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Development of a new tool for the long term in vitro ecotoxicity testing of nanomaterials using a rainbow-trout cell line (RTL-W1)

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ABSTRACT

The current wide use of manufactured nanomaterials (MNs) is leading to the release of nanoparticles (NPs) to water bodies. Aquatic organisms, including fish, are exposed to low concentrations of NPs for long periods of time being necessary to develop laboratory toxicity tests reflecting realistic conditions. Additionally, today there is a demand of in vitro assays respecting the 3Rs principle. Thus, the main aim of this work was to stablish an in vitro tool for the assessment of long-term NPs ecotoxicity. Considering the key role of liver in detoxification, a rainbow trout liver cell line, RTL-W1, was used. CuO NPs were chosen to validate this tool taking into account their important production level. Cells were exposed for 21 days to 25 or 100 μg CuO NPs/ml. Every seven days cells were split and one fourth of them transferred to a new plate with appropriate concentrations of NPs in culture medium. Lower concentrations of CuO NPs did not cause any deleterious effect, whereas higher concentrations led to significant mortality after 14 days and to the intracellular accumulation of Cu particles. Identical results were observed in cells exposed to $CuSO_4$ at the same Cu concentrations. Therefore, the observed toxic effects might be mainly due to Cu^{2+} ions.

1. Introduction

Manufactured nanomaterials (MNs) are currently used in a number of consumer products so that they can enter the environment and be spilled into the aquatic compartments, through waste water treatment plants and industrial effluents, agricultural run-offs, accidental spills, etc. As a consequence, in recent years, engineered nanoparticles (NPs) are emerging as a potential new type of environmental pollutant (Ruiz et al., 2015). Hence, the release of NPs into the aquatic environment could be threatening the survival of inhabiting organisms (Farré et al., 2009; Handy and Ramsden, 2011).

Among metal-based NPs, CuO NPs probably show one of the highest production volumes, since they are employed in a number of fields. For instance, they are used as additives in lubricants, polymers and plastics, metallic coating inks, or antimicrobial coatings, and they are also applied in textiles due to their biocidal activity, or in cosmetics. In addition, environmental remediation and electronics take advantage of their particular physico-chemical properties (Nanotech project, 2014; Park et al., 2007; Perelshtein et al., 2009; Cioffi et al., 2005; Ren et al., 2009; Rubilar et al., 2013). The precise concentration of CuO NPs in the aquatic environment is difficult to establish but some modelling studies revealed that they can appear at concentrations of 0.01 mg of Cu/l in marine environments highly contaminated by this metal (Bryan and Langston, 1992).

Although Cu is an essential element for living organisms, taking part in many biological processes, for instance as co-factor for redox active enzymes (like cytochrome *c* oxidase and superoxide dismutase), it results in high toxicity when present in excess amounts because it can interfere with homeostasis of other metals, cause DNA damage, and generate reactive oxygen species (ROS) that can adversely modify proteins, lipids and DNA (Aruoma et al., 1991; Banci et al., 2010; Boyles et al., 2016; Halliwell and Gutteridget, 1984; Xie et al., 2006).

The toxicity of CuO NPs has been extensively studied using both in vivo and in vitro approaches (Dai et al., 2015; Isani et al., 2013; Ivask

Abbreviations: BSA, Bovine serum albumin; CFDA-AM, 5-carboxyfluorescein diacetate, acetoxy methyl ester; CYP, cytochrome P450; DLS, dynamic light scattering; EDTA, ethylenediaminetetraacetic acid; EDX, energy dispersive X-ray spectroscopy; FBS, fetal bovine serum; ITS, intelligent testing strategy; MEM, Minimum Essential Medium; NP, nanoparticle; NRU, neutral red uptake; P/S, penicillin/streptomycin; PBS, phosphate buffer saline; RMANOVA, repeated measurements analysis of variance; ROS, reactive oxygen species; SEM, standard error of the mean; SDS, sodium dodecyl sulphate; TEM, transmission electron microscopy

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et al., 2014; Ivask et al., 2015; Mancuso and Cao, 2014; Pedroso et al., 2013; Rossetto et al., 2014), and it has been reported that these NPs are toxic to both vertebrates and invertebrates (Aruoja et al., 2009; Buffet et al., 2011). Actually, some studies identified CuO NPs as the most cytotoxic among various metal oxide NPs (Karlsson et al., 2008).

In the context of aquatic ecotoxicology, fish are the most widely used vertebrates in risk assessment and regulation (Fent, 2001). Therefore, understanding the actions of NPs on fish is essential for an appropriate evaluation of the health of the aquatic environment. Nevertheless, in vivo testing is extremely time-consuming and costly, requiring much maintenance and a high number of animals, which is ethically debated. Therefore, the European Commission encourages the development and application of alternatives to animal tests (Castaño et al., 2003; Lee et al., 2008; Scholz et al., 2013). The use of in vitro methods with fish cells has been seriously considered for assessing the hazardous effects of chemicals on living systems, since many studies have demonstrated a good in vivo/in vitro correlation and it is generally accepted that the first interaction of a toxicant with an organism occurs at the cellular level (Schirmer, 2006). The changes provoked by NPs in cells can then possibly be translated to higher levels of organization, finally reflecting the effects on the whole organism (Babich et al., 1991; Castaño et al., 2003; Fent, 2001; Rusche and Kohlpoth, 1993; Zahn et al., 1995).

An important gap of in vitro ecotoxicological experiments is that they are mainly conducted for short exposure times, which do not reflect real-life situations with continuous release of products over a long period. Actually, there is a general lack of knowledge about MNs long term toxic effects. In this sense, in vitro systems using long-term exposure protocols, as those described in this article, can be a powerful tool to initially determine the mechanisms underlying the potential cytotoxic effects of NPs after prolonged exposure at levels that are not toxic in acute experiments.

Rainbow trout (*Oncorhynchus mykiss*) is probably the most commonly studied cold water fish species (Wolf and Rumsey, 1985) and it is considered as one of the most sensitive species for acute toxicity testing (Fent, 2001). Therefore, the use of rainbow trout derived cell lines can be of relevance for in vitro studies dealing with pollutants ecotoxicity. In addition, since liver is one of the target organs for NPs toxic action and bioaccumulation (Connolly et al., 2016), we decided to use a rainbow trout cell line of hepatic origin for our studies: RTL-W1 cells, which are probably biliary epithelial cells in origin (Malhão et al., 2013) derived from the normal liver of an adult male rainbow trout. RTL-W1 cells retain important catabolic activities as xenobiotic reductase and glutathione-*S*.transferase activities and those dependent on cytochrome P450 1A (CYP1A), (Lee et al., 1993; Nehls and Segner, 2001; Smeets et al., 1999; Thibaut et al., 2009).

However, the main drawback of using fish cell cultures in ecotoxicological studies is that cell lines are much less sensitive than whole organisms (Bols et al., 1985; Castaño et al., 1996; Segner and Lenz, 1993). Nevertheless, the sensitivity of cytotoxicity tests can be increased by the selection of appropriate cytotoxic endpoints (Castaño et al., 2003). Regarding this aspect, it is important to evaluate changes in cell viability (a cellular general response) with a number as high as possible of assays covering different mechanisms of toxic action. Taking this into account, in the present work we have applied a system normally used in our laboratory based on the utilization of three indicator dyes per sample, reflecting effects on general metabolic activity of the cells, on lysosomal functioning and on plasma membrane integrity, what provides a broad overview of the sensitivity of the cells (Lammel and Navas, 2014). Independently of the limitations of in vitro approaches, the information gained can be used at different levels in an integrated testing strategy (ITS), for instance in order to prioritize chemicals for further testing in higher tier more costly in vivo assays. Actually, data obtained in vitro have demonstrated to be useful for the toxicity ranking of chemicals or MNs (Farcal et al., 2015).

According to what has been said above, we carried out the present work with the main aim of determining the long term (21 days) cytotoxic effects of CuO NPs on RTL-W1 cells. For the selection of the appropriate concentrations to be applied, first 24 h acute cytotoxicity assays were performed. Influence of CuO NPs on RTL-W1 cell viability and subcellular morphology were explored at different time points along the 21 days-experiment. Inasmuch there is general consensus that dissolution of CuO NPs is an important factor that influences their toxicity (Aruoja et al., 2009; Ivask et al., 2014), impacts of CuO NPs on cells were compared with those produced by $CuSO_4$ salt, used as control source of ions and applied at the same Cu concentrations and treatment periods as NPs. Although the system developed in our laboratory focused on testing CuO NPs toxicity on a specific fish cell line, a similar approach could be used to observe the effects of other MNs on a variety of cell lines from different origin.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents were purchased from Sigma-Aldrich (Madrid, Spain), at least than otherwise stated. For cell culture, L-Glutamine (200 mM), penicillin and streptomycin (P/S) (10,000 U/ml each), trypsin-ethylenediaminetetraacetic acid (EDTA) (200 mg/ml) and Leibovitz's (L-15) cell culture medium were purchased from Lonza (Barcelona, Spain). Phenol-red free serum-free Minimum Essential Medium (MEM) was supplied by Gibco (Life Technologies, Madrid, Spain). Among the reactants used for determining cytotoxicity, resazurin and 5-carboxyfluorescein diacetate, acetoxy methyl ester (CFDA-AM) were purchased from Invitrogen (Madrid, Spain). For the electron microscopy analyses, paraformaldehyde (16%) and glutaraldehyde (25%) were supplied by Electron Microscopy Sciences (Hatfiled, UK) and Spurr's resin was provided by TAAB Laboratories Equipment Ltd. (Aldermaston, UK).

2.2. Nanoparticles and nanoparticle suspension preparation

Uncoated spherical CuO NPs, of 15–20 nm in diameter size according to the manufacturer's information, were supplied by PlasmaChem GmbH (Berlin, Germany). Initial CuO NP dispersions at 10 mg/ml were freshly prepared in milli-Q water prior to exposure experiments. The suspensions were sonicated for 20 min in an ice-water bath using a probe sonicator (Vibra cell VCX130, Sonics & Materials Inc., Newtown, CT, USA) at 80% amplitude in continuous mode with a 2 mm microtip. For the exposure experiments, initial dispersions of CuO NPs were mixed at a proportion 3:1 with a solution of bovine serum albumin (BSA, 80 mg/ml in water) used as dispersant agent. This mixture was then diluted into L-15 medium to reach a concentration of 100 μg/ml. This suspension was vortexed for 5 s and thereafter sonicated for 10 min in a water-bath sonicator (S 40H Elmasonic, Elma, Germany) immediately before use. $CuSO_4{\cdot}5H_2O$ was used as a control source of Cu^{+2} ions.

2.3. Physico-chemical characterization of CuO NP suspensions

2.3.1. Dynamic light scattering (DLS)

The hydrodynamic size frequency distributions of CuO NPs in both the initial suspensions in milli-Q water and in culture medium at 100 μg/ml were determined by means of DLS, using a Zetasizer Nano-ZS device (Malvern Instruments Ltd., Malvern, UK). Milli-Q water and L-15 medium with BSA were used as background controls. Since the same initial CuO NP dispersion in water was employed for the preparation of all dispersions during the three long-term experiments (21 days of length), DLS measurements were carried out in fresh CuO NP initial dispersions (time 0), and after 1, 7, 14 and 21 days. In addition, given that CuO NP suspensions in L-15 medium were renewed every 6 or 7 days, those suspensions at 100 μg/ml were analysed by DLS

immediately after their preparation and after 1, 6 or 7 days of incubation under culture conditions. For the conversion of intensity readouts to particle number or volume, CuO refractive index (2.630) was used. Three independent measurements from three different dispersions were carried out, with each measurement consisting of four individual readings of six runs, each of 10 s duration.

2.3.2. Transmission electron microscopy (TEM)

Transmission electron microscopy analysis was performed to characterise the morphology and size distribution of fresh CuO NP suspensions in milli-Q water and in cell culture medium and to detect any change in shape and/or size along exposure (observations at 24 h, 7, 14 and 21 days for suspensions in milli-Q water, and 24 h, 6 and 7 days for exposure dispersions). TEM analysis was carried out using a JEM 2100 HT (JEOL Ltd., Tokyo, Japan) operated at an accelerating voltage of 200 kV with integrated energy dispersive X-ray spectroscopy (EDX) (Oxford Inca, UK). The size of the particles (Feret diameter) in the TEM micrographs was determined by Image J (National Institutes of Health, USA). The samples were prepared by dropping five 10 µl aliquots of CuO NP dispersions onto carbon-coated Cu grids. CuO NP samples in L-15 medium were prepared for TEM analysis at a concentration of 200 µg/ml (following the steps previously indicated), to make easier the localization of NPs along the grids. (DLS measurements evidenced that size distributions of 200 µg CuO NPs/ml dispersions were similar to those obtained from 100 µg/ml dispersions).

2.3.3. Cell culture and exposure experiments

RTL-W1 rainbow trout (*Oncorhynchus mykiss*) cell line is a generous gift from Drs. Lee and Bols, who obtained it from normal liver tissue (Lee et al., 1993). Cells were cultured in 75 cm^2 Cell Star cell culture flasks (Greiner Bio-One GmbH, Germany) in Leibovitz's L-15 culture medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S) solution and 1% L-Glutamine (supplemented medium). Flasks were incubated at 20° C and split twice a week using PBS/EDTA and Trypsin-EDTA.

2.4. Short-term exposure to CuO NPs

RTL-W1 cells were seeded in 96-well plates (Greiner Bio-One GmbH) at an initial cell number of 5×10^4 cells/well in 100 µl culture media. After 24 h, medium was retired, cells were washed with phosphate buffer saline (PBS) and exposed to serial 1/2 dilutions of fresh CuO NP dispersions (nominal concentration range 0.78–100 µg/ml) or $CuSO_4{\cdot}5H_2O$. After 24 h of exposure, treated cells were analysed for cytotoxic effects by three different assays (see below, *Cytotoxicity assays*) carried out on the same set of cells. Cells cultured in L-15 medium with BSA served as control. Cells treated with sodium dodecyl sulphate (SDS, ranging from 65.84 to 500 µM), were used as positive cytotoxicity control. Three independent experiments were performed in order to determine the toxic concentrations to be used in the long term exposure experiments.

2.5. Long-term exposure

Cells were seeded in 24 well plates, at a density of 28×10^4 cells/well (in a total volume of 0.560 ml) and maintained at 20 °C for 24 h to obtain a confluent cell monolayer. Thereafter, cells were exposed to those CuO NPs concentrations used in the short term experiments that were the nearest to the IC_{10} and IC_{50} calculated in these experiments: 25 and 100 µg CuO NPs/ml L-15 supplemented medium.

In parallel, other seeded plates were exposed to $CuSO_4{\cdot}5H_2O$ dissolved in cell culture medium. Exposure concentrations of $CuSO_4$ were those leading to the same Cu concentration as in the CuO NP treatments (78.5 and 313.9 µg $CuSO_4{\cdot}5H_2O$/ml).

At days 7 and 14, cells were rinsed with PBS, trypsinized with trypsin-EDTA and ¼ of cells were sub-cultured in a new 24 well plate. They were maintained in L-15 medium without treatment for 24 h to allow them to attach to the bottom of the wells. Thereafter, medium was replaced by new medium with the corresponding CuO NPs or $CuSO_4{\cdot}5H_2O$ concentrations. Four plates were initially established for each experiment ($n = 3$), and cytotoxicity was evaluated after 1, 7, 14 and 21 days, each time in a different plate.

2.6. Cytotoxicity assays

The method established by Dayeh et al. (2013) was followed, as reported in Lammel et al. (2013) and in Lammel and Navas (2014). After the corresponding exposure period, the medium was retired, cells washed with PBS and the cytotoxicity assays performed. This system employs resaruzin (alamarBlue assay), 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM assay) and neutral red (in the neutral red uptake, NRU, assay) reagents to monitor, on the same set of cells, metabolic activity, cell membrane integrity and lysosomal damage, respectively. Fluorescence intensity was measured in a microplate reader (Tecan Genios, Tecan Group Ltd., Männendorf, Switzerland) at specific excitation and emission wavelengths for each assay. Fluorescence readouts were corrected by subtracting the fluorescence of the cell-free control and normalized with respect to cells treated only with supplemented medium + BSA.

2.7. Interference

Before performing cytotoxicity assays, we tested if NPs dispersed in L-15 medium or adsorbed to cell monolayers may interfere with the fluorescence signal in the cytotoxicity assays (due to autofluorescence or to fluorescence quenching phenomena). A detailed protocol for interference is described by Lammel et al. (2013). Cell-free wells or cells monolayers were exposed to CuO NPs at the same concentration range (0.78–100 µg/ml) used in the cytotoxicity assays. Fluorescence emitted by dispersed MNs in cell culture medium or adhered to the cells after 24 h of treatment was measured simulating the same conditions of the cytotoxicity assays, but without adding the corresponding fluorophores (resaruzin, CFDA-AM or NR). Non-treated cells served as a reference.

AlamarBlue is a commercial solution made up of the redox-sensitive resazurin (7-hydroxy-3H-phenoxazin-3-one 10 oxide) dye. Since CuO has oxidizing properties, it must be assessed if CuO NPs are able to acellularly affect resaruzine reduction to resorufine. For this purpose, CuO NPs in phenol-red free MEM medium (from 0.78 to 100 µg/ml) were co-incubated with the substrate at 20 °C for 30 min, and emitted fluorescence was measured and compared with a control in which NPs were not added.

To explore the possible interference of CuO NPs with the reaction products (resorufin, 5-CF and NR), exposed cells were incubated with the maximum product concentration that can be expected to be formed in the respective assays and with 10% of this maximum concentration (resorufin at 0.1 and 1 µM, respectively; 5-CF at 4 and 0.4 µM, respectively; or NR at 33 µg/ml). Fluorescence was registered at time 0 and 30 min (for resorufin and 5-CF compounds) or 1 h (for NR) after incubation at 20 °C. Similar assays were performed to explore possible interferences of $CuSO_4$ salt, at Cu concentrations similar to these reached in the CuO NPs suspensions.

2.8. Microscopic monitoring of cultured cells

2.8.1. Phase-contrast microscopy

Cell growth in plate wells was observed using an inverted phase-contrast microscope. (Zeiss Axiovert 25, Carl Zeiss AG, Germany). Images of RTL-W1 cells were taken at 1, 7, 14 and 21 days of treatment by using a digital camera (Canon EOS 1000D, Canon Inc., Amstelveen, The Netherlands).

2.8.2. Transmission electron microscopy (TEM)

For the TEM analyses, cells were seeded as previously described in Cell Star 24-well plates but 12 mm poly-L-lysine coated glass coverslips (BioCoat, Discovery Labware Inc., USA) were introduced into the wells allowing the cells to grow on them. In the case of the cells treated for 14 and 21 days, the coverslips were introduced in the plate only 7 days before the end of the treatment, at the moment of splitting the cells. After exposure, the coverslips with the cells on their surface were washed with Milloning's buffer (pH 7.3), fixed in 4% paraformaldehyde - 2.5% glutaraldehyde and postfixed in 1% osmium tetroxide. Thereafter, they were washed with milli-Q water, gradually dehydrated in acetone and finally embedded in Spurr's resin, according to the method described by Lammel et al. (2013). The resin embedded cells were cut into ultrathin sections (60 nm) using a Leica Ultracut E ultramicrotome (Leica Microsystems, Germany) equipped with a diamond knife. The samples were stained in 1% uranyl acetate and Reynolds' lead citrate. Cells were visualized using a JEM 1400 (JEOL Ltd., Tokyo, JP) microscope operated at an accelerating voltage of 120 kV with EDX (Oxford Inca).

2.8.3. Assessment of CuO NPs dissolution

A series of experiments were carried out in order to assess the leaching of Cu from CuO NPs. Suspensions of CuO NPs were centrifuged in order to separate nanoparticles, that accumulate in the pellet, from released Cu, that remain in the supernatants (Fernández-Cruz et al., 2013; Naha et al., 2016; Song et al., 2014). Suspensions were prepared at 25 and 100 μg/ml (19.98 and 79.90 μg/ml of Cu) as described in the section *Nanoparticles and nanoparticle suspension preparation*. Aliquots of 12 ml were prepared and incubated at 20 °C up to a maximum of five days. At time 0 and after incubation times of 24, 48, 72, 96 and 120 h these aliquots were centrifuged (12,000 × *g*, 60 min) to separate dissolved Cu from non-dissolved CuO NPs. It must be emphasized that time 0 actually corresponds to 2 h, this was the time needed to obtain the final pellet and supernatant from the moment in which the CuO NPs were added to the medium to generate the suspension In order to assess whether centrifugations were efficient in removing NPs from medium, DLS measurements were performed in the supernatants. In no case NPs were detected. Supernatants were carefully separated from the pellets and both were digested prior to their analysis by inductively coupled plasma – mass spectrometry (ICP-MS) or - optical emission spectrometry (ICP-OES) as a function of the expected concentration. Samples were digested with 1 ml of HNO_3 and 0.5 ml of HF in polypropylene open tubes with reflux caps and heated by means of a DigiPrep graphite heating block digestion system (SCP SCIENCE, USA) with controlled temperature (10 min at 75 °C, addition 0.5 ml of H_2O_2 drop to drop, ramp of 10 min from 75 to 115 °C, and hold for 120 min). A Thermo iCAP-Q (Thermo Scientific, Bremen, Germany) device equipped with a quadrupole mass analyser and an electron multiplier detector was used for ICP-MS (as described in Hernández-Moreno et al., 2016) and an Agilent 5100 Synchronous Vertical Dual View with a vertical torch and CCD detector was used for ICP-OES. Limits of detection (LOD) and limits of quantification (LOQ) were calculated as being 3 and 10 times the standard deviation of the blank, respectively.

Three independent experiments were performed for each time point and CuO NPs concentration. The amount of Cu released to media was calculated as well as the corresponding percentage (Table 3).

2.9. Statistical analysis

Data are represented as mean ± standard error of the mean (SEM) of at least three independent experiments carried out at different moments ($n = 3$). In each of these three experiments, each concentration was tested in triplicate in the culture plates for the short-term exposures, whereas four replicates per plate were used in each of the long-term experiments). All statistical calculations were performed using Sigma Plot version 12.5 (Jandel Scientific, CA, USA). Normality of the distributions was checked by means of the Shapiro-Wilk test ($p < 0.05$). The program checks automatically for homoscedasticity of variances. In the short term experiments, significant differences between effects caused by treatments with respect to control cells were checked by means of one-way repeated measures analysis of variance (RMANOVA) followed by Dunnett's as post hoc test. Since cytotoxicity of controls was set at 100% no error was generated in these cells. Thus, effects observed in cells exposed to CuO NPs were compared with those detected in cells receiving the lowest CuO NPs concentration. Those were not statistically different from controls but showed a SEM that reflected the variability of the assays. The concentration causing 50% of inhibitory effect (IC_{50}) was calculated by fitting the fluorescence results to a regression equation for a four- parameter logistic curve: $y = [(\max - \min)/[1 + (x/IC_{50})^{-b}]] + \min$, (where max is the maximal response observed, b is the slope of the curve, and min is the minimal response).

In long-term experiments, significant differences between cell viability at different time points with respect to the controls at time 0 were detected by means of RMANOVA followed by Dunnett's post hoc test. In all cases, significant differences were considered when $p < 0.05$.

3. Results

3.1. Physico-chemical properties of CuO NPs

Details about the size frequency distribution of CuO NP suspensions measured by DLS are shown in Table 1.

When the intensity of the signal was taken into account in the DLS measurements, CuO NP suspensions in milli-Q water showed a main peak of 169.8 nm at time 0. This peak included 92% of the total distribution. The remaining 8% of the distribution corresponded to a peak of 4584.3 nm. This distribution did not show variations along time, and after 21 days still a main peak (92% of the distribution) of 146.4 nm was observed together with a minor peak (8%) corresponding to a diameter of 4574.2 nm. In CuO NPs suspensions in L-15 medium at time 0, the main peak (82%) was similar to this detected in milli-Q water (170.0 nm). Particles of only 24.0 nm (10%) and 7.5 nm (8%) were detected. This pattern changed with time, and after 7 days in L-15 medium, most of the distribution (67%) corresponded to 13.6 nm particles. In addition, particles of 68.5 nm (24%) and of 2578 nm (9%) could be detected.

When the size distribution was presented as a function of the number of particles, in the milli-Q water suspensions a single peak of 51.3 nm appeared at time 0 and did not show strong variations along time, so that after 21 days a single peak of 41.4 nm was observed. In L-15 medium, a peak of 7.2 nm was detected at time 0 and of 7.6 nm after 7 days. When presented as a function of the volume of particles, the size distribution exhibited three peaks in the suspensions in milli-Q water at time 0: a main peak of 70.9 nm (90%) and two other peaks corresponding to bigger particles of 387.2 nm (3%) and 4960.5 nm (7%). After 21 days, it was still possible to observe particles of 70.8 nm (85%) and of 4857.6 nm (4%). They were accompanied by a third peak of 25.5 nm (11%). CuO NP suspensions in L-15 exhibited a single peak of 7.0 nm at time 0 and of 7.5 nm after 7 days of incubation.

According to the suppliers, CuO NPs showed a spherical shape with approximately 15–20 nm in size. When analysed by TEM, CuO NPs exhibited a near-spherical shape in milli-Q water at time 0 and formed agglomerates of different sizes (Fig. 1A). The mean diameter of individual particles was 19.26 ± 0.41 nm (Fig. 1B), with > 90% of them exhibiting a size < 40 nm. After 21 days preserved at 4 °C, NPs did not show any change in shape or size (Fig. 1C and D). In cell culture medium at time 0 also aggregates were observed, being the mean diameter of individual particles of 23.83 ± 1.85 nm. Mean size of CuO NP agglomerates was 247.64 ± 80.97 nm. (Fig. 1E and F). When incubated for 24 h under culture conditions in L-15 medium, CuO NPs underwent a reduction in size (Fig. 1G) and attempts to identify NPs

Table 1
Hydrodynamic size distribution of CuO NPs dispersed in Milli-Q water or in L-15 medium with dispersant (BSA) along time in long-term experiments (time 0, day 1, 6 and 7, for exposure dispersions, and time 0, day 1, 7, 14 and 21, for CuO NPs in milli-Q water), according to intensity, number and volume.

Sample	Time (days)	Z-ave (d. nm)	PDI	Average hydrodynamic diameter (nm ± SEM) (%)						
				Intensity			Number	Volume		
				Peak 1	Peak 2	Peak 3	Peak 1	Peak 1	Peak 2	Peak 3
CuO NPs (10 mg/mL) in milli-Q water	0	160.1 ± 17.6	0.4 ± 0.1	169.8 ± 17.7 (92)	–	4584.3 ± 164.8 (8)	51.2 ± 3.6	70.9 ± 4.2 (90)	387.2 ± 84.0 (3)	4960.5 ± 107.6 (7)
	1	148.3 ± 11.5	0.4 ± 0.1	172.3 ± 11.5 (90)	–	4264.2 ± 500.4 (9)	43.2 ± 1.9	80.2 ± 3.4 (74)	137.9 ± 112.1 (19)	4554.7 ± 435.0 (7)
	7	144.9 ± 7.8	0.4 ± 0.0	164.5 ± 1.7 (91)	–	4073.8 ± 601.5 (9)	55.7 ± 5.5	85.9 ± 6.5 (82)	462.1 ± 214.2 (10)	4627.3 ± 243.7 (8)
	14	141.4 ± 1.0	0.4 ± 0.0	175.1 ± 11.7 (93)	–	4456.4 ± 223.2 (7)	38.4 ± 1.7	84.7 ± 6.3 (64)	31.3 ± 1.6 (30)	4475.4 ± 458.4 (6)
	21	124.6 ± 7.5	0.4 ± 0.0	146.4 ± 1.7 (92)	–	4574.2 ± 6.7 (8)	41.4 ± 0.5	70.8 ± 4.1 (85)	25.50 ± 4.2 (11)	4857.6 ± 8.3 (4)
CuO NPs (100 µg/mL) in L-15 medium + BSA	0	124.6 ± 16.7	0.5 ± 0.0	170.0 ± 15.7 (82)	24.0 ± 3.0 (10)	7.5 ± 0.0 (8)	7.2 ± 0.0	7.0 ± 0.1	–	–
	1	48.5 ± 25.1	0.5 ± 0.1	112.6 ± 1.5 (53)	240.5 ± 61.8 (25)	45.6 ± 7.2 (22)	7.4 ± 0.2	7.7 ± 0.2	–	–
	6	53.9 ± 12.0	0.3 ± 0.0	18.1 ± 2.0 (85)	90.3 ± 8.2 (14)	5374.7 ± 51.6 (1)	7.4 ± 0.1	7.2 ± 0.2	–	–
	7	16.1 ± 1.3	0.4 ± 0.0	13.6 ± 0.4 (67)	68.5 ± 12.0 (24)	2578.9 ± 879.9 (9)	7.6 ± 0.2	7.5 ± 0.1	–	–
L-15 medium + BSA	0	13.8 ± 0.5	0.38 ± 0.0	18.85 ± 2.0 (85)	54.2 ± 8.5 (12)	4582.3 ± 350.8 (3)	5.4 ± 0.2	6.0 ± 0.0	–	–
	1	17.3 ± 2.8	0.4 ± 0.0	16.2 ± 3.2 (74)	84.2 ± 10.3 (23)	4558.8 ± 112.1 (3)	5.8 ± 0.3	6.3 ± 0.3	–	–
	6	13.7 ± 0.2	0.4 ± 0.0	17.0 ± 2.5 (80)	68.9 ± 5.2 (18)	4321.7 ± 83.2 (2)	5.7 ± 0.2	5.6 ± 0.2	–	–
	7	14.8 ± 0.9	0.4 ± 0.0	15.2 ± 2.0 (72)	78.3 ± 5.6 (25)	4330.0 ± 191.7 (3)	5.6 ± 0.4	5.6 ± 0.3	–	–

L-15 medium with BSA was measured as background control. Three independent experiments were performed ($n = 3$), the values are represented as mean ± SEM. Numbers in brackets indicate the percentage of each peak in the global distribution. *Z*-ave, *Z* average; PDI, polydispersion index.

after 6 days incubation were not successful. EDX analysis of the particles in L-15 medium at time 0 confirmed the presence of Cu and O (Fig. 1H).

3.2. Interference of CuO NPs with assays components

No autofluorescence was detected for CuO NP dispersions in culture medium or in 24 h exposed cells under assay conditions.

No interference with the fluorescences emitted by each of the fluorophores used in the three cytotoxicity assays was observed either for CuO NP dispersions in culture medium, or in exposed cells for 24 h under assay conditions, after the corresponding washing steps.

AlamarBlue is a commercial solution made up of the redox-sensitive resazurin dye. Since CuO is an oxidizing agent, we assessed if CuO NPs are able to acellularly affect reduction of resaruzine to resorufine. No acellular reduction of resazurin was observed upon incubation of the dye (1.25% v/v) with increasing concentrations of CuO NPs (from 0.78 to 100 µg/ml). We did not detect any interference of CuO NPs with reaction products (resorufin, 5-CF or NR dye) at the corresponding emission and excitation wavelengths, at none of the tested CuO NP or reaction product concentrations. In the same way, $CuSO_4$ did not show any kind of interference.

3.3. Cytotoxicity of CuO NPs in RTL-W1 cell line

3.3.1. Short-term (24 h) cytotoxicity

After 24 h of CuO NPs exposure, a dose-dependent decrease in cell viability was observed with the three cytotoxicity assays applied (Fig. 2). Calculated IC_{50} and IC_{10} values appear in Table 2. The corresponding IC_{50} values for alamarBlue, CFDA and NRU assays were 91.9, 97.7 and 60.0 µg of CuO NPs/ml, respectively. When only Cu content was taken into account, the corresponding IC_{50} values were 68.8, 80.4 and 50.1 µg Cu/ml, respectively. IC_{10} values were similar for the three cytotoxicity assays ranging between 22.1 and 30.2 µg CuO NPs/ml (or between 17.4 and 24.3 µg Cu/ml). In the case of $CuSO_4$ exposure, cell viability was above 80% for all the assayed concentrations (0.78–100 µg $CuSO_4$/ml), so that calculation of IC_{50} was not possible. IC_{10} values were 14.3, 23.5 and 13.33 µg Cu/ml, for the alamarBlue, CFDA-AM and NRU assays, respectively. NOEC and LOEC values were also calculated and are shown in Table 2.

3.4. Long-term (21 days) cytotoxicity

Those concentrations used in 24 h exposure experiments that were most close to the calculated IC_{50} and IC_{10} mean values (calculated from results of the alamarBlue, CFDA-AM, and NRU assays) were used as target concentrations for long-term exposures. Therefore, RTL-W1 cells were exposed to 100 (~EC_{50}) and 25 (~EC_{10}) µg CuO NPs/ml. In parallel, cells were exposed to $CuSO_4$ (used as control ion source), at the same Cu relative concentrations as those used for CuO NPs treatment (313.9 and 78.5 µg of $CuSO_4 \cdot 5H_2O$/ml, respectively).

Similar cell viability was observed for CuO NPs and $CuSO_4$ treatments along 21 days (Fig. 3). Chronic exposure to the low concentrations (~EC_{10}) of CuO NPs or $CuSO_4$ did not affect cell viability up to 21 days of treatment. Cells exposed to CuO NPs or $CuSO_4$ at a concentration close to the 24 h IC_{50} (100 µg/ml and 313.9 µg/ml, respectively) did not show a significant reduction of viability with respect to time 0 after 7 days of exposure. A significant ($p < 0.05$) increase of cytotoxicity was detected with all the three assays used after 14 days of exposure. At this time point, the reduction in cell viability was more pronounced when measurements were performed using the NRU assay, what would indicate that toxicity is preferentially exerted at the lysosomal level. After 21 days of exposure at these concentrations, cell viability decreased to values close to cero with the three cytotoxicity assays used.

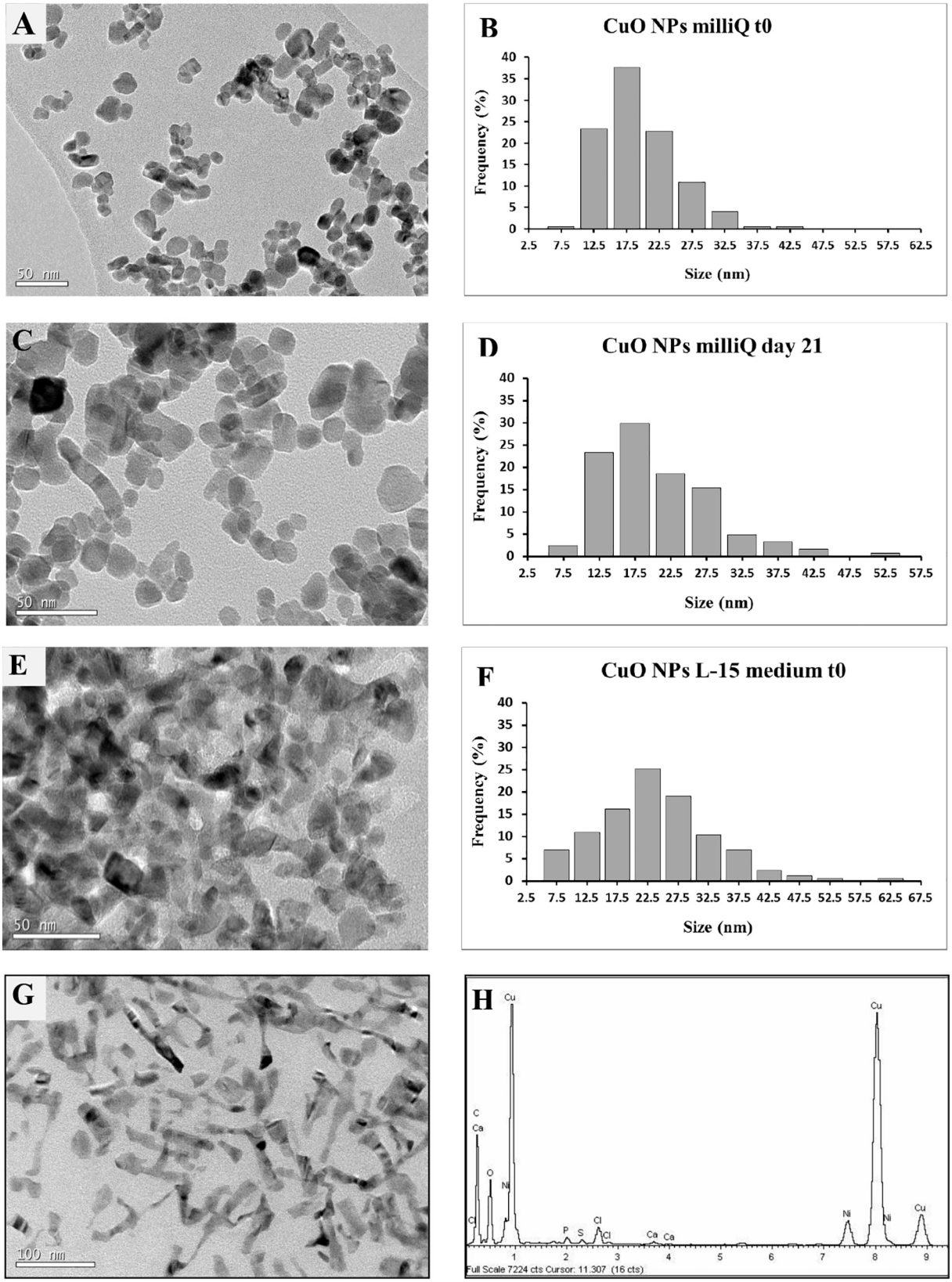

Fig. 1. Micrographs and size frequency distribution of CuO NPs suspensions. (A) TEM micrographs of CuO NPs dispersed in Milli-Q water (10 mg/ml) at time 0 and (B) size frequency distributions. (C) TEM micrographs of the same dispersions after 21 days at 4 °C, and (D) size frequency distributions. (E) CuO NPs dispersions in L-15 medium (200 μg/ml) at time 0. A higher agglomeration than in Milli-Q water is observed. (F) Size frequency distribution calculated from discrete nanoparticles inside the agglomerates. (G) CuO NPs in L15 medium after 24 h incubation at 20 °C. (H) EDX spectrum from an area of the agglomerate is shown in G. Similar spectrum is observed in CuO NPs suspensions in Milli-Q water.

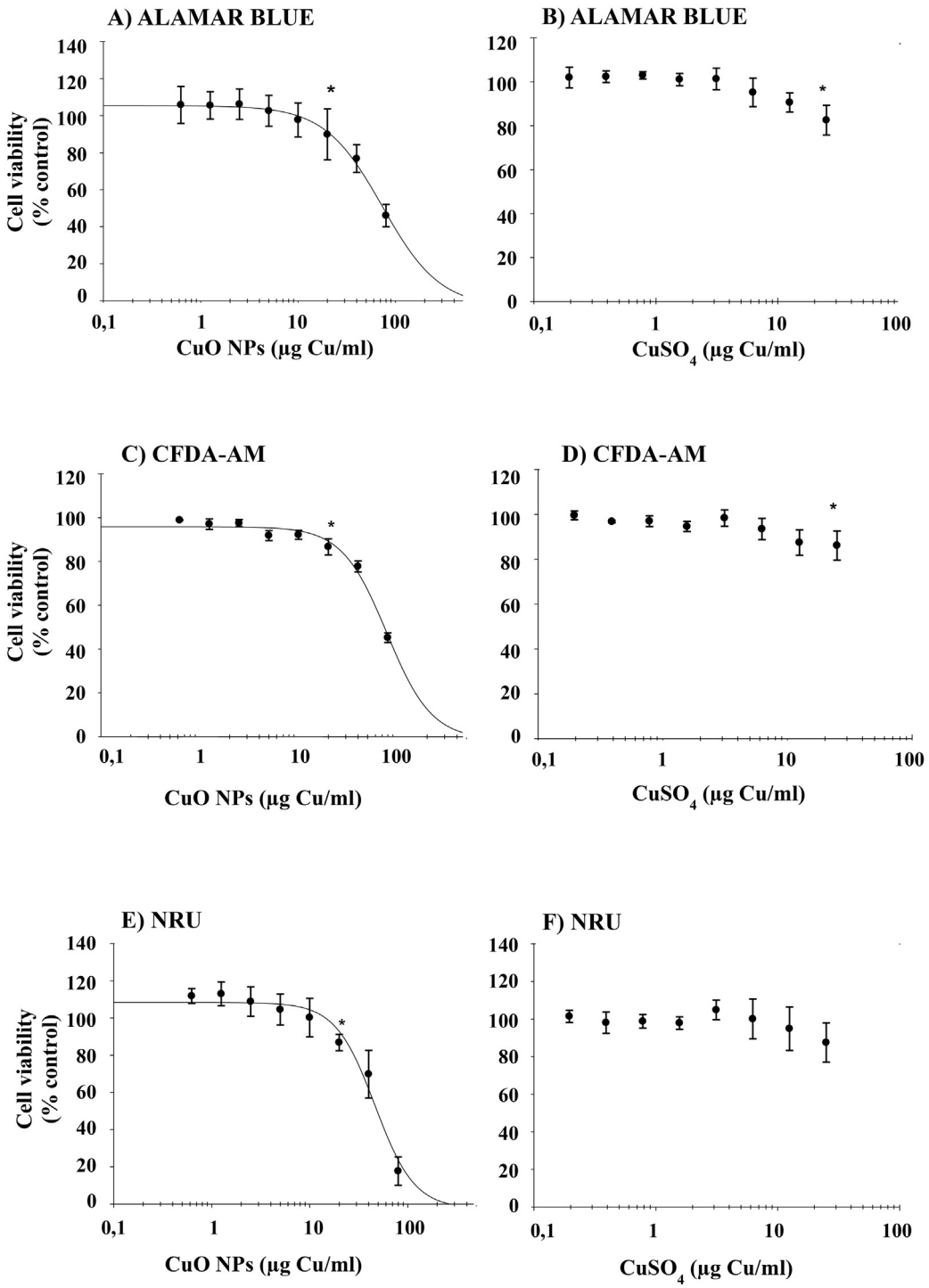

Fig. 2. Effects of CuO NPs and $CuSO_4$ on RTL-W1 cells viability after 24 h exposure. Cell viability is expressed as percentage respect to control cells. (A) Cell viability estimated by means of the alamarBlue assay after exposure to CuO NPs. (B) alamarBlue results after exposure to $CuSO_4$. (C, D) Cell viability estimated through the CFDA-AM assay. (E, F) Cell viability estimated with the NRU assay. Results are the mean ± standard error of the mean (SEM) of at least three independent experiments. Asterisks represent statistically significant differences with respect to cells treated with the lowest CuO NP concentration ($p < 0.05$).

3.5. Long-term subcellular effects of CuO NPs and $CuSO_4$ on RTL-W1 cells

Cells treated with 25 µg of CuO NPs/ml or 78.5 µg of $CuSO_4$/ml (selected low concentrations), did not show any meaningful change in cellular growth or subcellular morphology along the 21 days of treatment, and no Cu particle accumulation or recruitment was observed (micrographs are not shown, but cell structure was similar to that observed in the control, in Fig. 4A and B). This is in accordance with the results obtained from cytotoxicity assays. However, cells treated with 100 µg CuO NPs/ml for only 1 day exhibited already swollen mitochondria with unstructured cristae (Fig. 4C and D). After 7 days of exposure, some images suggested the existence of pinocytosis processes (Fig. 4D and E) and particle inclusions could be detected inside phagolysosomes. At this time point, some cells appeared severely damaged (Fig. 4F). After 14 days of exposure, routine analyses by inverted phase-contrast microscopy showed altered cell growth and only remains of

Table 2
IC_{50}, IC_{10}, NOEC and LOEC values calculated from dose-response curves obtained after treatment of RTL-W1 cells with CuO NPs or $CuSO_4$ for 24 h.

μg CuO NPs/μl (μg Cu/μl)	CuO NPs				$CuSO_4$			
	IC_{50} mean ± SEM	IC_{10} mean ± SEM	LOEC	NOEC	IC_{50} mean ± SEM	IC_{10} mean ± SEM	LOEC	NOEC
AlamarBlue	91.87 ± 2.96 (68.77 ± 10.92)	22.14 ± 7.39 (17.42 ± 6.19)	25.00 (20.00)	12.50 (10.00)	NE	68.88 ± 20.91 (14.32 ± 5.93)	100 (20.00)	50 (10.00)
CFDA-AM	97.67 ± 4.51 (80.42 ± 3.69)	30.21 ± 5.14 (24.34 ± 4.02)	25.00 (20.00)	12.50 (10.00)	NE	114.84 ± 34.60 (23.49 ± 10.08)	100 (20.00)	50 (10.00)
NRU	60.00 ± 8.60 (50.14 ± 7.18)	24.51 ± 5.14 (19.03 ± 4.38)	25.00 (20.00)	12.50 (10.00)	NE	40.51 ± 15.46 (13.33 ± 8.13)	NP	NP

Three independent experiments were performed ($n = 3$). IC values are expressed as mean ± S.E.M. of CuO NPs or $CuSO_4$ nominal concentrations and Cu estimated concentration (in brackets). NE, non-estimated due to lack of effect at the assayed concentrations.

degraded cells could be observed by TEM. These cells accumulated intracellular crystals into membranous structures (maybe degraded mitochondria), (Fig. 4G and H). Analysis based on EDX confirmed the presence of Cu inside the aforementioned deposits (Fig. 4H). After 21 days of CuO NP treatment at the highest concentration, no cells were observed, probably due to the high mortality detected (close to 100%) that would lead to detachment of cells from the coverslip.

Similar subcellular effects to those produced by CuO NP treatment were observed for the high (313.9 μg/ml) $CuSO_4$ concentration. After 24 h, an increase in mitochondria volume and number were evidenced (Fig. 5A and B), and localized fragments of the plasma membrane appeared unstructured (Fig. 5C). After 7 days of exposure a higher proportion of cells than in the case of CuO NP exposure exhibited extensive damage (Fig. 5D, E, F). Upon longer exposures (14 days), similar intracellular crystals to those formed with the highest CuO NP treatment were observed (Fig. 5G, H and I) and after 21 days it was not possible to detect any cell.

3.6. Assessment of CuO NPs dissolution

The amounts of Cu (μg) detected by means of ICP in supernatants and pellets obtained by centrifugation of CuO NPs suspensions are shown in Table 3. Already at time 0, in the CuO NPs suspension at 100 μg/ml (79.90 μg Cu/ml leading to a nominal Cu amount of 958.80 μg in 12 ml of suspension) the amount of Cu in the supernatant was 516.75 ± 39.02 μg which supposed the 60.80% ± 4.14 of the total Cu determined by ICP. It must be taken into account that (as indicated in the materials and methods section) actually from the beginning of the procedure to the time point in which we were able to obtain supernatants and pellets the time lapse was of approximately 2 h. The amount of Cu in the pellet, in principle mostly of nanoparticle nature, was 333.22 ± 35.45 μg. After 24 h the percentage of Cu in supernatants arrived to 90.53% ± 2.02. After 48 h the 95.81% ± 0.8 of the Cu appeared in the supernatant and from the third day (72 h) it could be considered the 100%.

The CuO NPs suspension at 25 μg/ml (19.98 μg Cu/ml and nominal Cu amount of 239.76 μg in 12 ml of suspension) showed a similar behaviour so that at time 0 (actually, considering the time necessary for preparing the sample and centrifuging approximately 2 h) the percentage of Cu in the supernatant was 65.9% ± 3.96. However, already after 24 h the percentage of Cu in the supernatant was 100% (Table 3).

4. Discussion

In the present study we have described the setup of an in vitro protocol to study the effects of prolonged exposure of fish cell lines to low concentrations of NPs. This method has been tested with CuO NPs on RTL-W1 cells, but it could be also used to test long term toxicity of other MNs on different types of adherent cell lines.

As a first step, since biological impacts of MNs are dependent on several NP features apart from chemical composition (as size, shape, solubility and aggregation), a comprehensive characterization of CuO NP physicochemical properties under culture conditions along the exposure time was performed. Regarding this matter, mean Feret diameter of CuO NPs calculated from TEM micrographs at time 0 (19.26 nm in milli-Q water and 23.83 nm in L-15 medium) was lower than the hydrodynamic size obtained by DLS when measurements were expressed as intensity of the signal (169.8 nm in milli-Q water and 170.0 nm in L-15 medium). The DLS measurements evidenced the tendency of NPs to agglomerate in aqueous media thereby giving the size of clustered particles rather than this of individual particles (Siddiqui et al., 2013). TEM and DLS analyses of CuO NP dispersions in milli-Q water showed that they were stable up to 21 days at 4 °C (Fig. 1 and Table I). By the contrary, the peaks observed in the size frequency distribution of CuO NP dispersions in L-15 medium detected by DLS at time 0 and at 24 h (attributed to CuO particles) were not present at days

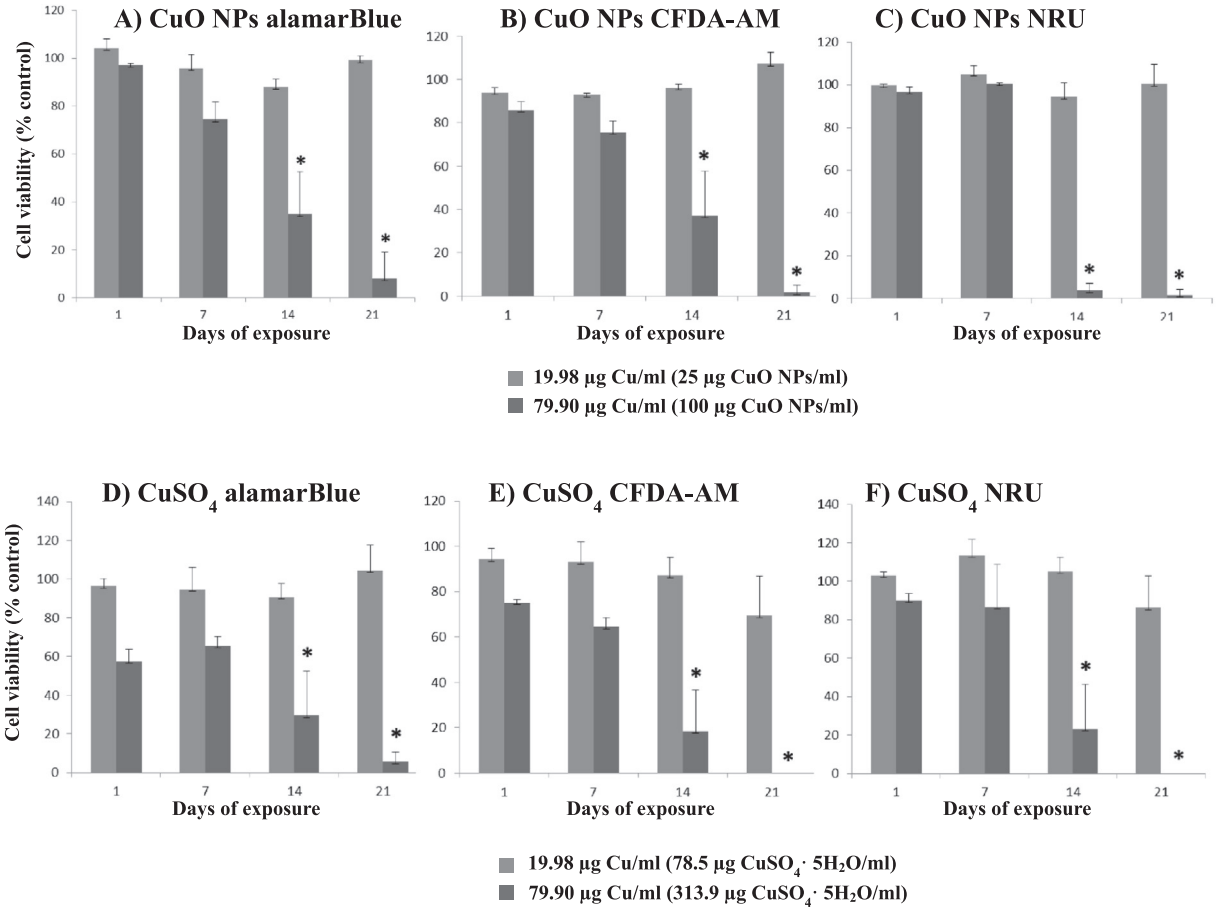

Fig. 3. Effects of CuO NPs and $CuSO_4$ on RTL-W1 cells viability after prolonged exposure. (A) Cell viability variations along time in cells exposed to CuO NPs estimated by means of the alamarBlue assay. (B) Cell viability in cells exposed to CuO NPs estimated by means of the CFDA-AM assay. (C) Cell viability estimated through the NRU assay. (D) Cell viability variations along time in cells exposed to $CuSO_4$ estimated by means of the alamarBlue assay. (D) Cell viability variations estimated through the CFDA-AM assay. (E) Cell viability estimated through the NRU assay. Results are the mean ± standard error of the mean (SEM) of at least three independent experiments. Asterisk indicates significant differences with respect to the controls ($p < 0.05$) along the exposure time at the corresponding concentration.

6 or 7. This observation supports the idea that NPs dissolved in L-15 medium and it is consistent with the fact that TEM images showed a reduction in NP size after 24 h and that it was not possible to detect any particle at day 6 by means of TEM. The ICP analyses carried out on pellets and supernatants obtained by centrifugation of CuO NPs suspensions at different time points confirmed the high solubility of CuO NPs, showing that after 24 h suspensions at 100 and 25 µg/ml exhibited only 9.47% and 0% of Cu in particle form, respectively. The high solubility of CuO NPs dispersed in the amino acid-rich L-15 medium could be due to the chelating action of proteins and amino acids that subtract Cu^{2+} ions from the dissolution equilibrium (Wang et al., 2012).

Interestingly, when DLS readouts were expressed as particle number or volume, a single peak of 7.2 nm and 7.0 nm, respectively, was maintained for 7 days. Stable peaks of 5.4 nm and 6.0 nm (considering particle number and volume, respectively) were observed in L-15 medium alone with BSA. It is difficult to know if these peaks observed in the CuO NPs suspensions should be associated to NPs or if they were those already seen in the medium. We must consider the possibility that bigger agglomerates detected when DLS results were expressed as a function of intensity masked these other peaks, corresponding to particles of lower size. However, the fact that the ICP analyses showed that after seven days most of the Cu appeared in soluble form suggests that these peaks would correspond actually to the medium peaks.

In order to determine the most appropriate concentrations to be applied in the long-term protocol, the short term (24 h) toxicity of CuO NPs on RTL-W1 cells was assessed, and IC_{50} and IC_{10} values were calculated for each cytotoxicity end-point (cellular metabolism, plasma membrane integrity, and lysosomal functionality). Although NOEC and LOEC values (12.5 and 25 µg of CuO NPs/ml, respectively) were identical comparing the three different assays, if we consider IC_{50} values, short term toxicity appears to be exerted mainly at lysosomal level, since IC_{50} obtained from NRU assay (60.00 µg CuO NPs/ml) is lower than those obtained from alamarBlue (91.87 µg CuO NPs/ml) and CFDA-AM (97.67 µg CuO NPs/ml) assays. Inhibition of lysosomal activity by CuO NPs has been previously described by Wang et al. (2012 and 2011). In addition, IC_{50} values for RTL-W1 cells were in the range described for most of the studied organisms (10–100 mg/l, Ivask et al., 2014). Curiously, when CuO NP and $CuSO_4$ toxicities were compared as a function of Cu concentration, similar IC_{10} values were obtained (Fig. 2, Table 2). This is in accordance with the ICP results that evidenced that only after 2 h NPs were dissolved in the culture media, so that cells incubated with CuO NPs were mostly exposed to Cu^{2+} ions, which could be the main responsible of the observed toxicity. Many studies have suggested that dissolution is an important factor influencing harmful effects of metal NPs in biological systems (Griffitt et al., 2009; Hoheisel et al., 2012; Song et al., 2015), although it cannot solely explain the total observed toxicity (Song et al., 2014; Karlsson et al., 2008; Piret et al., 2012; Wang et al., 2012; Zhang et al., 2014).

CuO NP concentrations used in acute exposures most close to the mean IC_{10} and IC_{50} calculated values were used as target concentrations for long term exposures (25 and 100 µg of CuO NPs/ml, respectively), reflecting, respectively, low (non-toxic) and mid (toxic) doses. Exposure

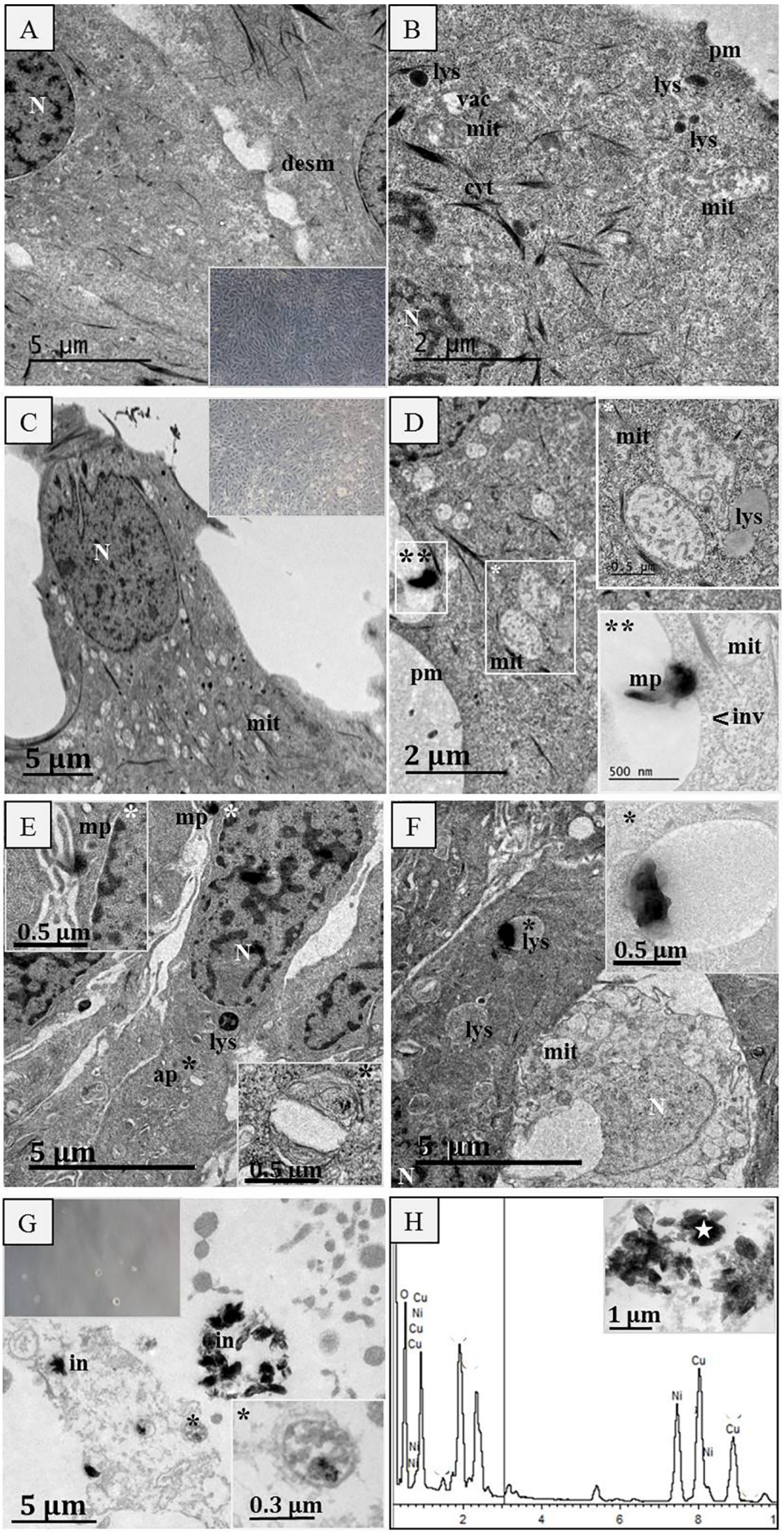

Fig. 4. TEM images showing subcellular structures of RTL-W1 cells along 14 days of CuO NP treatment. (A and B) TEM micrographs of control cells cultured in L-15 medium for 21 days. (C and D) Cells treated with 100 µg CuO NPs/ml for 24 h showing numerous swollen mitochondria (magnified in * insert) and some membrane protrusions containing electrodense material (detailed in ** insert). These protusions were associated with surrounding membrane invaginations. (E and F) Cells treated with 100 µg CuO NPs/ml for 7 days showing membrane electrodense protusions (E, detailed in white asterisk insert) and autophagosomes resulting from possible mitophagy process (E, magnified in * insert). Some cells were seriously damaged and had numerous swollen mitochondria (F). Electrodense material containing Cu appeared included inside lysosomes (F, detailed in * insert). (G) Rests of cells treated for 14 days with 100 µg CuO NPs/ml. (H) EDX analysis of a deposit similar to those shown in G confirmed the presence of Cu. Abbreviations: N, nucleus; desm, desmosomes; lys, lysosomes; ap, autophagosome; vac, vacuoles; mit, mitochondria; cyt, cytoskeleton; pm, plasma membrane; mp, membrane protrusion; inv., invagination of the plasma membrane; in, electrodense inclusions.

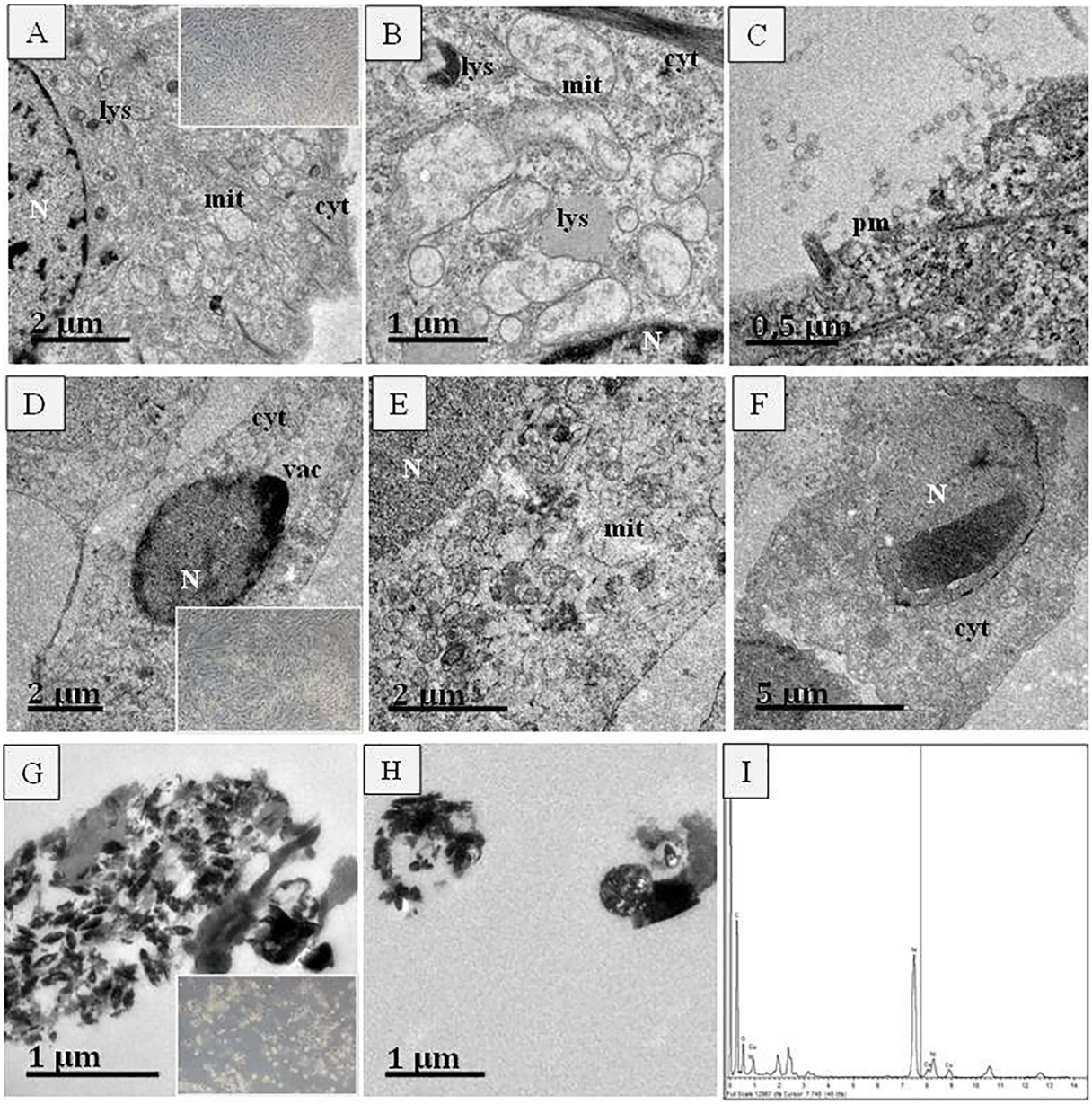

Fig. 5. TEM images showing subcellular structures of RTL-W1 cells exposed to $CuSO_4$ treatment. Phase contrast microscopy micrographs of cell culture at different culture times are placed on the corners of some TEM micrographs. (A, B and C) Cells treated with 313.9 µg of $CuSO_4$/ml for 24 h. An increase in lysosome number and mitochondria size were observed, and some areas along the plasma membrane were unstructured. (D, E and F) Cells treated with 313.9 µg of $CuSO_4$/ml for 7 days. Cells were seriously affected, with unstructured mitochondria and damaged nucleus. (G and H) Rests of cells treated for 14 days with 313.9 µg of $CuSO_4$/ml. Crystals are accumulated inside remains of degraded cells. (I) EDX analysis of a deposit confirming the presence of Cu. Abbreviations: N, nucleus; lys, lysosomes; mit, mitochondria; cyt, cytoskeleton; pm, plasma membrane.

to $CuSO_4$ was carried out in parallel applying concentrations that yielded similar Cu levels in the culture medium as those present in the CuO NP treatments (78.5 and 313.9 µg of $CuSO_4$ $5H_2O$/ml, respectively). The selected low levels of CuO NPs or $CuSO_4$ did not yield any significant impact on RTL-W1 cell viability up to 21 days. However, at the higher concentrations selected, both CuO NPs and $CuSO_4$, reduced drastically cell viability after 14 days of treatment, arriving to near to zero at day 21. Strikingly, although a CuO NP concentration close to the 24 h- EC_{50} value was applied in the long term experiments, the expected reduction of approximately 50% in cell viability was not observed after 24 h of treatment in the long term studies. This effect could be related to the differences between 24 h and 21 days experimental procedures: short term experiments were carried out in 96 well plates with lower cell culture medium volumes and exposure surface than the 24 well plates used in the long term experiments, what might influence cellular metabolism, NP uptake, and detoxification pathways.

No significant differences in cell viability were detected among different cytotoxicity assays up to 14 days exposure, suggesting that the highest concentration produced extensive and general cell toxicity. This hypothesis is corroborated by TEM images that showed cells completely degraded and evidenced accumulation of Cu containing crystals inside. Taking into account that changes in cell ultrastructure along time were similar for cells exposed to CuO NP or to $CuSO_4$, and that the CuO NPs disappeared almost completely from cell culture medium after 72 h exposure, we could say that the main toxic effects of CuO NPs were exerted by Cu^{+2} dissolved from the particles.

Metal NPs can be taken up via endocytosis (Wang et al., 2012) and endocytic vesicles may be converted to lysosomes. Once inside

Table 3
Copper (μg) determined by ICP in the supernatant and pellets obtained after centrifugation of CuO NPs suspensions (100 μg/ml and 25 μg/ml) at time 0 and after the corresponding incubation time at 20 °C. 25 μg/ml of CuO NPs were equivalent to 19.98 μg Cu/ml that corresponded to 239.76 μg of Cu (nominal) in the 12 ml aliquots that were prepared for the analysis of Cu content. 100 μg/ml of CuO NPs were equivalent to 79.90 μg Cu/ml that corresponded to 958.80 μg of Cu (nominal) in 12 ml. Three independent experiments were performed for each time point. The data presented as mean ± standard error media (SEM) (n = 3).

CuO NP suspension concentration	Nominal Cu (μg) in 12 ml	Time (h)	Total Cu (μg) in 12 ml by ICP	Cu (μg) in the supernatant	% of Cu in the supernatant	Cu (μg) in the pellet	% of Cu in the pellet
25 μg/ml	239.76	0	183.87 ± 3.21	121.17 ± 7.21	65.90 ± 3.96	62.70 ± 7.73	34.10 ± 3.96
		24	227.56 ± 7.99	227.56 ± 7.99	100	0	–
		48	207.91 ± 7.22	207.91 ± 7.22	100	0	–
		72	226.21 ± 30.78	226.21 ± 30.78	100	0	–
		96	197.59 ± 8.53	197.59 ± 7.08	100	0	–
		120	207.35 ± 10.66	207.35 ± 10.93	100	0	–
100 μg/ml	958.80	0	849.97 ± 17.54	516.75 ± 39.02	60.80 ± 4.14	333.22 ± 35.45	39.20 ± 4.14
		24	903.78 ± 17.46	818.20 ± 4.24	90.53 ± 2.02	85.58 ± 20.18	9.47 ± 2.02
		48	952.07 ± 52.47	912.17 ± 52.79	95.81 ± 0.8	39.90 ± 7.43	4.19 ± 0.8
		72	897.83 ± 14.90	882.33 ± 13.91	98.27 ± 0.27	15.50 ± 2.58	1.73 ± 0.27
		96	884.57 ± 59.65	880.97 ± 59.42	99.59 ± 0.3	3.60 ± 0.85	0.41 ± 0.19
		120	839.15 ± 62.06	837.64 ± 62.02	99.82 ± 0.09	1.51 ± 0.28	0.18 ± 0.004

lysosomes, the low pH can facilitate the release of Cu^{+2} ions leading to what it has been called a Trojan horse effect (Thit et al., 2015). These Cu^{+2} ions cannot be effectively controlled by the mechanisms that normally regulate their concentration inside the cell and the intracellular Cu toxic concentration could be rapidly reached (Ivask et al., 2014). In the present work, some TEM images of RTL-W1 cells treated with CuO NPs suggested endocytosis events (Fig. 4) and EDX analysis confirmed the presence of Cu in electrodense inclusions inside primary lysosomes. It is also possible that Cu^{+2} ions enter the cell via one or more specific Cu carriers such as Ctr-type transporters or Na^{+} channels (Minghetti et al., 2008; Grosell and Wood, 2002), by which $CuSO_4$ might exert its toxic effect on cells. The prolonged treatment of RTL-W1 cells with low concentrations of CuO NPs did not lead to a decrease in cell viability, indicating that cellular defense mechanisms were able to counteract the toxic effects of Cu^{+2} ions. However, the increase in Cu concentrations provoked a clear toxic effect, probably as a result of Cu accumulation, which overwhelms the natural defenses of the cell, leading to its precipitation in form of crystals and causing intensive cell damage at day 14. Some Cu precipitates seemed to be localized in putative degraded mitochondria (Fig. 4G) and, after seven days of CuO NP treatment (only at high dose), some images suggested mitophagy of mitochondria containing small electrodense deposits (Fig. 4E). Mitophagy is the selective degradation of mitochondria by autophagy and often occurs on defective mitochondria following damage or stress. Previous cellular localization studies have shown that CuO NPs were located also in the mitochondria of A549 cells, altering mitochondrial structures, disrupting the electron transport chain, increasing cytoplasmic ROS production and pro-apoptotic proteins, resulting in irreversible DNA damage (Wang et al., 2012).

In summary, the present work describes a rapid, high-throughput, versatile and a high performance system to study the long term effects of low doses of MNs on cell lines. This approach constitutes a very valuable tool for estimating the toxicity of MNs and the results can be applied in a variety of scenarios supporting the 3Rs principle (for instance in determining appropriate concentrations to be used in higher tier in vivo assays or for a more accurate estimation of the mechanisms underlying the toxic action of MNs, what it can be essential for their risk assessment). The long term exposure permits increasing the sensitivity of the assay and the application of three different cytotoxicity assays on the same set of cells gives important complementary information on the mechanisms of toxicity. Although this system can be applied to other cell lines and MNs, in the present work it has shown its utility in determining the toxicity of CuO NPs on RTL-W1 cells. It was evidenced that cells were able to counteract the low concentrations of MNs, even after 21 days exposure, but that higher concentrations, equivalent to 24 h IC_{50}, caused deleterious effects whose intensity increased with the time of exposure.

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