA, or MEGA, MTS assays were performed according to the manufacturer's protocol, and the plate was read at 490 nm optical density to measure the absorbance of the formazan product using a microplate reader (Thermo Scientific Multiskan™ FC). Since some polyphenols can interfere with formazan formation in the MTS assay (Akter et al., 2019; Wang, P. et al., 2010;), we tested MEGA and MEPCA in this assay prior to the cell studies to ensure that no spurious signals were observed due to polyphenol addition. Viability of untreated cells was normalized to 100% to calculate the viability of the cells treated with different concentrations of ^{NP}CuO and polyphenol compounds. Cell viability data for these experiments are presented in the Supplementary Material (Tables S11–S18 and Figures S11–S18).

2.9 Statistical analysis

For all studies, data are expressed as means of at least three independent trials with error bars representing standard deviations. Statistically significant differences within and between the groups were evaluated by performing ANOVA and 2-tailed unpaired *t*-tests. Results with p values of ≤ 0.05 were considered to be statistically significant. To determine EC_{50}/IC_{50} values, a graph of percent non-viable cells was plotted with respect to ^{NP}CuO, ^{w}CuO, ^{l}CuO, dissolved copper, H_2O_2, MEPCA, or MEGA concentrations on a semi-log plot

and fit to a sigmoidal dose-response curve using OriginPro 8.1 software. EC_{50} values were calculated by fitting all points of four trials with a single curve.

3. Results

3.1 Activity of polyphenols with $^{NP}CuO/H_2O_2$–mediated DNA damage

To evaluate polyphenol effects on $^{NP}CuO/H_2O_2$-mediated DNA damage, plasmid gel electrophoresis assays were performed. Focusing on damage prevention using a single biomolecule (DNA) allows a more complete mechanistic examination of biologically relevant oxidative damage prevention than can be obtained in a cellular environment. Since gel electrophoresis readily separates damaged (nicked) from undamaged (supercoiled) plasmid DNA, sample treatment is straightforward and assessment time is short. After staining, intensities of the damaged and undamaged plasmid DNA bands are quantified, so that polyphenol antioxidant abilities can be directly determined and compared. Treating plasmid DNA with ^{NP}CuO (500 μM) and H_2O_2 (50 μM) for 150 min at pH 7 results in ≥ 85% DNA damage. These conditions result in an average of one strand break per plasmid to facilitate DNA damage quantification, and this DNA damage is used as the positive control. As reported, the manufacturer-added ^{NP}CuO dispersant has little effect on observed $^{NP}CuO/H_2O_2$ DNA damage effects (Angelé-Martínez et al., 2017).

Once initial DNA damaging conditions were established, ten polyphenol compounds were evaluated for their ability to prevent $^{NP}CuO/H_2O_2$-mediated DNA damage. Hydrogen peroxide alone and MEPCA (800 μM) alone do not damage DNA (Figure 2, lanes 2 and 3, respectively); however, more than 90% of plasmid DNA is nicked (top band) when treated with ^{NP}CuO (500 μM) and H_2O_2 (50 μM; lane 4). In lanes 5 to 15, DNA treated with $^{NP}CuO/H_2O_2$ and increasing concentrations of MEPCA (0.5–800 μM) show decreased DNA damage, as indicated by the increasing intensity of supercoiled DNA (bottom band). Percent DNA damage inhibition plotted with respect to MEPCA concentration results in a best-fit, sigmoidal dose-response curve (Figure 2B) used to determine the MEPCA concentration at which 50% of DNA damage is inhibited (IC_{50} value). The IC_{50} value of 7.5 ± 0.5 μM for MEPCA indicates that it is a potent antioxidant, even at low micromolar concentrations. Blood concentrations of dietary polyphenols are typically between 0.3–10 μM (Reddy et al., 2005; Scalbert and Williamson, 2000; Sugisawa and Umegaki, 2002; van het Hof et al., 1998; Yamamoto et al., 2003; Yang et al., 1998), so MEPCA shows significant antioxidant effects at biologically relevant concentrations.

Table 1 summarizes the results obtained for each of the polyphenol compounds evaluated using this DNA damage assay (all gel electrophoresis results are provided in Figures S1–10 and Tables S1–S10 of the Supplementary Material). Four of these polyphenols, MEPCA, propyl gallate, epicatechin gallate, and MEGA prevent $^{N}PCuO/H_2O_2$-mediated damage. EGCG prevents a maximum of ~50% DNA damage only at very high concentrations (~800 μM). PCA, VA, and EC show no activity. Little correlation is observed between polyphenol DNA damage prevention ability (IC_{50} value) and polyphenol oxidation potential (Perron et al., 2008) ($R^2 = 0.42$) or the pK_a of the first phenolic hydrogen pK_a (Perron and Brumaghim, 2009) ($R^2 = 0.43$; Supplementary Material, Figure S11).

In contrast, adding EGC alone to plasmid DNA (Figure 3, lane 3) results in a significant amount of damaged DNA after 150 min. With $^{NP}CuO/H_2O_2$ and EGC addition (lanes 5–15), DNA is increasingly damaged as EGC concentration increases until it is so damaged with strand breaks at multiple sites that only a diffuse band at lower molecular weight is observed. Thus, EGC acts as a prooxidant to enhance DNA damage caused by $^{NP}CuO/H_2O_2$. GA also enhances $^{NP}CuO/H_2O_2$-mediated DNA damage but to a lesser degree than EGC (Table 1).

3.2 ROS detection using EPR spectroscopy

To more directly ascertain the ROS formed by $^{NP}CuO/H_2O_2$ under various conditions and to determine the effects of polyphenol addition on these ROS, EPR spectroscopy studies were performed. $^{NP}CuO/H_2O_2$ conditions generate primarily $^1O_2$, $O_2^{\bullet-}$, and $^{\bullet}OH$, likely by different mechanisms (Cu^+ reduction of hydrogen peroxide, electron transfer from ^{NP}CuO conduction band, polyphenol oxidation, etc.) (Angelé-Martínez et al., 2017; Bandara et al., 2005; Bondarenko et al., 2012; W. Chen et al., 2012; Gunawan et al., 2011; Hong et al., 2013; Jose et al., 2011; Y. Li et al., 2012; Pascholiano et al., 2008; Qin and Li, 2011; Shi et al., 2012; Song et al., 2016; Srikanth et al., 2015). Due to the short lifetime of these ROS, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was used as a spin trap in these experiments, since it can form distinguishable, long-lived adducts with $^{\bullet}OH$ and $O_2^{\bullet-}$ (Supplementary Material, Figure S12). The spin trap 2,2,5,5-tertramethyl-1-pyrroline-*N*-oxide (TEMP) was also used because it forms an adduct with $^1O_2$ (TEMPO; Supplementary Material, Figure S13) that can be distinguished from $^{\bullet}OH$ and $O_2^{\bullet-}$ adducts (Alia et al., 2001; Fufezan et al., 2002). Using DMPO and TEMP allows trapping of generated ROS to determine the relative amounts formed under different conditions. These EPR experiments were conducted with representative polyphenols that have differing effects on $^{NP}CuO/H_2O_2$-mediated DNA damage: MEPCA as an antioxidant, VA with no effect, and EGC as a prooxidant.

The room temperature EPR spectrum of $^{NP}CuO/H_2O_2$ shows a DMPO-OH adduct resonance derived from $^{\bullet}OH$ generation (Figure 4A). However, the EPR spectra of the superoxide salt K_2O under the same conditions also results in only DMPO-OH adduct resonances. Thus, although $^{\bullet}OH$ adduct is the only ROS detected under $^{NP}CuO/H_2O_2$ conditions, $O_2^{\bullet-}$ may also form and decompose rapidly to generate $^{\bullet}OH$ (Angelé-Martínez et al., 2017).

Addition of MEPCA, the polyphenol compound that most effectively prevents ^{NP}CuO-mediated DNA damage, to a mixture of ^{NP}CuO and H_2O_2 almost completely suppresses the DMPO-OH resonance intensity (Figure 4B). The remaining weak resonance observed upon MEPCA addition(Figure 4B) is due to superoxide generation and decomposition into DMPO-OOH, which in turn decomposes into DMPO-OH (Finkelstein et al., 1979).

In contrast, the EPR spectrum showed very little effect on the observed DMPO-OH resonance intensity upon VA addition (Figure 4C), whereas the EPR spectrum in the presence of the prooxidant EGC (Figure 4D) shows a DMPO-OH resonance with a two-fold increase over that of $^{NP}CuO/H_2O_2$ alone (Figure 4A). Generation of $^{\bullet}OH$ under these conditions directly follows the trend observed for polyphenol prevention of ^{NP}CuO-mediated DNA damage, suggesting that *in vitro* polyphenol antioxidant and prooxidant

activity with $^{NP}CuO/H_2O_2$ can be evaluated accurately by both DNA gel electrophoresis and by measuring the intensity of the DMPO-OH resonance using EPR spectroscopy.

Since copper chelation by polyphenols is well known (Galleano et al., 2010), to determine whether these polyphenol compounds prevent or promote ROS formation by interacting with dissolved copper or with the nanoparticle surface, additional EPR experiments were carried out with washed CuO nanoparticles (^{w}CuO). Thoroughly washing the nanoparticles before suspending them in aqueous solution results in a supernatant with less than 2.5 μM dissolved copper (from the original 5 mM stock), as measured using the bathocuproine method (Angelé-Martínez et al.). By removing dissolved copper from the ^{NP}CuO suspension supernatant, any ROS generated are likely due to H_2O_2 reaction with the nanoparticle surface, and polyphenol prevention of this ROS generation will likely be due to surface interactions (Angelé-Martínez et al., 2017).

In the EPR spectrum of $^{w}CuO/H_2O_2$ (Figure 4E), the DMPO-OH resonance is again observed, with an intensity slightly higher than that observed for the non-washed $^{NP}CuO/H_2O_2$ (Figure 4A). Upon MEPCA addition, this DMPO-OH resonance is completely suppressed, but a remaining weak resonance is observed (Figure 4F) that is similar to the resonance observed upon MEPCA addition to $^{NP}CuO/H_2O_2$. This resonance has been assigned previously to $O_2^{\bullet-}$ and $^{\bullet}OOH$ (Finkelstein et al., 1979; Hu and Jiang, 1996), indicating that MEPCA suppresses $^{\bullet}OH$, $O_2^{\bullet-}$, and $^{\bullet}OOH$ formation both at the nanoparticle surface and from dissolved copper in solution.

Prevention of ^{NP}CuO cytotoxicity requires that polyphenol compounds are antioxidants in the reducing environment of the cytoplasm and nucleus (Akthar et al., 2012; Bulcke and Dringen, 2015; Jing et al., 2015; Sharifi et al., 2012; Soliman et al., 2013; Zou et al., 2013). Thus, the ability of MEPCA to prevent hydroxyl radical production by $^{NP}CuO/H_2O_2$ was also examined in the presence of ascorbate, a reducing agent that can reduce Cu^{2+} to hydroxyl-radical-forming Cu^{+}) (Kadiiska and Mason, 2002; Perron et al., 2011). The EPR spectrum of ^{NP}CuO with H_2O_2 and 1.25 equivalents of ascorbate per copper ion shows the DMPO-OH adduct resonance as well as an additional resonance from a second ROS (Figure 5A). These additional resonances are similar to the weak resonances observed in Figures 4B and 4F and are consistent with superoxide formation (Finkelstein et al., 1979; Hu and Jiang, 1996) with subsequent decomposition into the hydroperoxide adduct (DMPO-OOH) and then to DMPO-OH (Finkelstein et al., 1979; Noda et al., 2008; Yamaguchi et al., 2000). The ascorbyl radical signal is not observed under these conditions.

Addition of MEPCA to the $^{NP}CuO/H_2O_2$/ascorbate solution has a significant effect on ROS generation (Figure 5B). The DMPO-OH resonance intensity is reduced by approximately 7-fold from the spectrum without MEPCA (Figure 5A), and no DMPO-$O_2^{\bullet-}$ resonance is observed. In addition, a new resonance from ascorbyl radical is observed in the spectrum as a doublet centered at g = 2.004 (Barbehenn et al., 2003; Boatright and Crum, 2016; Mouithys-Mickalad et al., 1998; Pietri et al., 1990; Warren and Mayer, 2008).

The EPR spectrum of $^{w}CuO/H_2O_2$/ascorbate shows resonances for ascorbyl radical, DMPO-OH, and DMPO-O_2 (Figure 5C). The intensities of the DMPO-OH and DMPO-O_2

resonances are similar to those observed in the $^{NP}CuO/H_2O_2$/ascorbate sample (Figure 5A), but the ascorbyl radical resonance is only observed in the presence of ^{w}CuO, not ^{NP}CuO. MEPCA addition to the $^{w}CuO/H_2O_2$/ascorbate system (Figure 5D) reduces the intensity of the DMPO-OH by 5-fold, the ascorbyl radical by 2-fold, and the DMPO-O_2 resonance to zero compared to samples without MEPCA.

The same EPR experiments were repeated using the TEMP spin trap, but no $^1O_2$ was detected. However, resonances similar to those reported for DMPO-O_2 are observed in the presence of $^{NP}CuO/H_2O_2$ and $^{NP}CuO/H_2O_2$/ascorbate (Figure S21). Thus, from these EPR results, MEPCA clearly suppresses $^{\bullet}OH$ and $O_2^{\bullet-}$ generation at the ^{NP}CuO surface or from dissolved copper with or without addition of ascorbate. Suppression of $^{\bullet}OH$ and $O_2^{\bullet-}$ formation by MEPCA demonstrates its ability to quench different ROS, either in the presence of the reducing agent ascorbate, on the surface of the nanoparticles, or by the dissolved ions from ^{NP}CuO.

3.3 Investigating $^{NP}CuO/H_2O_2$-mediated L929 cell death

Human lung and skin fibroblast cells play a pivotal role in many diseases including lung cancer, skin cancer, pulmonary hypertension, and pulmonary fibrosis and are often used in cell toxicology studies (Kalluri and Zeisberg, 2005). Mouse fibroblasts (L929) cell cultures were used to assess the toxicity of ^{NP}CuO at different doses (15.63–625 μM) after 24 h exposure. Cell viability decreased in a dose-dependent manner following ^{NP}CuO exposure (Figure 6A). At low concentrations (<15.63 μM), ^{NP}CuO treatment resulted in insignificant toxicity to L929 cells. For higher ^{NP}CuO exposures (31–313 μM), cell viability decreased with increasing concentration, falling to 0.2% of control at ≥ 313 μM. The effective concentration of ^{NP}CuO required to kill 50% of cells (EC_{50}) was calculated as 154 ± 15 μM (Table S12 and Figure S15, Supplementary Material).

^{w}CuO toxicity to L929 cells was also examined at 16–625 μM after 24 h (Figure 6A). Similar to ^{NP}CuO, cell viability upon ^{w}CuO exposure is concentration dependent, with an EC_{50} value of 170 ± 15 μM for ^{w}CuO (Table S13 and Figure S16, Supplementary Material). While cell viability was lower at every concentration for the washed nanoparticles than the unwashed particles, the overall IC_{50} value was just 10% higher for the unwashed particles (170 ± 15 μM vs. 154 ± 15 μM), and within the margin of error. Since the washing reduced the dissolved copper concentration by 2000-fold (5 mM to 2.5 μM, Angelé-Martínez et al.), the dissolved copper ions from solution must not play a significant role in toxicity, especially for the washed particles..

To confirm the similarity of ^{NP}CuO and ^{w}CuO particle sizes and their stability in cell medium, both types of nanoparticles were characterized. TEM images showed that ^{NP}CuO are roughly spherical, with a diameter of 50–60 nm (Angelé-Martínez et al., 2017). To test ^{NP}CuO stability in water and EMEM cell culture medium, a suspension was incubated in cell culture medium at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air for 24 h. The mean hydrodynamic diameter (weighted by intensity) of ^{NP}CuO in EMEM cell culture medium and in water measured by DLS is ~139 ± 2 nm and ~175 ± 14 nm, respectively (Table S19 and Figure S20, Supplementary Material). Similarly, the mean hydrodynamic diameter (weighted by intensity) of ^{w}CuO in EMEM cell culture medium and in water

measured by DLS is ~154 ± 11 nm and ~186 ± 13 nm, respectively (Table S11 and Figure S14, Supplementary Material). The size distributions of ^{NP}CuO and ^{w}CuO suspended in sterile, deionized water and EMEM cell culture medium, as measured by DLS analysis, showed larger values than the particle size measured by TEM. This indicates that the ^{NP}CuO and ^{w}CuO form aggregates in water and EMEM cell culture medium that are ~2 to 3 times larger than the primary particle sizes. ^{NP}CuO and ^{w}CuO appear to be moderately dispersed in water and EMEM cell culture medium; however, ^{NP}CuO and ^{w}CuO in EMEM medium formed smaller aggregates compared to ^{NP}CuO and ^{w}CuO in water. Proteins in the medium are known to form a corona on the surface of the nanoparticles which can affect aggregation and how they interact with cells (Monopoli et al., 2012). In addition, the composition of the medium may affect the rate and degree of copper dissolution from both types of nanoparticles. The washed particles had similar diameters to the unwashed particles both in deionized water and EMEM medium (slightly larger size for the washed particles but close to the margin of error), which suggests similar cellular uptake would be expected with and without washing.

Since dissolved copper can decrease cell viability by causing DNA damage and cell death (Aruoma et al., 1991) and by directly altering the expression of apoptotic genes (Chan et al., 2008), we tested whether copper released from ^{NP}CuO into the cell media contributes to L929 cytotoxicity. Release of copper ions from the surface of ^{NP}CuO was demonstrated when ^{NP}CuO were suspended in DMEM, and this released copper is responsible for some of the nanoparticles' toxic effects (Midander et al., 2009). To examine the contribution of dissolved copper on ^{NP}CuO toxicity in our system, cells were treated with $CuCl_2$ solutions (0.17–6.6 μM), and L929 cell viability assays were performed to compare the cell-damaging effects of dissolved copper in the ^{NP}CuO supernatant with those of $CuCl_2$ solutions at comparable dissolved copper concentrations, as calculated based on our previous work (Angelé-Martínez et al., 2017). Less than 30% of non-viable cells were observed when L929 cells were incubated with different concentrations of $CuCl_2$ solutions for 24 h (Table S14 and Figure S17, Supplementary Material). Our data suggest that even if copper ions were released from ^{NP}CuO into the cell culture media, they do not significantly contribute to cytotoxicity or the oxidative damage associated with ^{NP}CuO nanoparticle exposure. This result is consistent with previous findings demonstrating that dissolved copper from ^{NP}CuO is insufficient to produce mortality in zebrafish (Griffitt et al., 2007).

We also investigated the effect of the organic dispersant in the supernatant of ^{NP}CuO suspensions. This is the supernatant after the first centrifugation to separate the nanoparticles from the dissolved copper, but with dissolved copper removed (^{l}CuO). High concentrations of ^{l}CuO (12 mM) showed less than 30% non-viable L929 cells (70% viable) (Table S15 and Figure S18, Supplementary Material). Thus, at the much lower concentrations used in our experiments (typically 62.5 μM) the effect of this dispersant should be negligible.

L929 cell viability in the presence of H_2O_2 was also examined after 24 h. As shown in Figure 6B, increased cell killing results from H_2O_2 addition up to 1000 μM, at which less than 0.8% of treated cells survive. The EC_{50} value for H_2O_2 alone was measured to be 152 ± 9 μM (Table S16 and Figure S19, Supplementary Material). When ^{NP}CuO and H_2O_2 were combined, L929 cell survival also decreased in a dose-dependent manner (Figure 6B), and

cells are killed much more effectively by $^{NP}CuO/H_2O_2$ than by H_2O_2 or ^{NP}CuO alone. In fact, ^{NP}CuO or H_2O_2 alone at 50 μM concentration causes no change in cell viability, but the combination of $^{NP}CuO/H_2O_2$ at 50 μM each cause almost 90% cell death. These results demonstrate that $^{NP}CuO/H_2O_2$ system is highly toxic to L929 cells, and this combination causes greater-than-additive cell killing compared to either ^{NP}CuO or H_2O_2 alone.

3.4 Effect of MEPCA and MEGA on $^{NP}CuO/H_2O_2$-mediated L929 cell death

Since MEPCA was the only polyphenol compound to prevent ^{NP}CuO-mediated DNA damage at blood polyphenol concentrations (0.3–10 μM (Reddy et al., 2005; Scalbert and Williamson, 2000; Sugisawa and Umegaki, 2002; van het Hof et al., 1998; Yamamoto et al., 2003; Yang et al., 1998); MEPCA IC_{50} = 7.5 μM), we tested this polyphenol as well as its gallol analog, MEGA, for their ability to inhibit $^{NP}CuO/H_2O_2$-mediated L929 cell death (Tables S18 and S19 and Figures S21 and S22 in the Supplementary Material, respectively). MEGA has no effect on L929 viability at these concentrations (Schlickmann et al., 2017), and protocatechuic acid and its esters show no tocxicity to L929 cells up to 50 μM (Daré et al., 2020). Upon treatment with $^{NP}CuO/H_2O_2$ (153.5 and 80 μM, respectively), only 17% of cells were viable. MEPCA or MEGA addition (1 μM) reduced $^{NP}CuO/H_2O_2$ cytotoxicity, resulting in 81% and 71% cell viability, respectively (Figure 7A). Increasing polyphenol concentrations to 10 μM decreases this viability rescue, and above 50 μM, MEPCA or MEGA treatment shows no significant rescue of $^{NP}CuO/H_2O_2$ toxicity.

4. Discussion

4.1 Polyphenol effects on $^{NP}CuO/H_2O_2$–mediated DNA damage and ROS generation

Plasmid DNA damage assays evaluate both antioxidant and prooxidant polyphenol activity, and both behaviors were observed in these studies. The catechol-containing MEPCA is the most effective polyphenol for prevention of ^{NP}CuO-mediated DNA damage, with an IC_{50} value within the physiological range of blood polyphenol concentrations (0.3–10 μM) (Reddy et al., 2005; Sugisawa and Umegaki, 2002; van het Hof et al., 1998; Yamamoto et al., 2003; Yang et al., 1998). Catechol-containing polyphenols more effectively prevent DNA damage than their gallol analogs. The more potent ECG differs from EGCG only in the presence of a catechol group on the B-ring (Figure 1) instead of the gallol group in EGCG, indicating that the presence of a catechol group confers greater antioxidant behavior. Similarly, the carboxylic-acid-containing GA is a prooxidant under these conditions, whereas its methyl ester PCA has no activity. In addition, gallol-containing GA and EGC are prooxidants, promoting DNA damage. In bacteria, gallol-containing polyphenols are observed to promote DNA damage, induce apoptosis, and inhibit growth (Chuang et al., 2010; Hanasaki et al., 1994; Heim et al., 2002; Liu et al., 2013; Long et al., 2010; Nemeikaite-Ceniene et al., 2005; Yamashita et al., 1999; Yoshino et al., 1999). Carboxylic acid functional groups on the phenol ring also leads to lower polyphenol DNA damage prevention ability. MEPCA, the methyl ester of the carboxylate-containing PCA, is the most effective polyphenol tested, whereas PCA does not prevent $^{NP}CuO/H_2O_2$-mediated DNA damage. Likewise, the methyl ester MEGA inhibits DNA damage from $^{NP}CuO/H_2O_2$, but the corresponding carboxylate GA increases DNA damage under similar conditions.

The antioxidant and prooxidant activity of the polyphenols with $^{NP}CuO/H_2O_2$-mediated DNA damage differs somewhat from that observed for Cu^+-mediated DNA damage (Table 1). Some differences are expected due to varying DNA assay times (150 min for ^{NP}CuO and 30 min for Cu^+) and H_2O_2 concentrations (400 μM for ^{NP}CuO and 50 μM for Cu^+). In addition, ascorbate is added to reduce Cu^{2+} to Cu^+ for the copper-mediated DNA damage studies but not for the ^{NP}CuO-mediated DNA damage assays. Nonetheless, MEPCA, PREGA, ECG, MEGA, and EGCG exhibit primarily antioxidant effects under both $^{NP}CuO/H_2O_2$- and Cu^+/H_2O_2-mediated DNA damage conditions, although PREGA, ECG, and MEGA show slight prooxidant effects at lower concentrations (0.1–10 μM) in the Cu^+/H_2O_2 system (Table 1). MEPCA and PREGA have the same IC_{50} values under both DNA-damaging conditions, but 2- to 3-fold higher concentrations of ECG, MEGA, and EGCG are required to prevent the same amount of DNA damage by $^{NP}CuO/H_2O_2$ or Cu^+/H_2O_2.

In the presence of Cu^{2+}, EGCG, ECG, EGC, and catechin promote copper-mediated DNA damage (Furukawa et al., 2003). Resveratrol also causes slight $^{\bullet}OH$-mediated DNA damage in the presence of Cu^{2+} that is enhanced upon H_2O_2 addition (Burkitt and Duncan, 2000). Thus, polyphenol effects on $^{NP}CuO/H_2O_2$-mediated DNA damage (Table 1) differ somewhat from their effects on either Cu^+- or Cu^{2+}-mediated DNA damage. Since DNA-damaging $^{NP}CuO/H_2O_2$ effects result from ROS generated by both dissolved copper and on the nanoparticle surface (Angelé-Martínez et al., 2017), differences in antioxidant or prooxidant activity between ^{NP}CuO- and copper-mediated DNA damage may be a consequence of different mechanisms of ROS generation at the ^{NP}CuO surface or differing interactions between the polyphenols and dissolved copper compared to the nanoparticle surface.

Although interactions of polyphenols with ^{NP}CuO are not well investigated, density functional theory calculations of Cu^+ binding to flavonoid compounds in the gas phase demonstrate the importance of the catechol group on the B-ring for copper binding; in the absence of a carbonyl group on the C-ring (Figure 1) the preferred Cu^+ binding site is between the deprotonated phenolic oxygens on the B ring (Kazazic et al., 2006). UV spectra of flavones and flavonols change upon Cu^{2+} addition and return to the original spectra after addition of Cu^{2+} chelators EDTA or DTPA, demonstrating the metal chelation ability of the catechol group in the B-ring and suggesting that polyphenol-copper binding affects DNA damage (Brown et al., 1998). VA has no effect on ^{NP}CuO-mediated DNA damage, likely because its lone phenolic group reduces metal binding ability (Khokhar and Owusu-Apenten, 2003; Melidou et al., 2005) so that it cannot effectively bind copper in solution or on the nanoparticle surface.

Similar to trends observed for $^{NP}CuO/H_2O_2$-mediated DNA damage, catechol-containing polyphenols more effectively prevent Cu^+-mediated DNA damage than gallol-containing polyphenols (Perron et al., 2011). Under copper-mediated DNA damage conditions, this catechol-antioxidant activity is explained by the formation of a stable Cu^{2+} complex with high affinity constant that does not reduce to hydroxyl-radical-generating Cu^+ (Reaction 1) (Bhattacharya and Patel, 1985; Perron et al., 2011). The prooxidant activity of dopamine, EC, ECG, EGC, GA, MEGA, and PREGA in Cu^+-mediated DNA damage assays is explained by the formation of a polyphenol-Cu^{2+} complex, but it this case, the complex

then undergoes intramolecular electron transfer, yielding an unstable Cu^{+} complex that dissociates to make Cu^{+} available for $^{\bullet}OH$ generation (Perron et al., 2011). Thus, copper binding, either in solution or at the nanoparticle surface may be responsible for both the observed antioxidant and prooxidant effects. In addition, ROS other than $^{\bullet}OH$ radical are generated by ^{NP}CuO surface reactions and possible electron transfer from the ^{NP}CuO conduction band (Angelé-Martínez et al., 2017), and these differences in ROS generation may also contribute to differences between polyphenol effects on $^{NP}CuO/H_2O_2$- and Cu^{+}/H_2O_2-medated DNA damage.

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH + OH^{-} \quad [1]$$

4.2 ROS detection using EPR spectroscopy

EPR results with $^{NP}CuO/H_2O_2$ and $^{NP}CuO/H_2O_2$/ascorbate indicate that MEPCA prevents generation of $^{\bullet}OH$ and $O_2^{\bullet-}$ radicals. Because this reactivity is observed with both $^{NP}CuO/H_2O_2$ and $^{w}CuO/H_2O_2$, these results suggest that MEPCA interacts with the copper ions on the surface of the nanoparticle. A similar effect was observed upon adding a series of polyphenols to ZnO samples irradiated with UV light; H_2O_2 generation was reduced compared to non-polyphenol-treated nanoparticles (Markham et al., 1954).

Mechanisms for $^{\bullet}OH$ generation by ^{NP}CuO include reduction of H_2O_2 by electron transfer from the ^{NP}CuO conduction band (Reaction 2) or by electron transfer to Cu^{2+} to form hydroxyl-radical-generating Cu^{+} (Reaction 3) (Pascholiano et al., 2008). Generation of $O_2^{\bullet-}$ also has been postulated to occur by electron transfer from the ^{NP}CuO conduction band to O_2, where the limiting step is O_2 adsorption on the nanoparticle surface (Reaction 4) (Bandara et al., 2005; Pascholiano et al., 2008; Shi et al., 2012), or from reduced Cu^{+} (Reaction 5) (Rowley and Halliwell, 1983). Once $O_2^{\bullet-}$ is generated, $^{\bullet}OH$ may form through the Cu^{2+}-assisted Haber-Weiss process (Reaction 6). As corroborated by EPR results, the ability of EGC to promote DNA damage by $^{NP}CuO/H_2O_2$ results from its ability to increase $^{\bullet}OH$ (or preliminary $O_2^{\bullet-}$) generation under these conditions. Polyphenol effects on ROS generation by $^{NP}CuO/H_2O_2$ and $^{NP}CuO/H_2O_2$/ascorbate do not correlate with their first-phenolic-hydrogen pK_a (Perron and Brumaghim, 2009) or their oxidation potentials (Perron et al., 2008), indicating complexity in both radical generation and copper/^{NP}CuO-polyphenol interactions. The strong correlation between EPR results and polyphenol antioxidant/prooxidant activity suggests that this method can be used to screen polyphenols for their affects on ^{NP}CuO-mediated DNA damage.

$$e^{-}_{cb} + H_2O_2 \rightarrow {}^{\bullet}OH + {}^{-}OH \quad [2]$$

$$e^{-}_{cb} + Cu^{2+} \rightarrow Cu^{+} \quad [3]$$

$$e^{-}_{cb} + O_2 \rightarrow O_2^{\bullet -} \quad [4]$$

$$Cu^{+} + O_2 \rightarrow Cu^{2+} + O_2 \bullet^{-} \quad [5]$$

$$O_2 \bullet^{-} + H_2O_2 \rightarrow O_2 + {}^{\bullet}OH + {}^{-}OH \quad [6]$$

4.3 Cell viability analysis

Our results demonstrate that $^{NP}CuO/H_2O_2$ induces cytotoxicity in L929 cells significantly more than either component alone. This cytotoxicity is likely due to production of intracellular ROS by ^{NP}CuO under hydrogen-peroxide induced oxidative stress. Both MEPCA and MEGA significantly reduce $^{NP}CuO/H_2O_2$ cytotoxicity at 1 μM, a concentration within the physiological polyphenol range of 0.3–10 μM in blood (Reddy et al., 2005; Scalbert and Williamson, 2000; Sugisawa and Umegaki, 2002; van het Hof et al., 1998; Yamamoto et al., 2003; Yang et al., 1998). The IC_{50} measured for $^{NP}CuO/H_2O_2$-mediated DNA damage prevention assay is significantly higher than 1 μM (7.5 for MEPCA and 185 for MEGA); however, these cellular and DNA damage assays are not directly comparable because ^{NP}CuO and the polyphenols must be taken up and trafficked in cells, unlike in the DNA assays. In addition, MEPCA and MEGA can interact with cells through multiple pathways aside from direct DNA damage inhibition (Reddy et al., 2005; Sugisawa and Umegaki, 2002; van het Hof et al., 1998; Yamamoto et al., 2003; Yang et al., 1998).

Our results are qualitatively consistent with other studies showing antioxidants can rescue cells exposed to copper or ^{NP}CuO. For example, addition of sulphoraphane (6 μM) to mouse BALB 3T3 cells exposed to 10 μg/mL ^{NP}CuO reduced cytotoxicity (34% to 63% viable cells) and also reduced glutathione oxidative stress biomarkers (Akthar et al., 2012). Similarly, addition of the polyphenol resveratrol at high concentrations (100 μM) partially reversed ^{NP}CuO-induced cell killing in Hep-2 airway epithelial cells (33% to 58% viable cells) and reduced cellular oxidative stress biomarkers (Fahmy and Cormier, 2009). Our results are not directly comparable to these prior cell studies because we used different cell lines and included H_2O_2 in the challenge, a common source of oxidative stress that is known to produce ROS in the presence of copper (Reaction 1). Under these conditions, we have demonstrated that the MEPCA and MEGA polyphenols have a substantially larger effect at lower concentrations (1 μM increases cell viability by 64% and 54% for MEPCA and MEGA, respectively). Overall, the results show that hydrogen peroxide significantly increases ^{NP}CuO cytotoxicity and that the polyphenols MEPCA and MEGA protect against $^{NP}CuO/H_2O_2$-mediated DNA damage and cytotoxicity.

5. Conclusions

The activity of polyphenols to affect ^{NP}CuO-mediated DNA in the presence of H_2O_2 ranges from potent antioxidant activity (MEPCA), to no activity (PCA), to potent prooxidant activity (EGC). Polyphenol effects on $^{NP}CuO/H_2O_2$-mediated and Cu^{+}-mediated DNA damage are substantially similar. ^{NP}CuO-generated ROS were monitored by EPR spectroscopy and correlate with DNA assay results for the three observed polyphenol effects (antioxidant, no activity and prooxidant), indicating that this method can be used as a

screening tool for polyphenol prevention of DNA damage by ^{NP}CuO and H_2O_2. In addition, we found that combining H_2O_2 with ^{NP}CuO significantly increases cytotoxicity compared to either component alone.

Our work demonstrates that two polyphenols, MEPCA and MEGA, prevent $^{NP}CuO/H_2O_2$-mediated L929 cytotoxicity at physiologically relevant (1 μM) concentrations. The differences in behavior observed for polyphenol prevention or promotion of $^{NP}CuO/H_2O_2$–mediated DNA damage and polyphenol prevention of $^{NP}CuO/H_2O_2$ cytotoxicity highlight the need for additional investigation into polyphenol-nanoparticle interactions as well as their biological effects to identify polyphenols that prevent ^{NP}CuO toxicity under oxidative stress conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

^{NP}CuO	copper oxide nanoparticles
^{w}CuO	washed copper oxide nanoparticles
^{l}CuO	leachate of copper oxide nanoparticles
DMEM	dulbecco’s modified Eagle’s medium
DMPO	5,5-dimethyl-1-pyrroline-*N*-oxide
EC	epicatechin
ECG	epicatechin gallate
EGC	epigallocatechin
EGCG	epigallocatechin gallate
EPR	electron paramagnetic resonance spectroscopy
FBS	fetal bovine serum
GA	gallic acid
L929	mouse fibroblast (L929) cells
MEGA	methyl-3,4,5-trihydroxybenzoate

MEPCA	methyl-4,5-dihydroxybenzoate
MOPS	3-(*N*-morpholino)propanesulfonic acid
PCA	protocatechuic acid
PREGA	propyl gallate
ROS	reactive oxygen species
TEMP	2,2,5,5-tertramethyl-1-pyrroline-*N*-oxide
TEMPO	singlet oxygen adduct of TEMP
VA	vanillic acid

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Research Highlights

- Polyphenol antioxidants were tested for their effects on DNA damage and cytotoxicity by CuO nanoparticles (^{NP}CuO) and hydrogen peroxide
- Half of the ten tested polyphenols prevent $^{NP}CuO/H_2O_2$-mediated DNA damage, but two increase DNA damage
- Levels of reactive oxygen species generated by $^{NP}CuO/H_2O_2$ with polyphenols correlate with their DNA damaging effects
- Combining ^{NP}CuO and H_2O_2 is significantly more cytotoxic than either component alone
- Polyphenols MEPCA and MEGA prevent $^{NP}CuO/H_2O_2$-induced cell death in mouse fibroblast cells

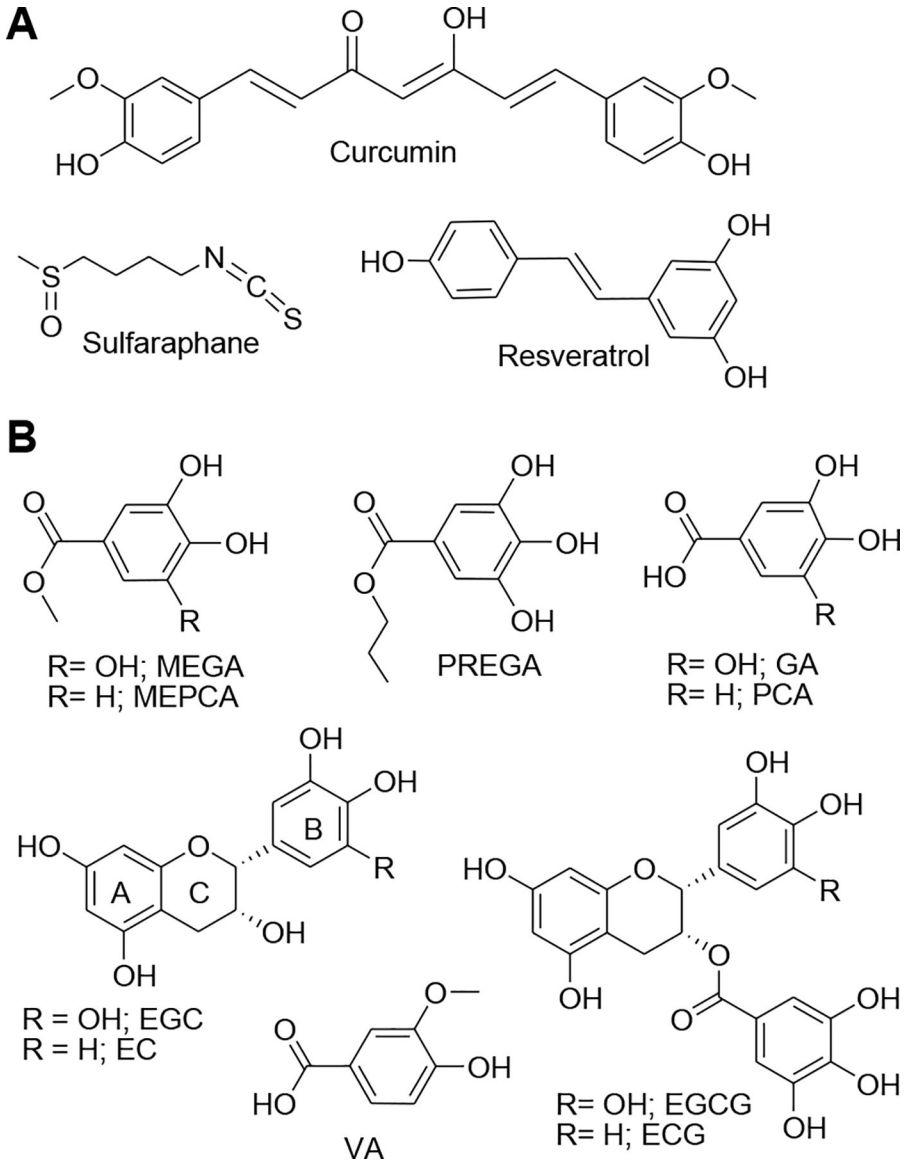

Figure 1.

A) Molecular structures of sulforaphane, curcumin, and resveratrol used to prevent nanoparticle toxicity, and B) polyphenolic compounds evaluated for their abilities to prevent ^{NP}CuO-mediated damage in this study.

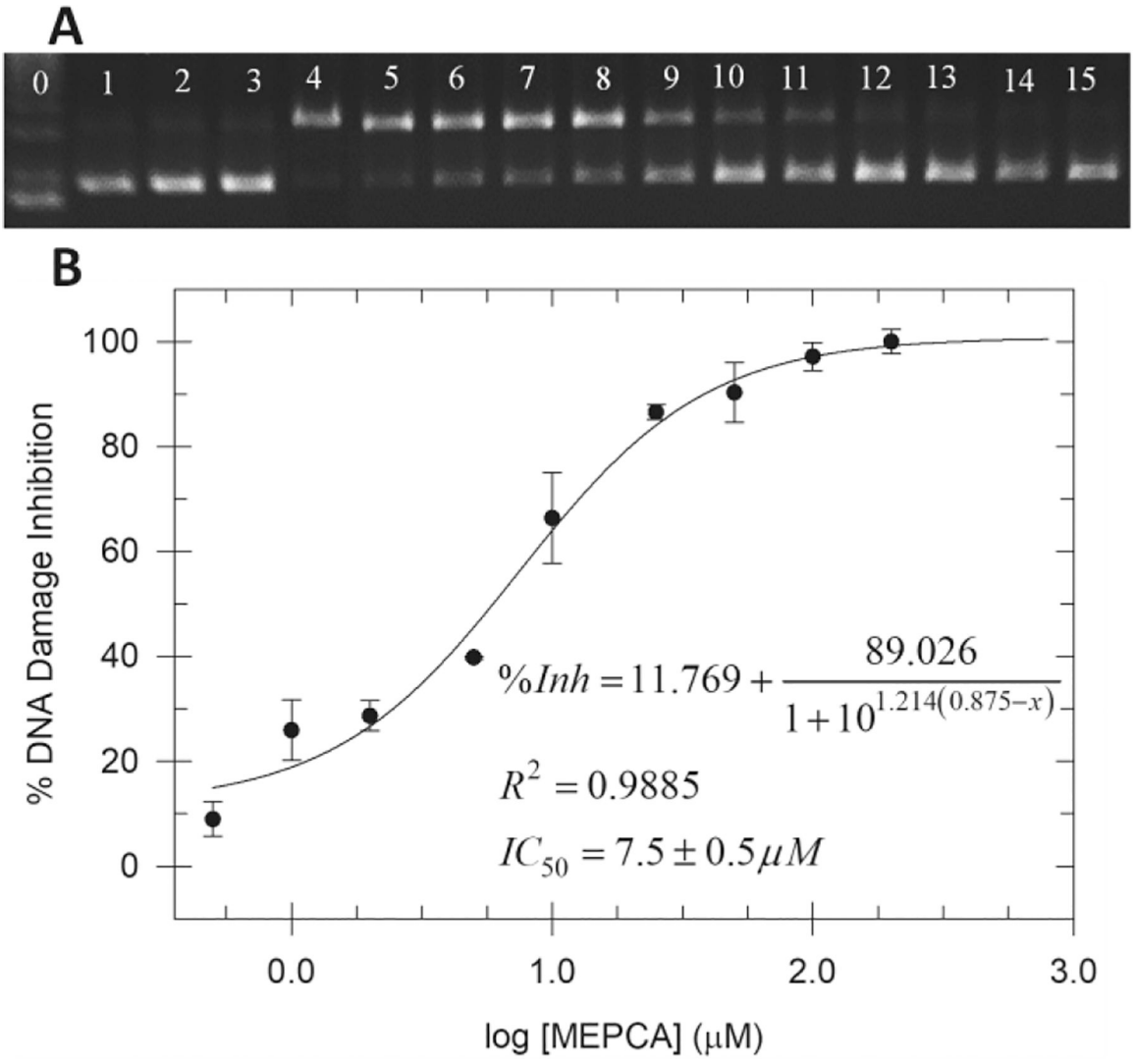

Figure 2.

A) Gel electrophoresis image of plasmid DNA treated with ^{NP}CuO (500 μM), H_2O_2 (50 μM), and MEPCA (0.5–800 μM) at pH 7 (MOPS buffer) for 150 min. Lane 0: 1 kb molecular weight ladder; lane 1: plasmid (p); lane 2: p + H_2O_2 (50 μM); lane 3: p + MEPCA (800 μM); lane 4: p + ^{NP}CuO (500 μM) + H_2O_2 (50 μM); lanes 5–15: p + ^{NP}CuO (500 μM) + and H_2O_2 (50 μM) + increasing concentrations of MEPCA (0.5, 1, 2, 5, 10, 25, 50, 100, 200, 400, and 800 μM, respectively). Damaged (nicked) plasmid DNA is in the top band, undamaged (supercoiled) DNA is in the bottom band, and the brightest band in lane 0, just below the undamaged DNA band, is the 3 kb DNA marker. B) Percentage of DNA damage inhibition with respect to MEPCA concentration in the presence of ^{NP}CuO and H_2O_2 fit with a sigmoidal dose-response curve.

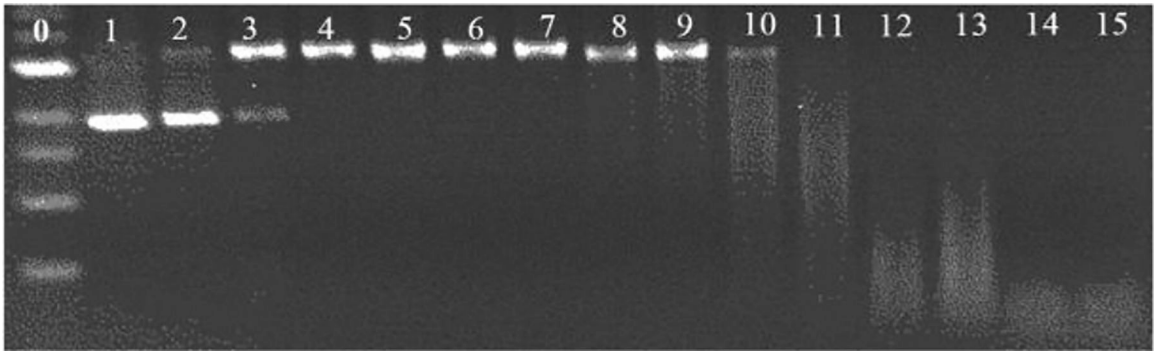

Figure 3.

Gel electrophoresis image of plasmid DNA treated with ^{NP}CuO (500 μM), H_2O_2 (50 μM), and EGC (0.5 – 800 μM) at pH 7 (MOPS buffer) for 150 min. Lane 0: 1 kb molecular weight ladder **(with bands labeled in** Figure S10); lane 1: plasmid (p); lane 2: p + H_2O_2 (50 μM); lane 3: p + EGC (800 μM); lane 4: p + ^{NP}CuO (500 μM) + H_2O_2 (50 μM); lanes 5–15: p + ^{NP}CuO (500 μM) + H_2O_2 (50 μM) + increasing concentrations of EGC (0.5, 1, 2, 5, 10, 25, 50, 100, 200, 400, and 800 μM, respectively). Damaged (nicked) plasmid DNA is in the top band, undamaged (supercoiled) DNA is in the bottom band, and the diffuse bands at lower molecular weights indicate significant DNA damage with many strand breaks per plasmid.

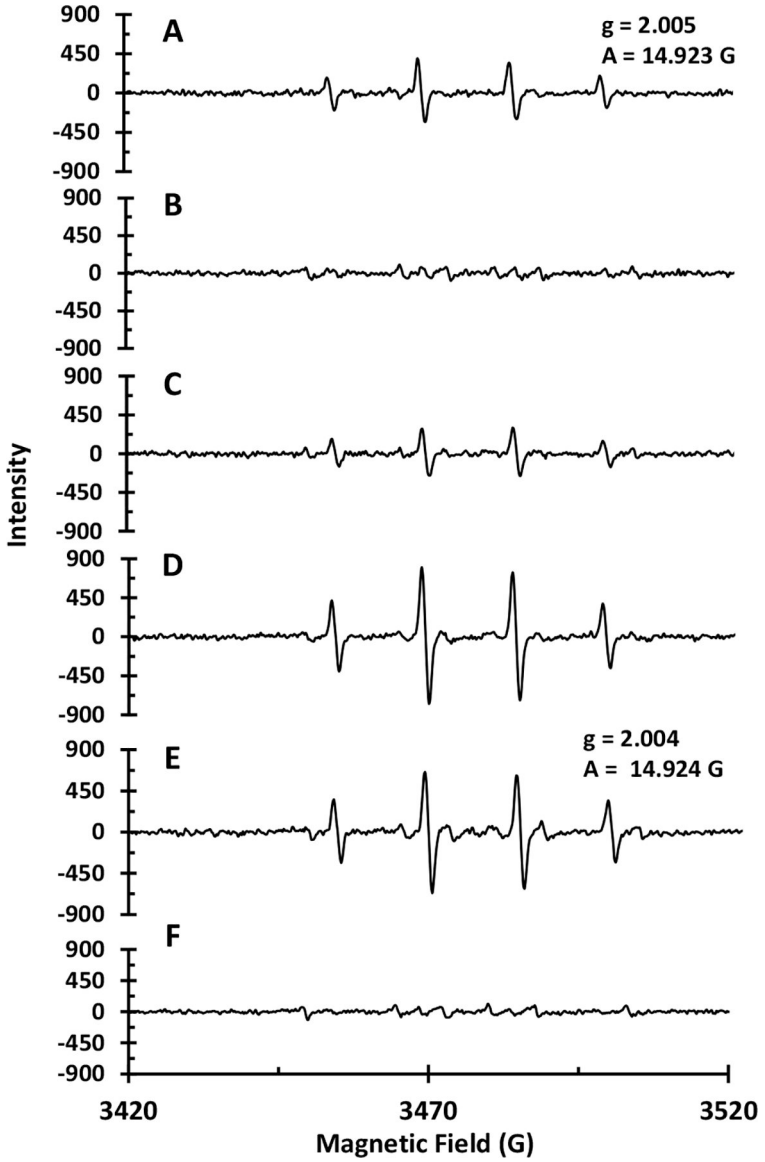

Figure 4.

EPR spectra of A) ^{NP}CuO (300 μM) and H_2O_2 (22.5 mM); B) ^{NP}CuO (300 μM), H_2O_2 (22.5 mM), and MEPCA (600 μM); C) ^{NP}CuO (300 μM), H_2O_2 (22.5 mM), and VA (600 μM); D) ^{NP}CuO (300 μM), H_2O_2 (22.5 mM), and EGC (600 μM); E) ^{w}CuO (300 μM) and H_2O_2 (22.5 mM); and (F) ^{w}CuO (300 μM), H_2O_2 (22.5 mM), and MEPCA (600 μM). All spectra were collected at room temperature and pH 7 (MOPS, 10 mM) with DMPO (30 mM) as a spin trap.

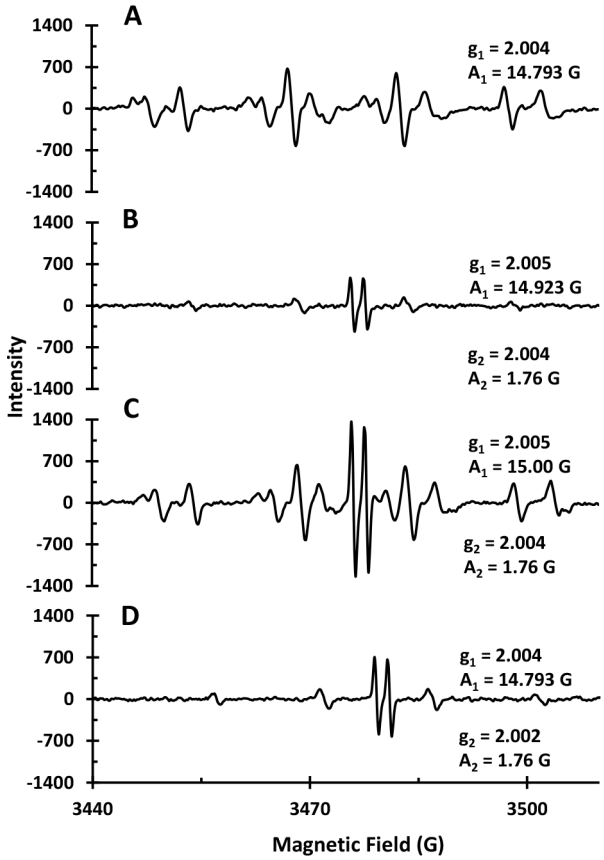

Figure 5.

EPR spectra of: A) ^{NP}CuO (300 μM), ascorbate (375 μM), and H_2O_2 (22.5 mM); B) ^{NP}CuO (300 μM), ascorbate (375 μM), H_2O_2 (22.5 mM) , and MEPCA (600 μM); C) ^{w}CuO (300 μM), ascorbate (375 μM), and H_2O_2 (22.5 mM); and D) ^{w}CuO (300 μM), ascorbate (375 μM), H_2O_2 (22.5 mM), and MEPCA (600 μM). All spectra were acquired at room temperature and pH 7 (MOPS, 10 mM) with DMPO (30 mM) as a spin trap.

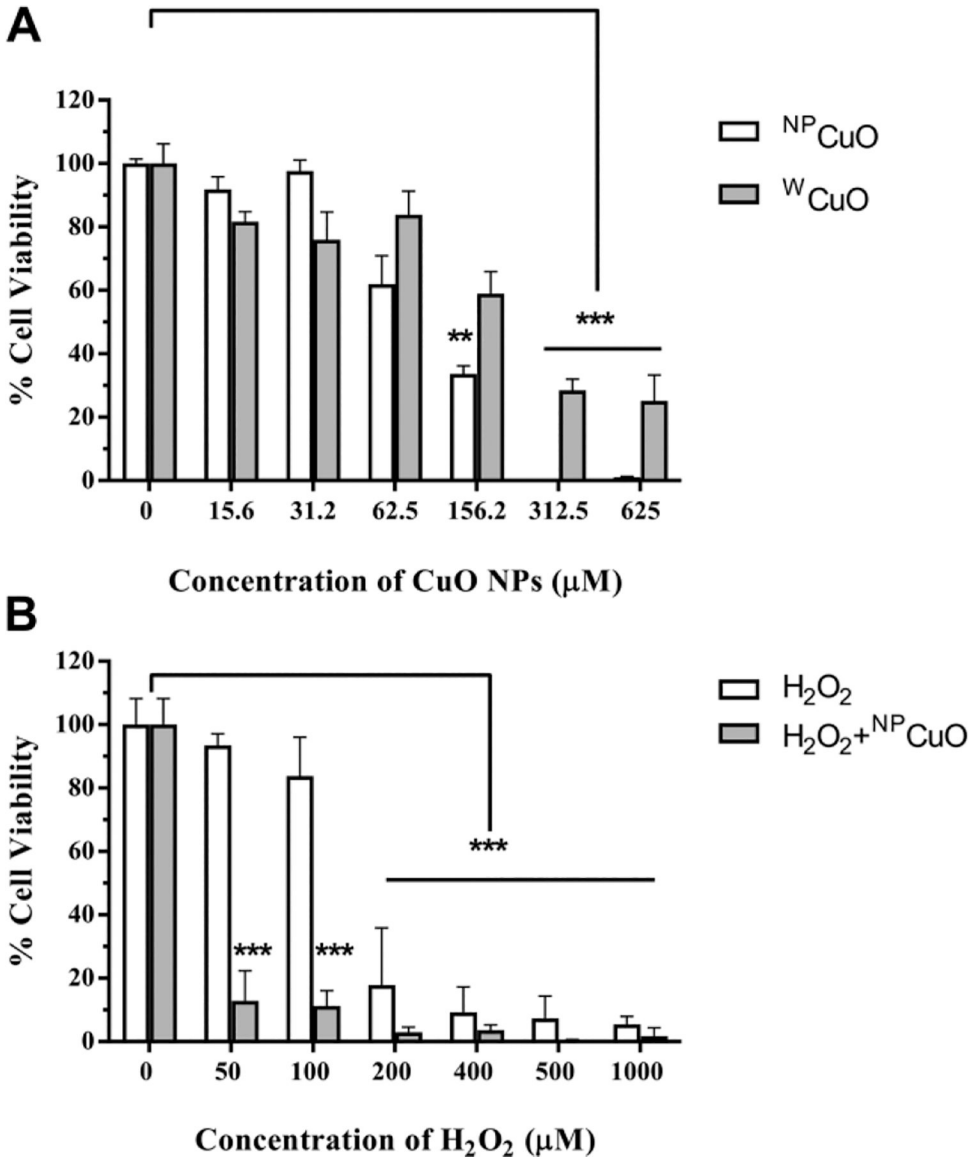

Figure 6.

L929 cell viability measured with the MTS assay after exposure to different conditions: cell viability after exposure to A) different concentrations of ^{NP}CuO or ^{W}CuO nanoparticle suspensions for 24 h for ^{NP}CuO or ^{W}CuO concentrations from 15.6–625 μM and B) cell viability after 24 h exposure to ^{NP}CuO (62.5 μM) and varying H_2O_2 concentrations (50–1000 μM). Data are expressed as means ± standard deviations (n = 4); ** *p*-value <0.01 and *** *p*-value <0.001.

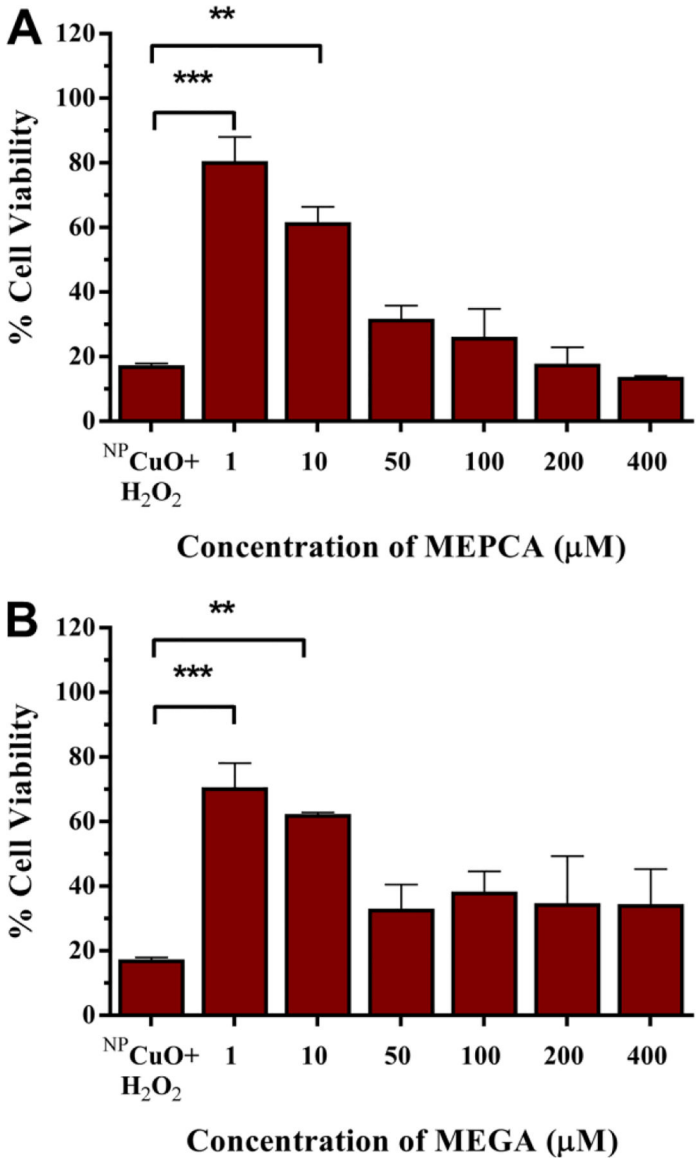

Figure 7.

L929 cell viability measured by MTS assay after exposure to $^{NP}CuO/H_2O_2$ (153.5 μM and 80 μM, respectively) and A) MEPCA (1–400 μM) and B) MEGA (1–400 μM). Data expressed as means ± standard deviations (n = 4); ** *p*-value <0.01, and *** *p*-value <0.001.

Table 1.

Polyphenol affects on DNA damage by ^{NP}CuO or Cu^{+} and H_2O_2.

Polyphenol	IC_{50}[a] (μM) with $^{NP}CuO/H_2O_2$	IC_{50} (μM) with Cu^{+}/H_2O_2[b]
MEPCA	7.5 ± 0.5	8.24 ± 0.03
PREGA	112 ± 19	125.90 ± 0.02 (prooxidant 0.2–10 μM)[c]
ECG	120 ± 22	53.04 ± 0.02 (prooxidant 0.1–4 μM)[c]
MEGA	185 ± 8	102.3 ± 0.1 (prooxidant 0.2–10 μM)[c]
EGCG	~800[d]	225.9 ± 0.1
PCA	No activity	~480
VA	No activity	No activity
EC	No activity	Prooxidant only 0.2–500 μM
GA	Prooxidant	Prooxidant 4–10 μM; damage prevention ≥ 300 μM[c,d]
EGC	Prooxidant	Prooxidant only 0.2–1000 μM

[a] Concentration that inhibits 50% DNA damage

[b] Cu^{+} concentration = 6 μM; H_2O_2 concentration = 50 μM (Perron et al., 2011)

[c] Polyphenols are prooxidants at lower concentrations and antioxidants at higher concentrations

[d] A complete IC_{50} curve was not obtained due to concentration limitations