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Metal oxide nanoparticles induce cytotoxic effects on human lung epithelial cells A549

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Increasing in production and exposure to engineered nanoparticles (NPs), make necessary to acquire information about NP potential adverse health effects. Many studies, focused on NP toxicity, highlighted their cytotoxic potential but there is still a lack of information about the biological mechanisms involved. The aim of this research is the comparison of cytotoxicity between two types of metal nanoxides (CuO and TiO_2) on A549 cells. After physico-chemical characterization, NPs were administered to cells. Cell-particle interactions, membrane integrity, viability and oxidative stress were investigated. CuO exposure resulted in a significant reduction of cell viability, while no effects were observed after TiO_2 exposure. Both NPs induced cell cycle alteration, with a significant increase in frequency of cells in G1 and G2/M phases for TiO_2 and CuO respectively. Confocal microscopy detected NPs at different cellular levels, and TEM imaging highlighted their ability to be internalized as aggregates by phagocytic processes or even as small agglomerates free in the cytoplasm.

1. Introduction

Nanometer-sized particles (<100nm) constitute a fraction of atmospheric particulate matter (PM) and, according to their origin, they are defined “ultrafine particles” (UFPs) or nanoparticles (NPs). UFP is usually adopted to describe airborne particles deriving from combustion emissions; NP indicates nanomaterials intentionally produced by industrial processes (Warheit et al., 2008). Because of their peculiar characteristics, frequently different from the bulk materials, NPs find application in many different fields such as cosmetic, technological and pharmaceutical, allowing a remarkable increase in their production and utilization in the recent years. Therefore the environmental and occupational exposure to NPs is likely increasing and may represent a source of health risk, which determine an urgent need to develop rapid, accurate and efficient testing strategies to assess their biological effects (Hu et al., 2009).Among the different exposure routes, inhalation results to be the more critical; there are significant differences between NPs and larger particles regarding their distribution pattern into the

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respiratory system. Moreover the common mechanisms of clearance often results to be ineffective (Oberdoster et al., 2005). So inhaled nanosized materials can be both kept within the respiratory system or translocated to other target organs. Furthermore with a reduction in size, the properties can change dramatically regarding electrical conductivity, magnetic characteristics, hardness, active surface area, chemical and biological activity (Karlsson et al., 2008). Chemical analysis of UFPs has highlighted the high presence of metal oxide nanoparticles at sites surrounding factories, when compared to clean areas (Rogaczewska and Matczak, 1985), and epidemiological studies have reported a correlation between the level of UFP and the increase in pulmonary disease including exacerbation of bronchial asthma (Weichenthal et al., 2007). It has been hypothesized that different metal oxide NPs are able to generate oxidative stress and affect cell viability through the red-ox potential associated to transition metals (Fahmy et al., 2009), but the role of particle nanostructure in eliciting toxic mechanism remains to be fully understood. The aim of this study is to compare the biological effects induced by commercially available metal oxide NPs (CuO and TiO_2) on A549 lung epithelial cells. In particular we focused on potential cytotoxicity of these nanoxides, on their ability to be internalized in cell compartments and alter the cell cycle. We also investigated the ultrastructural modifications induced by such particles with TEM analysis.

2. Materials and methods

2.1 Particle suspensions preparation and characterization

CuO (<50nm) and TiO_2 (<100nm) NPs (Sigma-Aldrich) were suspended in ultrapure water, sonicated (1min) and diluted in PBS+BSA (0,1% final concentration) to optimize suspension stability. Working concentrations (1, 5, 10, 50, 100 μg/ml) were obtained by adding NP suspensions directly to culture medium (OptiMEM1X, 1%FBS). .CuO and TiO_2 water suspensions have been observed by TEM (Jeol JEM-1220) to depict particle morphology, while Dynamic Light Scattering (ZetaPlus-Brookhaven Instruments Corporation) was used to assess either the mean size of NPs and their polydispersion into the aqueous medium. For CuO we analyze dissolved metal content in culture medium by AAS (Perkin Elmer-SIMAA6000) after centrifugation (12,000rpm; 30min)

2.2 Citotoxicity

A549 cells (American Type Culture Collection) were maintained in OptiMEM1X, 10% FBS at 37°C and 5%CO_2. Cells were seeded in 12-well plate and exposed for 3 and 24h to increasing concentrations of NPs. Cell viability was assessed by Neutral Red retention and MTT assays. In both cases the Optical Density (OD) measured respectively at 540 and 570 nm is proportional to cellular viability. Lactate dehydrogenase activity (intracellular and released in the medium) was measured as marker of cell proliferation and membrane permeability using TOX-7 kit (Sigma–Aldrich) according to the protocol. .The experiments were replicated at least 3 times; results were expressed as mean±SE. Statistical differences were tested by the non-parametric Kruskal–Wallis test (KW) followed by Dunn's method.

2.3 Cell cycle

After treatment (24h) intracellular DNA was labeled with PI (10μM) and 10,000 cells per sample were analyzed with EPICS XL-MCL flow cytometer to highlight cycle alteration. Data were analyzed using the EXPO32 ADC software (Beckman-Coulter). To verify organization of mitotic spindle indirect immunocytochemistry of β-tubulin was performed using a FITC-conjugated secondary antibody.

2.4 Particles internalization

Treated cells were severely washed in PBS and fixed in 4% paraformaldheide. Mitochondria were stained with Mitotracker Red and nuclei with SYBR green (Molecular Probes) to be viewed under a Leica TCSNT confocal laser scanning microscope. NPs were imaged by laser reflection technique. For TEM imaging, cells were fixed in 2.5% glutharalheide, post-fixed in 1% OsO_4 and embedded in EPON-Araldite. Unstained ultrathin sections of 50nm thikness were directely inserted in the TEM (Jeol JEM-1220) operating at 80KV and images taken by a CCD camera.

3. Results

3.1 Particle characterization

TEM analysis confirmed the presence of differently sized aggregates, constituted of NPs well below 100nm for TiO_2 and 50nm for CuO. CuO appeared irregularly shaped, while TiO_2 had a spherical morphology. Mean diameters of 301,1nm and 338,3nm for CuO and TiO_2 water suspensions with a Polidispersity Index of 0,204 and 0,251 respectively were measured with DLS. Cu^{++} dissolution, revealed by AAS, ranging from 0,22 to 0,49 (data expressed as average rates of copper ions in supernatant and nominal dose of particles loaded).

3.2 Cytotoxicity

After exposure to CuO, a time- and dose-dependent significant decrease in cell viability, either with NR (data not shown) or MTT test (Fig.1A) was observed. The dose-response curve for CuO-induced reduction in viability was estimated as an EC_{50} of 19,91 μg/ml. At the same testing conditions, no mortality was evident after TiO_2 exposure (Fig.1B).
Intracellular LDH significantly decreased after 24h exposure to CuO (data not shown), corroborating the data from cell viability assays ($r^{2=}$ 0,923; P<0,05). Released LDH did not resulte in responsiveness, probably for some interactions between CuO NPs and detection molecules. This hypothesis is supported by cell free system experiments that showed a dose-dependent optical density decrease, when incubating LDH standard (0,25 IU/ml) with CuO NPs for 24h (data not shown).

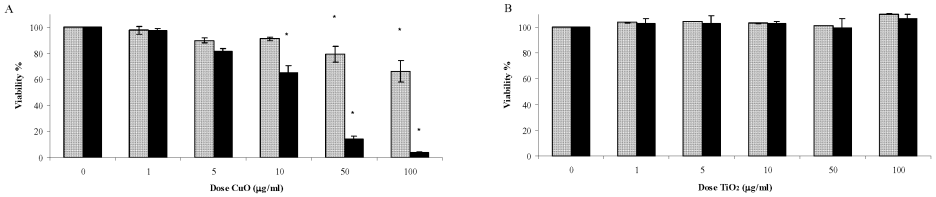

*Fig 1 .A549 cell viability by MTT assay after exposure to CuO (A) and TiO_2 (B) for 3h (grey bars) and 24h (black bars) *Significantly different from control (p<0,05).*

3.3 Cell cycle

The ability of NPs to interact with cells structures resulted also in a significant alteration of the cell cycle. Fig.2 shows A549 cell cycle profile after exposure to increasing concentration of both metal oxides. CuO induced an increase in the frequency of cell at G2/M phase (from 13,6% to 32,1%) while TiO_2 induced a slight increase in frequencies of cells at G1 phase, starting from 10μg/ml (from 63,1% to 73,8%), when compared to the control. Fluorescence microscopy of β-tubulin immunostained cells exposed to CuO (10μg/ml for 24h) confirmed the cycle arrest during early mitotic events (Fig. 3).

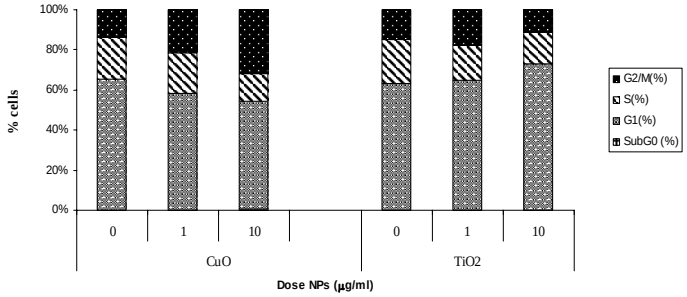

Fig.2 A549 Cell cycle frequencies distribution after exposure increasing concentration of CuO or TiO_2 NPs.

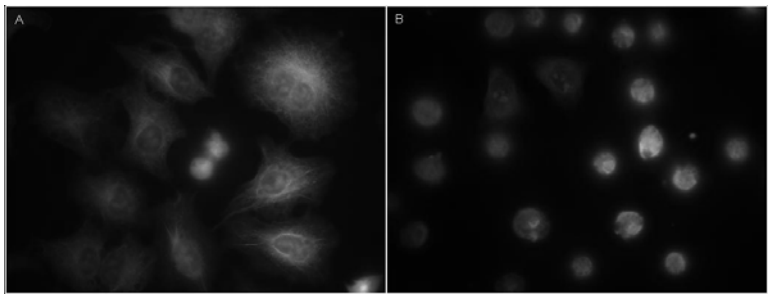

Fig.3 A549 cells immunostained for β-tubulin. A) Control cells; B) Cells exposed for 24h to 10 μg/ml CuO (x630).

3.4 Cell-particle interactions

Different microscopy techniques were applied to better localise the internalised particles. Fig.4 shows internalization of CuO (B) and TiO_2 NPs (C) into mitochondria and nuclei (white bright spots) detected by confocal reflection. Cell-NPs interaction and uptake was quantitatively detected by flow cytometer as increase in laser side scatter (SS) in exposed cells (data not shown). SS can be considerated a marker of an augmented cytoplasmic complexity due to NP interactions. TEM analysis evidenced the uptake and subcellular localization of metal oxides and the alterations induced by NPs. Both NPs were internalised into cytoplasm as free aggregates or phagocytised agglomerates (Fig.5B, C). In particular CuO showed an evident ability to modify the cell ultrastructure (D).

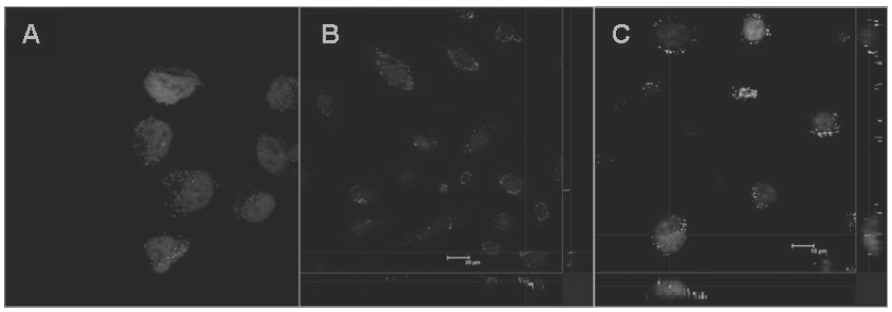

Fig.4 Particle uptake detected by confocal microscope with laser reflection of CuO metal nanoxide in mitochondria (B) and TiO_2 in nucleus (C) respect to the control (A) after exposure to 10μg/ml of NPs suspension.

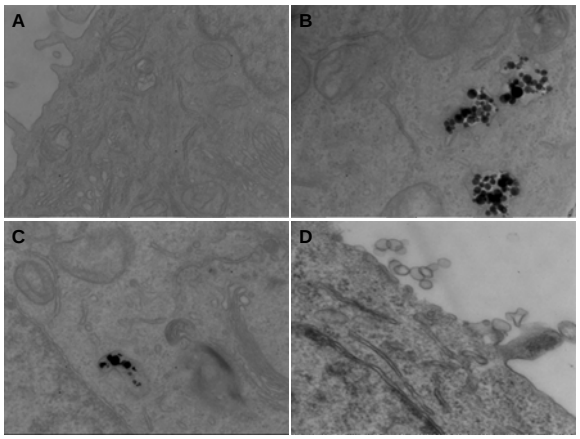

Fig.5 NPs (10μg/ml) cell uptake by TEM; A) Cell control (10k); B) Phagocitised TiO_2 NPs (20k); C) CuO in cytoplasm (20k) D)CuO-induced ultrastructure alteration (15k)

4. Discussion

Although it is reasonable to assume a correlation between metal oxides NPs exposure and increasing in pulmonary or respiratory diseases there is yet a lack of information about toxicity of these materials on biological systems. Current *in vitro* studies reported evidences of cytotoxicity for different metal nanoxides as CuO (Lanone et al., 2009) but results can differ depending on many factors such as cell types, NPs administration conditions and test sensitivity. In this work exposure of A549 cells to CuO and TiO_2 resulted in a significant time and dose dependent cytotoxicity for CuO, while TiO_2 showed negligible cytotoxicity. In agreement with Karlsson et al. (2008), toxic effects cannot probably be mainly attributed to the release in solution of Cu-ions, since NP supernatants failed to induce similar cytotoxic effects (data not shown). This hypothesis was supported by TEM and confocal evidences that showed efficient NP internalization at early exposure time even at mitochondrial level. Especially for CuO this led to progressive worsening of mitochondrial morpho-funcional conditions as reported

(Karlsson et al., 2009) and to generalized ultrastructural damages that determined cell death by necrotic changes. Confocal analyses also highlighted a co-localization of ROS and mitochondria after CuO exposure, suggesting a possible involvement of oxidative imbalance in the first steps of the cytotoxic events triggered by these NPs, even no quantitative cytometric data are till now available to support this hypothesis. The ability of copper to damage mitochondria and compromise the oxidative chain may be only one of the toxicity mechanisms, since other pathways may be involved. Indeed cell cycle progression resulted significantly affected as well as the spindle machinery assembly: cells treated with CuO arrested in G2/M phase and did not duplicate, as demonstrated by the intracellular LDH content, whose dose-dependently decreasing trend testified for a block in cell proliferation. Coupled with the microtubules disorganization, this effect may be ascribed either to DNA damage, already reported for CuO NPs, or disregulation of intracellular calcium homeostasis for the disruption of the membrane systems. As shown by TEM analysis, NPs, CuO in particular, are indeed able to induce lyses of the cell membrane as preliminary event, pointing to a rapid evolution of the cytotoxic pathways leading to the cell necrosis. However the mechanisms of NPs uptake and the molecular pathway during the initial cell responses are still unclear and much more investigative efforts are need to properly characterize the potential mechanisms of pulmonary toxicity of the NPs here investigated.

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