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Effect of particle size and dispersion status on cytotoxicity and genotoxicity of zinc oxide in human bronchial epithelial cells

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ABSTRACT

Data available on the genotoxicity of zinc oxide (ZnO) nanoparticles (NPs) are controversial. Here, we examined the effects of particle size and dispersion status on the cytotoxicity and genotoxicity of nanosized and fine ZnO, in the presence and absence of bovine serum albumin (BSA; 0.06%) in human bronchial epithelial BEAS-2B cells. Dynamic light scattering analysis showed the most homogenous dispersions in water alone for nanosized ZnO and in water with BSA for fine ZnO. After a 48-h treatment, both types of ZnO were cytotoxic within a similar, narrow dose range (1.5–3.0 μg/cm^2) and induced micronuclei at a near toxic dose range (1.25–1.75 μg/cm^2), both with and without BSA. In the comet assay, nanosized ZnO (1.25–1.5 μg/cm^2), in the absence of BSA, caused a statistically significant increase in DNA damage after 3-h and 6-h treatments, while fine ZnO did not. Our findings may be explained by better uptake or faster intracellular dissolution of nanosized ZnO without BSA during short treatments (3–6 h; the comet assay), with less differences between the two ZnO forms after longer treatments (>48 h; the *in vitro* micronucleus test). As ZnO is genotoxic within a narrow dose range partly overlapping with cytotoxic doses, small experimental differences e.g. in the dispersion of ZnO particles may have a substantial effect on the genotoxicity of the nominal doses added to the cell culture.

1. Introduction

The potential harmful effects of small particles depend on their physico-chemical properties that influence their bioavailability [1]. The very small size of nanoparticles (NPs) may favour their entry into the cells, which could eventually lead to stronger cytotoxic and genotoxic effects than observed with larger particles or extracellular ions [2,3]. On the other hand, differences in the conditions of *in vitro* toxicity studies can also modulate NP properties and thus influence the results [4,5]. For instance, the formulation of cell culture media and the dispersion protocol used may change the agglomeration status of NPs and thus affect their sedimentation and cellular contact and uptake or limit their surface area and reactivity, e.g. reactive oxygen species production [6–9]. Sonication and a protein source, e.g. bovine serum albumin (BSA) or fetal bovine serum (FBS), are commonly used to improve NP dispersion.

Accurate assessment of cytotoxicity is a critical prerequisite in genotoxicity studies which should be performed up to moderately cytotoxic doses, to avoid possible secondary effects of cytotoxicity [10]. Cytotoxicity is usually determined in separate experiments before the actual genotoxicity studies, to establish the dose-range to be tested in subsequent genotoxicity assays. If NP dispersions are variable and cytotoxicity occurs at a narrow dose range, the effective doses in the genotoxicity assay may be different than in the preliminary cytotoxicity experiment, resulting in too high or too low exposure, with a potential for false positive or negative outcome. The cytokinesis-block *in vitro* micronucleus (CBMN) test, an assay recently recommended for screening NPs for potential chromosomal damage [11,12], allows the calculation of cytokinesis block proliferation index (CBPI) or cytostasis – measures of cell cycle delay – as means to estimate cytotoxicity, simultaneously with micronucleus assessment.

Zinc oxide (ZnO) NPs, an example of partly soluble metal oxide NPs, are widely used in many industrial applications (*e.g.* in elec-

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tronics) and consumer products (e.g. cosmetics, textiles), mainly due to their wide band gap semiconductor properties and capacity to reflect, scatter and absorb UV radiation [13–16]. The antimicrobial properties of ZnO are utilized not only for biomedical applications [17] but also in food packaging where ZnO offers a more affordable solution than nanosilver [18]. Zinc is an essential element, but exposures to high levels of zinc can cause adverse health effects, such as metal fume fever after occupational exposure to ZnO particles [19,20]. The toxicity of nanosized ZnO has widely been documented in experimental animals and mammalian cells [21].

Several studies reported that ZnO NPs are genotoxic in various mammalian cells *in vitro*, particularly showing induction of primary DNA damage and micronuclei [22–48]. ZnO NPs induced chromosomal alterations in *Allium* root tip cells but not in Chinese hamster lung fibroblasts [49–51]. In Chinese hamster–human hybrid A_L cells, ZnO NPs produced a marginal increase in CD59 mutations which was abolished when endocytosis was prevented by Nystatin [52]. ZnO NPs were not genotoxic to bacteria [50,53–55]. In rats, an increase in 8-hydroxy-2'-deoxyguanosine was observed in serum after pulmonary exposure to ZnO NPs [56], but no increase in DNA damage was observed in liver or stomach tissue after oral exposure [50]. Oral treatment with ZnO particles of different sizes did not either induce micronuclei in mouse bone marrow or blood polychromatic erythrocytes [50,54].

ZnO NPs can be expected to be taken up by cells more efficiently, be dissolved faster in acidic cellular compartments (Trojan horse effect), and exert earlier or higher toxic effects than larger ZnO particles. A few studies have previously examined this possibility. In comparison with microsized ZnO, ZnO NPs were more cytotoxic to human colon carcinoma RKO cells [57] and induced more DNA damage in human nasal mucosa cells [28]. In human embryonic kidney HEK293 cells and mouse embryonic NIH/3T3 cells, only nanosized ZnO, but not bulk ZnO, was able to produce DNA damage, micronuclei, and anchorage-independent growth in soft-agar [25]. ZnO nanoparticles of 26 nm were more cytotoxic than larger ZnO NPs in human colorectal adenocarcinoma Caco-2 cells [58], and specific surface area determined the cytotoxicity of ZnO NPs in human lymphobalstoid WIL2-NS cells [59]. However, no clear difference was seen between nanosized and fine ZnO particles for cytotoxicity in mouse neural stem cells and human bronchoalveolar carcinoma A549 cells [60] or for cytotoxicity and DNA damage induction in human bronchoalveolar carcinoma A549 cells [31].

In the present study, the effects of particle size and the presence of BSA in the dispersion medium on the cytotoxicity and genotoxicity of ZnO in human bronchial epithelial cells BEAS–2 B were explored. Nanosized and fine ZnO particles were examined for the induction of primary DNA damage (alkaline comet assay) and chromosomal damage (CBMN test). Our findings show similar induction of micronuclei by both forms of ZnO after a 48-h treatment but an increase in DNA damage after 3 and 6 h only by nanosized ZnO in the absence of BSA, suggesting an early effect of both ZnO particle size and BSA.

2. Materials and methods

2.1. ZnO materials

Nanosized (30–35 nm) and fine (150–300 nm) ZnO particles were kindly donated by Umicore Zinc Chemicals (Angleur, Belgium). The physico-chemical characteristics of the ZnO particles tested, as provided by the manufacturer, are shown in Supplementary material in Table 1S.

2.2. Physico-chemical characterization of ZnO powders

The ZnO powders were characterized by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) using Zeiss ULTRAPlus FEG-SEM (Carl Zeiss NTS GmbH, Oberkochen, Germany) and Jeol JEM 2010 TEM (Jeol Ltd., Tokyo, Japan), respectively. Samples for SEM and TEM were prepared by gently crushing the ZnO powder, dispersing it in ethanol and applying a drop of the dispersion either onto a plain copper grid or onto a copper grid-holey carbon layer sample holder. Pictures of randomly selected particles were taken using an accelerating voltage of 3 kV for SEM and 200 kV for TEM.

The composition of each ZnO powder was analysed by an X-ray energy dispersive spectrometer (EDS ThermoNoran Vantage; Thermo Scientific, Breda, The Netherlands) attached to Jeol JEM 2010 TEM, whereas the phase structure and crystallite size of the ZnO particles were determined by X-ray diffractometry (PANalytical Empyrean Multipurpose XRD, PANalytical B.V., Almelo, the Netherlands). Crystallite sizes were determined from the peak at 2θ 36.2° (corresponding zincite structure indexes for the peak "[101]", intensity of the peak 100%) with the aid of the HighScore Plus software (PANalytical B.V.).

2.3. Dispersion of ZnO

Stock dispersions of nanosized ZnO (1 mg/ml) and fine ZnO (1 mg/ml) were prepared in two different vehicles – deionized (MilliQ) water and MilliQ water with 0.6 mg/ml (0.06%) BSA (Sigma-Aldrich, Steinheim, Germany) – and sonicated at 37°C for 20 min using a 37 kHz Elmasonic Ultrasound Cleaner (Elmasonic, Singen, Germany). The stock dispersions and solutions were then diluted in culture medium (with and without 0.06% BSA), followed by immediate sonication for another 20 min prior to addition to the cell cultures. Both forms of ZnO were evaluated at equivalent doses as mass of ZnO per unit surface area of the culture dish (μg/cm^2). For the convenience of the reader, the doses used are also given (Supplementary Material, Table 2S) as mass of ZnO per unit volume of culture medium (μg/ml).

2.4. Characterization of ZnO dispersions

The particle size distribution in stock dispersions of nanosized and fine ZnO (prepared as described above) was measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS system (Malvern Instruments, Malvern, UK). The broadness of the particle size distribution was reflected by polydispersity index (PDI; scale from 0 to 1, with 0 being monodispersion and 1 being polydispersion).

In addition, particles in the stock dispersions were documented by plating 0.5 ml of each sonicated stock dispersion onto a well of a 24-well plate and randomly selecting microscope fields for phase contrast imaging using Life Technologies™ EVOS® FL digital inverted microscope (Fisher Scientific, Pittsburgh, PA, USA) equipped with a 40× lens and a monochrome Sony™ ICX445 CCD camera (1/3″, 1280 × 960 pixels).

2.5. Cell system

Human bronchial epithelial cells (BEAS-2B) were purchased from American Type Culture Collection (ATCC #CRL-9609). The cells were maintained in serum-free Bronchial Epithelial Cell Growth Medium (BEGM) supplemented with bovine pituitary extract, hydrocortisone, human epidermal growth factor, epinephrine, transferrin, human insulin, retinoic acid, triiodothyronine, gentamicin and amphotericin B (BEGM BulletKit #CC-3170; Clonetics, Walkerwille, MD, USA). As recent studies have suggested that cell