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Cytotoxic, genotoxic and pro-inflammatory effects of zinc oxide nanoparticles in human nasal mucosa cells *in vitro*

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ABSTRACT

Despite increasing application of zinc oxide nanoparticles (ZnO-NPs) for industrial porpuses, data about potential toxic properties is contradictory. The current study focused on the cyto- and genotoxicity of ZnO-NPs in comparison to ZnO powder in primary human nasal mucosa cells cultured in the air–liquid interface. Additionally, IL-8 secretion as a marker for pro-inflammatory effects was measured. Particle morphology and intracellular distribution were evaluated by transmission electron microscopy (TEM). ZnO-NPs were transferred into the cytoplasm in 10% of the cells, whereas an intranuclear distribution could only be observed in 1.5%. While no cyto- or genotoxicity could be seen for ZnO powder in the dimethylthiazolyl-diphenyl-tetrazolium-bromide (MTT) test, the trypan blue exclusion test, and the single-cell microgel electrophoresis (comet) assay, cytotoxic effects were shown at a ZnO-NP concentration of 50 μg/ml ($P < 0.01$). A significant enhancement in DNA damage was observed starting from ZnO-NP concentrations of 10 μg/ml ($P < 0.05$) in comparison to the control. IL-8 secretion into the basolateral culture medium was increased at ZnO-NP concentrations of 5 μg/ml ($P < 0.05$), as shown by ELISA. Our data indicates cyto- and genotoxic properties as well as a pro-inflammatory potential of ZnO-NPs in nasal mucosa cells. Thus, caution should be taken concerning their industrial and dermatological application. Additionally, further investigation on repetitive NP exposure is needed to estimate the impact of repair mechanisms.

1. Introduction

Nano-sized particles (NPs) of many different metal and ceramic oxides are currently being commercially manufactured for applications including high-performance composite materials, photochemically active or wave-length selective surface coatings, cosmetics and process catalysts (Veranth et al., 2007). Being smaller than 100 nm in diameter, NPs have an extended surface compared to the bulk forms with regard to their mass. Thus, NPs exhibit specific physicochemical properties and functions (Colvin, 2003; Oberdörster et al., 2005). However, information about possible biological adverse effects is rare. Over the last 5 years, research on environmental and possible health hazards has been intensified. Results indicate the induction of cytotoxicity, inflammatory responses and oxidative stress by some NPs in human cell systems (Singh et al., 2009; Choi et al., 2010).

Nano-sized zinc oxide (ZnO) is used in industrial products including cosmetics, paints and medical materials. As a well-known photocatalyst, ZnO has received much attention in the degradation and complete mineralization of environmental pollutants (Yeber et al., 2000; Xu et al., 2007), comparable to titanium dioxide (TiO_2) NPs (Theogaraj et al., 2007). In combination with UV exposure, ZnO-NPs generate reactive oxygen species (ROS) like hydroxyl radicals or hydrogen peroxide in aqueous solutions, resulting in a high efficiency in the decomposition of organic compounds (Li and Haneda, 2003). Though ZnO-NPs are considered to be non-toxic by some authors (Colon et al., 2006; Qi et al., 2008), other studies describe possible side effects in several kinds of human cell systems. Exposure to ZnO-NPs has been associated with inflammatory responses and cytotoxicity (Jeng and Swanson, 2006). A genotoxic potential of ZnO-NPs has been demonstrated in Chinese hamster ovary (CHO) cells by Dufour et al. (2006) for concentrations of 105 μg/ml in the darkness and 54 μg/ml under UV exposure. DNA

Abbreviations: ZnO, zinc oxide; NP, nanoparticle; IL, interleukin; TEM, transmission electron microscopy; MTT, dimethylthiazolyl-diphenyl-tetrazolium-bromide; OTM, Olive tail moment.

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damage has been measured by the chromosome aberration test. However, information about the properties of NPs used in this study was rare (Singh et al., 2009). According to Sharma et al. (2009), an increased Olive tail moment was measured in a human epidermal cell line (A431) even in low concentrations of 0.8 μg/ml.

Summarizing the current literature, information about the genotoxic and cytotoxic potential of ZnO-NPs is inconsistent. Hence, the aim of the present study was to measure DNA damage and cytotoxicity induced by ZnO-NPs. We investigated primary human nasal mucosa cells for the first time, since these cells represent the initial contact region for particle exposure in the human organism. Additionally, the secretion of the pro-inflammatory cytokine IL-8 was determined in order to estimate the role of ZnO-NPs in the induction of immunological reactions. Due to the importance of the cell differentiation level as a determinant for IL-8 secretion, the air–liquid interface was chosen as an appropriate *in vitro* cell culture model favoring the development of well-differentiated nasal epithelial cells (Lopez-Souza et al., 2009).

2. Materials and methods

2.1. Isolation and culture of primary human nasal mucosa cells

Specimens from human mucosa of the inferior nasal turbinate of ten patients (3 female and 7 male patients, age range 44–68 years, median age 52 years) were obtained during surgery on the nasal airways. Since only mucosa resected for surgical reasons was investigated, there was no additional risk for the patient. The patients had signed an informed consent statement, and the study was approved by the Ethics Board of the Medical Department of the Julius-Maximilian-University of Wuerzburg. After cleaning from blood by washing in Minimum Essential Medium (MEM), the specimens were incubated in a mixture of 9 ml MEM supplemented with 0.1 mg/ml Protease XIV, 1 mg/ml DNAse DN25, Gentamycin 0.05 mg/ml, 100 U/ml Penicillin with 1 μg/ml Streptomycin, 0.250 U/ml Amphotericin B and 2 mM L-Glutamine for 24 h at 4 °C. Penicillin and Streptomycin were purchased at Biochrom AG (Berlin, Germany). All other chemicals were obtained from Sigma–Aldrich (Steinheim, Germany).

After 24 h the enzyme activity was stopped by adding 2 ml fetal calf serum (FCS; Linaris, Wertheim-Bettingen, Germany). Afterward, the specimens were scratched with a scalpel to detach the epithelial cells. The cell suspension was centrifuged at 500*g* and 4 °C for 5 min. For resuspension of the cell pellet, Airway Epithelial Cell Growth-Medium, (BEGM, PromoCell GmbH, Heidelberg, Germany) supplemented with antibiotics (100 U/ml Penicillin, 1 μg/ml Streptomycin) was used. Cells were counted after vital staining with trypan blue 0.4% (Sigma–Aldrich) using a light microscope (DM4000B, Leica Microsystems CMS GmbH, Wetzlar, Germany).

Nasal mucosa cells were cultured on semi-permeable membrane inserts (Corning® Transwell polycarbonate membrane inserts, 0.4 μm; 12 mm diameter, Corning Incorporated, New York, NY, USA). The inserts were covered with 100 μl collagen I (66 ng/ml; Sigma–Aldrich) and incubated for 30 min at 37 °C in a humidified incubator. 10^4 nasal mucosa cells were transferred onto each insert. Additionally, medium was added until 0.5 ml BEGM was apical to the membrane and 1.5 ml basolateral. Transwell membranes were cultured at 37 °C in a humidified incubator with 5% CO_2. The cells attached to the membrane within 2–3 h. Medium was changed every second day. After reaching 70%–80% confluence, the medium apical to the membrane was removed to provide an air–liquid interface. Nutrition was allowed by providing 1.3 ml BEGM with 10^{-7} M retinoid acid (Sigma–Aldrich) per insert under the membrane. The air–liquid interface condition was maintained for another week.

2.2. Chemicals

ZnO-NPs (<100 nm, surface area 15–25 m^2/g) and ZnO powder (<5 μm) were obtained from Sigma–Aldrich (Steinheim, Germany). Prior to cell exposure, 5 mg NPs were suspended in 870 μl sterilized distilled water. The stock suspension was sonicated (Bandelin, Sonopuls HD 60, Berlin, Germany) for 60 s at a high energy level of 4.2×10^5 kJ/m^3 using a continuous mode to create a high grade of dispersion according to the protocol of Bihari et al. (2008). 30 μl of 1.5 mg/ml bovine serum albumin (BSA) were added in order to stabilize the dispersion. Finally, 100 μl 10× concentrated PBS was added to achieve a physiological salt concentration at pH 7.4. 5 mg ZnO powder was suspended in PBS without prior sonication. Both stock suspensions were subsequently diluted with BEGM medium.

2.3. Characterization of nanoparticles

The morphology and size of nanoparticles in the stock dispersion was determined by transmission electron microscopy (TEM). After sonication and stabilization, the TEM samples were prepared by drop coating of the stock suspension on carbon-coated copper grids. The films on the grids were dried using a tissue paper prior to measurement. The particle characterization included shape, size, size distribution and tendency of aggregation. The evaluation was performed on a Zeiss transmission electron microscope EM 900 (Carl Zeiss, Oberkochen, Germany) at the Division of Electronmicroscopy in the Biocenter of the University of Wuerzburg. Additionally, size distribution of NP aggregates in the above mentioned cell culture medium was evaluated by dynamic light scattering (Malvern Instruments Ltd., Herrenberg, Germany). The size distribution of ZnO powder aggregates in cell culture medium was measured by laser diffraction spectrometry (Beckman Coulter LS 13320, Krefeld, Germany). A NP dispersion of 50 μg/ml was prepared according as described above and stored 1 h before characterization. The surface zeta-potential of NP dispersion in the above mentioned BEGM cell culture medium (pH 7.4) was assessed by a ZetaSizer 3000HSA (Malvern Instruments Ltd.). Soluble Zn^{2+} ions in the supernatant culture media were measured by atomic absorption spectrometry (SpectraAA 55 B, Varian GmbH, Darmstadt, Germany). Prior to measurement, medium samples were centrifuged in order to obtain particle-free probes.

2.4. Cell treatment

The treatment with ZnO-NPs and ZnO powder started immediately after sonication and dilution of the stock solutions. 500 μl of diluted nanoparticle or powder suspension were applied at end concentrations of 0.01, 0.1, 5, 10 and 50 μg/ml in the well. BEGM served as negative control, and directly alkylating methyl methane sulphonate (MMS, Sigma–Aldrich) at 100 μM was used as a reliable positive control of genotoxicity without cytotoxic effects. ZnO-NP or ZnO powder dilutions were exposed to the cells for 24 h at 37 °C in a humidified incubator with 5% CO_2. Subsequently, cells were detached from the wells by trypsinization with trypsin–EDTA 0.05% (Invitrogen, Karlsruhe, Germany) and centrifuged at 500*g* for 5 min. After removal of the supernatant, the cell pellet was resuspended at 10^5 cells/ml.

2.5. Cell preparation for TEM

For the ultrastructural study of nanoparticle cell invasion, cell pellets were obtained after 24 h of exposure. They were fixed in a fresh solution of 0.1 M sodium cacodylate buffer containing 2.5% glutaraldehyde and 2% formaldehyde followed by a 2 h fixation at 4 °C with 2% osmium tetroxide in 50 mM sodium cacodylate