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Developmental abnormalities and neurotoxicological effects of CuO NPs on the black sea urchin *Arbacia lixula* by embryotoxicity assay

Maria Maisano [a, *, 1], Tiziana Cappello [a, 1], Eva Catanese [a], Valeria Vitale [a], Antonino Natalotto [a], Alessia Giannetto [a], Davide Barreca [b], Elvira Brunelli [c], Angela Mauceri [a], Salvatore Fasulo [a]

[a] Department of Biological and Environmental Sciences, University of Messina, Messina, Italy
[b] Department of Chemical Sciences, University of Messina, Messina, Italy
[c] Department of Biology, Ecology and Earth Sciences, University of Calabria, Cosenza, Italy

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ABSTRACT

The embryotoxicity of CuO NPs was evaluated in the black sea urchin *Arbacia lixula* embryos, by using 24-well plates. Fertilized eggs were exposed to five doses of CuO NPs ranging from 0.07 to 20 ppb, until pluteus stage. CuO NPs suspensions in artificial seawater formed agglomerates of 80–200 nm size, and copper uptake was 2.5-fold up in larvae exposed to high NP concentrations in respect to control. Developmental delay and morphological alteration, including skeletal abnormalities, were observed, as well as impairment in cholinergic and serotonergic nervous systems. These findings suggest the potential of CuO NPs to interfere with the normal neurotransmission pathways, thus affecting larval morphogenesis. Overall, the embryotoxicity tests are effective for evaluation of nanoparticle effects on the health of aquatic biota. Furthermore, as the black sea urchin *A. lixula* demonstrated to be vulnerable to NP exposure, it may be a valid bioindicator in marine biomonitoring and ecotoxicological programmes.

1. Introduction

Nanotechnologies are gaining increased attention due to the wide variety of applications of nanoparticles (NPs) in various fields, including medicine, material sciences, engineering, cosmetics, and textile industries. The great majority of NPs derive from natural processes, such as forest fires, dust storms, erosion, volcanic eruptions (Wigginton et al., 2007). Yet, the rise in the use of man-made NPs and their consequent release into the environment might have repercussions on the ecosystem (Campos et al., 2014; Cupaioli et al., 2014; Gambardella et al., 2013; Pereira et al., 2014 Ren et al., 2014). In the last few decades, concerns have been raised over the toxic potential of NPs on the environment and human health, because of their small size and high catalytic properties (Asharani et al., 2008; Klaine et al., 2008; Muth-Köhne et al., 2013; Söderstjerna et al., 2014). Estuarine and coastal environments may represent the ultimate sink for different kinds of NPs (Canesi et al., 2012). Once released into the environment, abiotic factors including pH, salinity, and UV light may alter the surface properties of NPs, leading them to aggregation. It has been also demonstrated that NPs potential risk is higher than the bulk counterpart, because of their increased surface area and higher reactivity, which may enhance NPs bioavailability and toxicity (Bhatt and Tripathi, 2011; D'Agata et al., 2014a; Gomes et al., 2012).

Copper oxide (CuO) NPs are one of the most used metal nanomaterials nowadays. CuO NPs exert potential antiviral and antimicrobial properties (Bondarenko et al., 2013; Gabbay et al., 2006; Pandey et al., 2014), and therefore have an extensive range of applications, from the production of metallic coatings, wood preservation, air and liquid filtration, to inks production, skin products and textiles (De Rossetto et al., 2014; Fahmy and Cormier, 2009; Gomes et al., 2014). Copper plays an important role in metabolism, as it is a cofactor for numerous enzymes involved with redox reactions. Because Cu is an essential nutritional element, its

* Corresponding author. Department of Biological and Environmental Sciences, University of Messina, Viale F. Stagno d'Alcontres 31, 98166 Messina, Italy.
E-mail address: mmaisano@unime.it (M. Maisano).
[1] These authors contributed equally to the study.

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lack may lead to neural aberrations during embryonic development (Turski and Thiele, 2009), but evidences of its toxicity at high concentrations were also reported (Gomes et al., 2011; Perreault et al., 2012).

It has been demonstrated that aquatic organisms during early life stages are usually more susceptible to NPs toxicity (Bacchetta et al., 2012; Kadar et al., 2013; Lee et al., 2012), including CuO NPs (Ozel et al., 2013), than the adult counterparts. For this reason, fish embryotoxicity tests (FET) have been used as a simple, reliable, and highly sensitive diagnostic tool for the evaluation of embryonic abnormalities and for the monitoring of marine environmental pollution (Embry et al., 2010; OECD et al. 2014).

An alternative model organism for embryotoxicity tests is the sea urchin, a coelomate deuterostome, which shares many metabolic and physiologic similarities with mammals, besides a notable genomic homology with higher vertebrates, including humans (Check Hayden, 2010; Sea Urchin Genome Sequencing Consortium, 2006). Several aspects make it a suitable model for embryotoxicity test such as the large number of eggs available, their transparent envelope and the synchronous development (Manzo, 2004). Recent works, although few in number, reported the use of gametes, embryos and/or larvae of sea urchins to evaluate the effects of engineered NPs (Falugi et al., 2012; Gambardella et al., 2013; Manzo et al., 2013; Matranga and Corsi, 2012).

The aim of this work was to evaluate the embryotoxic effects of CuO NPs on the first developmental stages of the black sea urchin *Arbacia lixula* (Linnaeus, 1758), performing the embryotoxicity test in 24-well plates. The CuO NPs impact on the embryo-larval development was investigated trough morphological observations of both embryo and pluteus stages. Also, the cholinergic and serotonergic nervous systems were assessed in order to elucidate the neurotoxic potential of CuO NPs. *A. lixula*, together with the edible sea urchin *Paracentrotus lividus*, are the most abundant echinoids in the Mediterranean. They play an important ecologic role in the littoral ecosystem, where they control the algal communities growth (Gianguzza et al., 2014; Gianguzza and Bonaviri, 2013; Martin et al., 2011; Poleza et al., 2014; Wangensteen et al., 2013). Also, it was demonstrated that *A. lixula* and *P. lividus* show similar sensitivities to chemicals (Carballeira et al., 2012).

2. Materials and methods

2.1. Nanoparticles

The copper (II) oxide NPs powder was provided by COMETOX with a purity of 99.5%, 12 nm particle size, and 79.55 molecular weight.

CuO NPs stock solution was prepared in artificial seawater (ASW). It was sonicated in a bath sonicator (Falc sonicator, LCD series) for 2 h and then kept in constant shaking to reach a concentration of 400 ppb. For the embryotoxicity tests, fertilized eggs were exposed to five different concentrations of CuO NPs, (ranging from 0.07 to 20 ppb). Droplets of CuO NP suspensions were placed on slides and dried overnight in a stove at 60 °C. Slides were then coated with a Quorum Q150T ES carbon coater (Quorum Technologies, UK). Samples were analyzed under a Field Emission Scanning Electron Microscope (FESEM, FEI FESEM, FEI, Quanta 200F Company) equipped with EDAX Genesis software attachment. SEM micrographs along with the EDS spectrum were taken with an electron beam energy of 20 keV.

2.2. Sea urchin collection and embryotoxicity tests

A. lixula adult specimens were collected from the Mediterranean littoral coasts of Messina, Sicily, and brought immediately to the laboratory. Here, sea urchins were placed in a large tank containing aerated seawater for seven days at 18 ± 1 °C, pH 7.9–8.1, and salinity 36 ppt. Mature eggs and sperm were obtained by injecting 0.5 M KCl into the Aristotle's lantern. Sperm was collected in a sterile tube and activated by 1:50 dilution with ASW, while the ovules were placed directly in a sterile Petri dish. Finally, male gametes were added to eggs, maintaining a ratio of 100:1 and the successful fertilization was verified through the observation of the fertilization membrane by microscopical observation. From the 400 ppb CuO NPs stock solution, aliquots were taken and diluted in ASW to reach the final nominal concentration (0.07 ppb, 0.7 ppb, 7 ppb, 10 ppb and 20 ppb), and then added to each 24-well plate. ASW and $CuSO_4{}^*5H_2O$ 10 ug/L were used as negative and positive control, respectively. The positive control was prepared from the 100 mg/L $CuSO_4{}^*5H_2O$ stock solution. For each treatment, the experiment was conducted four times in duplicate. Approximately 500 fertilized eggs in a 2 ml volume were inoculated into each well, and the plates maintained at 18 ± 1 °C with a 12 h light–dark cycle for 72 h (Resgalla et al., 2012).

2.3. Quantification of copper content by atomic absorption spectroscopy

The organisms analyzed for copper quantification were growth in the same experimental condition described in the above section and, after treatment, swimming plutei have been collected by the upper phase of the solution, and transfer to microcentrifuge tubes. Plutei were centrifuged at 10,000 rpm × 5 min. The pellets were washed with an incubation medium without copper and centrifuged again at 10,000 rpm × 5 min. This procedure was repeated three times. Finally, the concentration of copper in the air-dried settled precipitate was determined according to Isani et al. (2013) utilizing atomic absorption spectroscopy (AAS). A calibration curve was obtained with standard solutions prepared by dilution in double-distilled water of a 1000 ppm commercial copper stock solution (Sigma–Aldrich Chemie GmbH). The detection limit was defined as the concentration of the element that could be distinguished from background-noise with a 95% confidence interval.

2.4. Morphological analysis

After 24 and 72 h post fertilization (hpf), embryos at the gastrula and pluteus stages were collected from each control and NP exposure plates for morphological observations. The embryo-larval development was then stopped by addition of 1 ml of 4% formaldehyde into each well of the 24-well plates used (Resgalla et al., 2012). Samples were mounted on slides and morphological characteristics were compared among the different concentrations of the NPs using a motorized Zeiss Axio Imager Z1 microscope (Carl Zeiss AG, Werk Göttingen, Germany), equipped with an AxioCam digital camera (Zeiss, Jena, Germany) for the acquisition of images.

2.5. AChE enzymatic activity assay

The plutei at 72 hpf were sonicated in phosphate buffer using the sonicator Microson, Ultrasonic Cell Disruptor XL (Misonix) for 2 min and centrifuged for 20 min at 12,000 rpm. The supernatant was used to measure the AChE activity by spectrophotometric assay according to the classical Ellman et al. (1961) method. The colorimetric reaction was recorded at 412 nm for 5 min, and compared with a standard curve, obtained by using known AChE amounts. Each measurement was conducted three times. Total proteins were measured by comparison to a calibration curve determined using the Pierce BCA Protein Assay Kit (Thermo Scientific). The AChE units were obtained by the ratio between the enzyme activity/min/mg protein.