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Comparative toxicity of CuO nanoparticles and $CuSO_4$ in rainbow trout

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ABSTRACT

This study compared the toxicity and accumulation of two different Cu compounds, CuO nanoparticles (NPs) and soluble $CuSO_4$, in erythrocytes and different tissues in rainbow trout (*Oncorhynchus mykiss*). The crystal structure of CuO NP analysed by XRD indicates that the NP are Tenorite, a monoclinic CuO. The *in vitro* toxicity results indicate that both Cu compounds increase the haemolysis rate in a dose-dependent way, but the effect was reduced treating cells with CuO NP. Moreover, both Cu compounds induce DNA damage and the entity of the damage, similarly to haemolysis, was more marked in cells treated with $CuSO_4$. *In vivo* results, obtained after intraperitoneal injection, showed that Cu concentrations were significantly higher in gills ($p < 0.0001$), kidney ($p = 0.007$) and liver ($p < 0.05$) of exposed fish with a significant increase in plasma Cu concentration 15 h after $CuSO_4$ treatment. Cu concentrations were significantly higher in fish exposed to $CuSO_4$ than CuO in kidney ($p < 0.05$) and gills ($p < 0.0001$). Significant DNA damage with respect to controls was detected only when Cu was injected as $CuSO_4$. The present data could serve to evaluate environmental Cu toxicity in fish depending on Cu speciation.

1. Introduction

Copper is an essential trace element for living organisms, acting as a cofactor for different enzymes (cytochrome c oxidases, superoxide dismutase, cerulopasmin, and monooxygenases) with a central role in cellular metabolism.

In aquaculture, Cu is regularly used in the form of $CuSO_4$ to control algal blooms and as a therapeutic chemical for ectoparasitic and bacterial infections (Heo, 1997; Griffin and Mitchell, 2007). However, relatively high Cu concentrations in the environment can be toxic to aquatic organisms due to metal participation in redox reactions. Soluble forms of Cu are highly toxic to fish (Grosell et al., 2007) and can accumulate when excess Cu is present in water and feed though dietary sources are tightly regulated (Clearwater et al., 2002). Cu can induce oxidative stress because it catalyses the formation of reactive oxygen species (ROS) via a Fenton-like reaction (Prousek, 2007), decreasing glutathione levels (Speisky et al., 2009) and interacting with antioxidant enzymes (Isani et al., 2003a).

A growing number of nanotechnology applications utilize metallic components, many of which can be toxic to aquatic organisms. Nanoparticles of metal oxides, such as ZnO, TiO_2 and CuO, are increasingly used in commercial and industrial products. Thus it is inevitable that nanoscale products enter the aquatic environment. Data on NP eco-toxicity (Baun et al., 2008; Handy et al., 2008; Griffitt et al., 2009; Shaw and Handy 2011; Al-Bairuty et al., 2013) are rapidly growing.

CuO NPs are increasingly used in industrial applications, or as consumer products, in medicine, and as pesticides (Kiaune and Singhasemanon, 2011). CuO has even been used for antimicrobial textiles (Gabbay and Borkow, 2006). When added to water, metal NPs can aggregate, sediment out of the water column, adsorb to nutrients, and disassociate to release soluble metal ions (Griffitt et al., 2009). Inhalation or ingestion is likely to be the major routes of NPs uptake in terrestrial organisms. In aquatic animals there may be other routes of NPs entry such as direct passage across gills and other external surface epithelia. According to Shaw and Handy (2011), endocytosis is a possible route for target cell entry in fish because NPs are too large to use ion transporters.

Erythrocytes are routinely used as a model to study the toxicity of new chemicals (Tiano et al., 2003; Prasanthi and Muralidhara Rajini, 2005) because numerous compounds persist in circulating

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blood before reaching target organs (Li et al., 2008). Moreover, fish erythrocytes possess nuclei, mitochondria and other organelles typical of somatic cells and can be easily isolated.

The main aim of this work was to compare the toxicity of Cu, both as $CuSO_4$ and CuO NP, in rainbow trout (*O. mykiss*) on cellular and organismal level. Experiments were carried out in three fundamental steps. Firstly, the physical characteristics of the CuO NP under study were determined by various analytical techniques. Then the *in vitro* influence of the two copper compounds on haemolysis and rainbow trout erythrocyte DNA was evaluated. Finally, *in vivo* experiments were performed to assess the time course of copper in plasma, erythrocytes and tissues after intraperitoneal injection of the two copper compounds.

2. Materials and methods

All reagents were of pure and analytical grade. CuO NP was purchased from Sigma-Aldrich. All experimental procedures were approved by the Ethical and Scientific Committee of Bologna University and were carried out in accordance with European legislation regarding the protection of animals used for experimental and other scientific purposes.

2.1. Characterization and preparation of CuO NP

CuO NPs were characterized by different techniques. GC–MS and HPLC-DAD-ESI–MS measurements were performed to detect any organic impurities. GC–MS analyses were carried out using an Agilent Technologies 5973N mass spectrophotometer and an Agilent Technologies 6890N gas-chromatograph. HPLC-DAD-ESI–MS spectra were obtained on a Hewlett Packard 1090 Series II HPLC associated with an 1100 MSD Hewlett Packard mass spectrophotometer.

The structure of the sample was characterized by X-ray diffraction (XRD), high resolution scanning electron microscopy (HR-SEM) and surface area analyses. XRD analyses were carried out on a Bruker D8 Advance diffractometer in Bragg-Brentano geometry at 40 kV and 40 mA using Cu-Kα radiation. High resolution observations were carried out on a field emission Zeiss Supra 40 scanning electron microscope. Surface area analysis was conducted by nitrogen adsorption on a Beckman coulter SA 3400 apparatus at an adsorption temperature of −196 °C. Before measurements, samples were treated under high vacuum at 130 °C for 2 h according to the Brunauer, Emmet and Teller (BET) method (Brunauer et al., 1938). Theoretical NP size was calculated from the surface area using the following equation: $D=6000/(\rho S)$. This equation assumes spherical NP where D is the equivalent particle diameter in nanometres, ρ the particle density in g/cm^3 and S the specific surface area in m^2/g.

A stock suspension was prepared in water at a concentration of 1 mg/ml and to avoid aggregation it was sonicated for 10 min just before use. Further dilutions were required to reach the final concentration used.

To investigate the tendency of the particles to aggregate, dynamic light scattering (DLS) and Z-potential measurements were performed on the particle suspensions using a Malvern Zetasizer Nano Instrument (model ZEN3600).

2.2. Experimental design

Specimens of rainbow trout (*O. mykiss*) were obtained from the "Eredi Rossi Silvio" fish farm, Sefro, (MC) Italy. The fish were kept in tanks containing water from the Scarsito river, a tributary of the Potenza river, and fed with commercial fish food obtained from Hendrix S.p.A. (Mozzecane, VR, Italy). Two sets of experiments were performed: *in vitro* studies to check the effect of dose and source of Cu on the erythrocyte haemolysis rate and DNA damage, and *in vivo* studies to determine the Cu concentration in tissues and erythrocyte DNA damage.

(a) *In vitro experiment*: Red blood cells were obtained from specimens (15 months old and 180–300 g weight). Blood was withdrawn by Li heparin sterile syringe from the caudal tail vein into an isotonic medium (0.1 M phosphate buffer, 0.1 M NaCl, 0.2% citrate, 1 mM ethylenediaminetetraacetic acid (EDTA), and pH 7.8). At least three trout were collected in each experiment and the blood obtained was always pooled to minimize individual variability. After removing plasma and buffy coat by centrifugation at 1000*g* for 10 min, erythrocytes were washed three times and suspended in isotonic buffer (pH 7.8) at different Cu concentrations for haemolysis test and comet assay.

(b) *In vivo experiment*: Forty-two specimens (8 months old and 100–200 g weight) were analysed. At day 0 (T_0) prior to Cu exposure, six specimens were sampled and the remaining 36 specimens were divided into three groups and intraperitoneally injected or not with a fixed amount of Cu (1 μg/g body weight) provided as CuO NP ($n=12$) or $CuSO_4$ ($n=12$). Cu was suspended or dissolved in saline solution (0.90% w/v NaCl). Trout serving as controls ($n=12$) received a corresponding amount (1 ml/kg) of saline solution. Trout were maintained in fresh water as reported above and were not fed during the Cu treatment. After 15 h (T_1, $n=4$ for each condition), 23 h (T_2, $n=4$ for each condition) and 38 h (T_3, $n=4$ for each condition), fish were randomly chosen, sacrificed and tissues (gills, liver, and kidney) immediately sampled for analysis. Blood samples were collected from the caudal vein of each specimen into a Li-heparin sterile syringe. Each blood sample was centrifuged as reported above to obtain plasma and stored at −20 °C until Cu determination, while erythrocytes were used both for comet assay and Cu analysis.

2.3. Haemolysis

To evaluate the haemolysis rate, the erythrocytes were suspended in the isotonic buffer pH 7.8 at room temperature. The degree of haemolysis was determined spectrophotometrically at a wavelength of 540 nm, as previously described (Falcioni et al., 1987), either in the absence or presence of the Cu compounds under study (CuO NP and $CuSO_4$). In particular, the haemolysis rate was determined using the following expression $100\times A/10\times A^*$; where A is the haemoglobin concentration present in the supernatant of the red cell suspension after centrifugation, and A^* is the haemoglobin concentration obtained after complete lysis with 10 volumes of distilled water at zero incubation time.

2.4. Single-cell gel electrophoresis (comet assay)

To evaluate the genotoxic effects of the two Cu compounds (CuO NP and $CuSO_4$), the alkaline comet assay was performed on nucleated trout erythrocytes as previously described (Moretti et al., 1998). After incubation, cells were suspended in 0.7% low melting agarose in PBS and pipetted on microscope slides pre-coated with a layer of 1% normal melting agarose. The agarose with the cell suspension was allowed to set on the pre-coated slides at 4 °C for 10 min. Subsequently, another top layer of 0.7% low melting agarose was added and allowed to set at 4 °C for 10 min. The slides were then immersed in lysis solution (1% sodium n-lauroyl-sarcosinate, 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl pH 10, 1% Triton X-100 and 10% DMSO) for 1 h at 4 °C in the dark to lyse the embedded cells and to permit DNA unfolding. After incubation in lysis solution slides were exposed to alkaline buffer (1 mM Na_2EDTA, 300 mM NaOH buffer, and pH > 13) for 20 min and subjected to 20 min electrophoresis at 25 V in the same alkaline buffer. The slides were finally washed with 0.4 M Tris–HCl buffer (pH 7.5) to neutralize excess alkali and remove detergents before staining with ethidium bromide. In each experiment images of 150 randomly selected cells (50 cells from each of three replicate slides) were analysed for each treatment using an Axioskop-2 plus microscope (Carl Zeiss, Germany) equipped with an excitation filter of 515–560 nm and a magnification of 20×. Imaging was performed using a specialized analysis system (Metasystem Altlussheim, Germany). The comet parameter considered was the % tail DNA obtained as TI/total comet intensity × 100 (where TI is the percentage of fluorescence in the comet tail). The alkaline version of comet assay reveals single and double-strand breaks as well as alkali-labile sites (Wozniak and Blasiak, 2003).

In the *in vitro* study, 10^6 erythrocytes/ml were incubated for 1 h at room temperature in isotonic buffer (see above) in the absence (control) or presence of different amounts of Cu as CuO NP or $CuSO_4$ and the comet assay was then carried out. In the *in vivo* study, blood from each group was pooled to minimize individual variability and the comet assay was carried out spreading 2.5×10^5 cells per slide (three slides per treatment). In all experiments, the DNA damage was expressed as the percentage of DNA in the tail (% tail DNA) as suggested by Kumaravel and Jha (2006). The % tail DNA was calculated as tail intensity/total comet intensity × 100.

2.5. Cu analyses

Cu concentration was determined in erythrocytes, plasma, liver, gills and kidney. Amounts of 0.2 g wet tissue were digested with 2 ml 65% HNO_3 and 0.5 ml 30% H_2O_2 in a microwave oven (Model 1200, Milestone, Italy) for 5 min at 250 W, 5 min at 400 W, 5 min at 500 W, and 1 min at 600 W. Cooled samples were transferred into 5 ml polyethylene volumetric flasks and Cu concentration was measured by a Varian SpectrAA-300 20-Plus atomic absorption spectrophotometer (Australia) equipped with a graphite furnace. Plasma was diluted 1:10 with ultrapure water before analysis and directly aspirated into the flame of an atomic absorption spectrophotometer (Model IL 11, Instrumentation Laboratory USA) equipped with a deuterium lamp. Two blanks were digested simultaneously during each run. The accuracy of the method was evaluated by calibration *versus* an international standard (CRM 278, Community Bureau of Reference-BCR, Brussels), which was run every 20 samples; the concentration values obtained fell within the confidence interval given by the BCR. The detection limits for Cu were 20 ng/ml (flame) and 1 ng/ml (furnace).

2.6. Statistical analysis

For *in vitro* haemolysis tests, differences between treatments were analysed using one-way ANOVA, followed by the Student–Newman–Keuls test. Cu concentrations in