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Toxicity of copper salts is dependent on solubility profile and cell type tested

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ABSTRACT

Copper (Cu) is considered an essential metal for living organisms. However, disruption of Cu homeostasis is toxic and can lead to disorders such as Menkes and Wilson's diseases. The brain appears to be a vulnerable target organ. This study investigated the toxicity of Cu based on its solubility profile and cell type tested. Human A-172 (glioblastoma), SK-N-SH (neuroblastoma) and CCF-STTG1 (astrocytoma) cells were assessed after exposure to different concentrations (0.5–500 μM) of copper sulfate ($CuSO_4$) or copper (II) oxide (CuO). Since Cu is a redox active transition metal, we hypothesized that oxidative stress would be the main mechanism underlying cell toxicity. Therefore, cell viability was correlated with the extent of reactive oxygen species (ROS) formation. Cell viability decreased at the higher concentrations of the Cu salts and CuO was more toxic compared to $CuSO_4$. The astrocytoma and glioblastoma cells were more vulnerable compared to the neuronal cells. Furthermore, it appears that oxidative stress only partially accounts for Cu-induced cell toxicity. Further studies are needed to better understand the unique susceptibility of glial cells and determine the physicochemical properties of insoluble Cu which accounts for its enhanced toxicity.

1. Introduction

Copper (Cu) is the 29th element on the periodic table and is classified as a transition metal. It is used widely in industry because of its malleable nature, alloying characteristics and electrical conductivity (Barceloux, 1999). Copper can exist in different oxidation states. These are mainly copper (I) and copper (II), which are referred to as cuprous and cupric, respectively. Exposure to copper can occur occupationally, as it is used in numerous applications such as machinery, electronics and copper smelting (Gaetke and Chow, 2003). Copper (II) sulfate ($CuSO_4$) is a soluble inorganic salt that is mainly used as a fungicide to control bacterial and fungal infections in a variety of crops (Michaud and Angela, 2003). Copper (II) oxide (CuO) is an insoluble compound used as a semiconductor in industry (Fortunato et al., 2012). It is also used in commercial products for its antimicrobial properties (Baek and An, 2011). Cu is used in household plumbing and corrosion of these pipes can cause levels to increase in the water supply. Exposure to Cu occurs through various routes. The most common is the oral route because the metal is present in many foods and drinking water (Armstrong et al., 1983). Although an essential metal, at high concentrations, Cu is toxic and the United States Environmental Protection Agency has set the limit of exposure for Cu in drinking water at 1.3 mg/L. This is equivalent to approximately 50 μM $CuSO_4$ and 25 μM CuO. Cu is mainly excreted in the bile and only about 3% is excreted through renal clearance. Either deficit or excess can lead to deleterious effects in the body and tight regulation of copper levels is essential for keeping the levels at an optimum. The brain and liver are the main organs susceptible to Cu toxicity (Linder and Hazegh-Azam, 1996; Tapiero et al., 2003).

Wilson's disease (WD) is a rare genetic disorder associated with accumulation of copper. The pathology is due to impaired function of a mutated copper transporter. The excess of copper can lead to hepatic cirrhosis and neurological problems due to progressive basal ganglia degeneration in the brain (Kitzberger et al., 2005; Strecker et al., 2006; Desai and Kaler, 2008). Cu has also been implicated in the pathogenesis of Alzheimer's disease (Molina et al., 1998; Lovell, 1998; Squitti et al., 2002; Sparks and Schreurs, 2003; Bush et al., 2003; Miller et al., 2005). The mechanism by which Cu accumulation can cause toxicity in the brain is unknown. However, since Cu is a redox active transition metal, oxidative stress has been suggested to be one of the mechanisms of toxicity (Deibel et al., 1996; Sayre et al., 1999; Perry et al., 2003; Becaria et al., 2003).

The hypothesis tested is that Cu toxicity depends on the metal salt used and neuroblastoma cells are more sensitive compared to glial cell lines. Furthermore, because Cu is redox-active, reactive oxygen species formation is the main mechanism responsible for cell death. Brain derived human cell lines were used to define the cell type most sensitive to a 24 h Cu exposure. Human neuroblastoma (SK-N-SH), astrocytoma (CCF-STTG1) and glioblastoma (A-172) cells were used as the type-specific cell culture model to assess cell proliferation, viability, and oxidative stress. Two

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different Cu salts, one soluble and one insoluble, were used to determine how the physicochemical characteristics of the metal salts may influence viability, and reactive oxygen species (ROS) formation. Our result show that CuO is more toxic compared to $CuSO_4$. In contrast to what we had hypothesized, cells of glial origin were more susceptible compared to the neuroblastoma cell line. Furthermore, cell viability did not always correlate with enhanced ROS formation which indicates that mechanisms other than oxidative stress may underlie Cu-induced toxicity.

2. Methods and materials

Human cell lines were purchased from ATCC. All tissue culture supplies were obtained from GIBCO. Cell proliferation and cell viability/cytotoxicity kit were purchased from Invitrogen.

2.1. Cell culture

Human cell lines were grown in ATCC recommended media as following: A-172 (DMEM), SK-N-SH (MEM), and CCF-STTG1 (RPMI-1640). The media was supplemented with 10% fetal bovine serum. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2. The $CuSO_4$ (Sigma) or CuO (Aldrich) solutions were prepared in sterile media immediately before dosing. Cells were seeded at a density of 10,000/well in 96-well plates. All cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. 24 h after plating, the cells were treated with the copper salts. The copper solutions (1 mM stock) were prepared fresh in sterile media prior to addition to the cells. The media was diluted to reach final concentrations of 0.5, 5, 50, 500 μM. After a 24 h exposure, Cu salts were removed, cells were washed twice with sterile phosphate buffered saline (PBS) and the plate was used for the ROS, cell proliferation, or MTT assays as described below. For the viability assay, cells were seeded at a density of 600,000 cells per plate (35 × 10 mm) and maintained for 24 h at 37 °C in a humidified atmosphere of 5% CO_2. The cells were exposed to copper salts as previously described. After 24 h incubation, supernatant was collected, trypsin (0.25%) was added to detach cells, samples were centrifuged, and the resulting pellets were analyzed. Therefore, the viability assay was conducted on both floating and detached cells.

2.2. Particle size characterization

Media or nanopure water, suspended with copper oxide nanoparticles (1 mM) or copper sulfate (1 mM), was analyzed for particle size characterization using Nicomp particle size analyzer model #370. After exposing cells to the copper salts, the stock solutions were immediately used for particle size characterization. The media contains 10% FBS. The water solution does not contain FBS and was used as a control. Particle size determination was conducted on at least three separate occasions.

2.3. Cell proliferation

The number of cells was determined by CyQuant cell proliferation assay kit (Invitrogen). The dye, when bound to the nucleic acid of the cell, shows strong fluorescence. After addition of dye, the cells were incubated and measured at excitation 480 nm and emission 520 nm using a Biotek fluorescent microplate reader.

2.4. Cell viability/cytotoxicity

Cell survival was measured using the viability/cytotoxicity kit (Invitrogen). This assay measures both live and dead cells simultaneously using fluorescence from two dyes. The live cells are measured by calcein AM, a nonfluorescent, cell-permeant dye which, upon enzymatic conversion by ubiquitous intracellular esterase activity inside the cell, becomes an intensely fluorescent dye. The dead cells are measured by EthD-1 dye which only enters cells with damaged membranes and binds to nucleic acid to produce bright red fluorescence. The optimal dye concentration used for this assay was 1 μM calcein AM and 2 μM EthD-1. Cell pellets were resuspended with 250 μl of PBS containing the dyes. Samples were incubated for 45 min after addition of dye. Fluorescent images of live and dead cells were captured using a Nikon fluorescent microscope equipped with a digital camera.

2.5. MTT assay

Cell viability was assessed by the MTT assay. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced to formazan in mitochondria of living cells. The absorbance of the colored solution is measured at 590 nm.

2.6. Measurement of reactive oxygen species formation

Reactive oxygen species (ROS) formation was assayed using 2′,7′-dichlorofluorescein diacetate (DCFH-DA). This dye rapidly enters cells and is desterified to the ionic dichlorofluorescein (DCFH). DCFH is oxidized to the fluorescent 2′,7′-dichlorofluorescein (DCF) by reactive oxygen species. A microplate fluorescent reader was used to measure the fluorescence at excitation and emission of 485 nm and 530 nm, respectively. The formation of reactive oxygen species was quantified using a 2′,7′-dichlorofluorescein (DCF) standard curve. Rate of ROS formation was determined as DCF formation in 1 h. The number of cells in the corresponding well was determined and the results are reported as nmol DCF formed/h/cell.

2.7. Statistical analysis

The data was analyzed using GraphPad Prism® software. The difference among groups was assessed using one-way ANOVA followed by the Tukey's post-hoc test. The asterisks denote significant levels compared to control ($^{*}p \leqslant 0.05$, $^{**}p \leqslant 0.01$, $^{***}p \leqslant 0.001$).

3. Results

3.1. Particle size analysis

The insoluble CuO was in a nanoparticulate form. Therefore, we measured particle size after suspending in water or media and assessed aggregation at 4 h or 24 h using a particle size analyzer. CuO nanoparticles were stable and did not aggregate significantly over time (Table 1). No particles were detected at any of the time points when $CuSO_4$ was used.

Table 1
Mean particle size analysis of copper oxide nanoparticles.

Suspended in following media	Time (h)		
	0 h	4 h	24 h
DMEM (A-172)	253 ± 120.7	463.7 ± 287.4	362.4 ± 226.3
RPMI-1640 (CCF-STTG1)	360 ± 192.3	408.3 ± 283.6	403 ± 275.3
MEM (SK-N-SH)	364 ± 172.7	396.8 ± 220.8	335 ± 181.4
Water	254.6 ± 119.6	280 ± 145.2	265 ± 149.1

Mean particle size of nanoparticle (1 mM) suspended in water or media and analyzed after 4 h or 24 h using particle size analyzer model #370.