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Comparative *in vitro* genotoxicity study of ZnO nanoparticles, ZnO macroparticles and $ZnCl_2$ to MDCK kidney cells: Size matters

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ABSTRACT

In the present study, we evaluated the roles that ZnO particle size and Zn ion release have on cyto- and genotoxicity *in vitro*. The Madin-Darby canine kidney (MDCK) cells were treated with ZnO nanoparticles (NPs), ZnO macroparticles (MPs), and $ZnCl_2$ as a source of free Zn ions. We first tested cytotoxicity to define sub-cytotoxic exposure concentrations and afterwards we performed alkaline comet and cytokinesis-block micronucleus assays. Additionally, the activities of both catalase (CAT) and glutathione S-transferase (GST) were evaluated in order to examine the potential impairment of cellular stress-defence capacity. The amount of dissolved Zn ions from ZnO NPs in the cell culture medium was evaluated by an optimized voltammetric method. The results showed that all the tested zinc compounds induced similar concentration-dependent cytotoxicity, but only ZnO NPs significantly elevated DNA and chromosomal damage, which was accompanied by a reduction of GST and CAT activity. Although Zn ion release from ZnO NPs in cell culture medium was significant, our results show that this reason alone cannot explain the ZnO genotoxicity seen in this experiment. We discuss that genotoxicity of ZnO NPs depends on the particle size, which determines the physical principles of their dissolution and cellular internalisation.

1. Introduction

High production and broad applicability of zinc oxide nanoparticles (ZnO NPs) (Keller et al., 2013; Piccinno et al., 2012) have highlighted an increased need for the careful assessment of their potential interactions with biological systems. The Food and Drug Administration (FDA) classifies ZnO as Generally Recognized As Safe (GRAS; FDA, 2016); however, many studies have indicated toxicity of its nanosized formulation (Pandurangan and Kim, 2015; Vandebriel and De Jong, 2012). Some authors reported that ZnO NPs are among the most cytotoxic NPs (Hu et al., 2009; Pujalté et al., 2015). In addition to the cytotoxicity, genotoxicity has been proven to represent an important risk of ZnO NP exposure (Magdolenova et al., 2014).

Genotoxicity testing is an important part of the safety assessment of products containing NPs (Doak et al., 2012; Guo and Chen 2015). Genotoxicity can occur even when cells are exposed to low concentrations of a genotoxic substance which does not induce cell death (Pfaller et al., 2010); however, this damage can accumulate in the genome or can be incorrectly repaired by cell repair mechanisms resulting in diverse genetic mutations and may even induce cancer development (Loeb, 2011).

Although both positive and negative results have been reported in various cell and animal test models, the existing data suggests that many NPs are genotoxic (Guo and Chen 2015). In many studies, observed DNA damage simply reflects cytotoxicity since high test concentrations of NPs that also induce cell death were selected. Namely, during cell death, cellular components including DNA, are being broken down, which can be detected and falsely interpreted as a genotoxic effect. Thus, both cytotoxicity and genotoxicity have to be tested together when the genotoxicity of a substance is assessed (Magdolenova et al., 2014).

It is known that oxidative stress plays an important role in NP-induced genotoxicity (Guo and Chen 2015; Magdolenova et al., 2014). NP interaction with cells can increase production of reactive oxygen species (ROS) or damage the cellular stress-defence system that normally regulates the level of intracellular ROS. An elevated ROS level can cause DNA strand breaks, oxidative DNA adducts, DNA cross-linkage and DNA-protein cross-links (Guo and Chen 2015).

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Toxicity of ZnO NPs is commonly ascribed to their high solubility (Heim et al., 2015; Pandurangan and Kim, 2015). When ZnO NPs dissolve, Zn ions are released, which can disrupt cellular Zn homeostasis, resulting in lysosomal and mitochondria damage and ultimately cell death. When ZnO NPs are endocytosed and sequestered in lysosomes the acidic environment increases Zn ion release (Pandurangan and Kim, 2015; Xia et al., 2008). While some authors report that ZnO NPs toxicity is a result of cellular intake of Zn ions (Heim et al., 2015; Pandurangan and Kim, 2015), the correlation of NP toxicity with the phenomenon of particle dissolution and the availability of released ions is not always demonstrated (Sharma et al., 2012).

Some *in vivo* studies have shown that different NPs, including ZnO, can be retained in the kidney (Li et al., 2012). Kidney cells were shown to be reliable for detecting a NP dose-response (Cowie et al., 2015). In our study we used the Madin-Darby Canine Kidney (MDCK) as an *in vitro* model of renal epithelial cells.

The aim of our work was to study whether sub-cytotoxic concentrations of ZnO particles induce DNA damage in the MDCK cells and whether the damage is dependent on the particle size (cell treatment with ZnO NPs *vs*. ZnO macroparticles; MPs). Since some current papers suggest that ZnO NP toxicity can be explained only by means of Zn ion release in the extracellular region (Heim et al., 2015; Pandurangan and Kim, 2015), we compared the effect of ZnO NPs with the equimolar concentrations of free Zn ions (ionized zinc from $ZnCl_2$). The amount of released Zn ions from ZnO NPs was assessed after the incubation in the cell-free cell culture medium. We hypothesise that if ZnO NP dissolution in the extracellular region is the main factor responsible for the toxic effects, the $ZnCl_2$ would provoke the most severe damage. In addition, we measured the potential of different Zn species to increase acellular production of ROS. The activity of cellular stress-related enzymes (GST, CAT) in the exposed MDCK cells was analysed in order to test the cellular defence capacity. Contrary to many recently published studies on ZnO NP genotoxicity *in vitro* (see Supplementary Table 1), we measured Zn ion concentrations with a method suited to discriminate between ions and NPs in cell medium adopted in our laboratory (Romih et al., 2017).

2. Materials and methods

2.1. Chemicals

$ZnCl_2$, methanol, KNO_3, H_2O_2 and N-lauroylsarcosine were purchased from Merck (Darmstadt, Germany). Agarose was purchased from Life Technologies (Carlsbad, USA). ZnO NPs (labelled size < 100 nm), ZnO MPs (labelled size < 1 μm), cell culture media and all the other chemicals were obtained from Sigma-Aldrich (Steinheim, Germany) unless otherwise specified.

2.2. Preparation and characterization of particle suspensions

123 mM stock suspensions of ZnO NPs and ZnO MPs were freshly prepared before experiments in deionized water (MilliQ, Millipore, Billerica, MA, USA [pH = 5.7, ρ = 18.5 MΩ·cm]). Before each experiment, stock suspensions were vortexed, sonicated in ultrasound bath (Sonis 2GT, Iskra PIO, Sentjernej, Slovenia) for 15 min and diluted in the corresponding cell culture medium to the final concentrations. The suspensions were characterised using transmission electron microscopy (TEM), dynamic light scattering (DLS) and zeta (ζ) potential measurements. The specimens for TEM analysis were prepared by drying the aqueous suspension of ZnO NPs or MPs at room temperature on a transparent carbon foil supported on a copper grid, and the images were obtained using a JEOL 2100 microscope (JEOL Ltd., Tokyo, Japan). The diameter of at least 500 ZnO NPs and MPs was measured. DLS measurements of the hydrodynamic size of the ZnO particles were performed in the 737 μM particle suspensions prepared in deionized water and in the 737 μM and 123 μM particle suspensions prepared in the cell culture medium. DLS is based on the determination of Brownian motion and accurate results can only be obtained if particles do not form a sediment in the sample. After initial mixing of particle suspensions, we left them for 20 min without shaking, in order to allow the largest particle aggregates/agglomerates, which can interfere with DLS measurement, settle on the bottom of the measuring flasks. After 20 min, we performed DLS measurements of the particles that remained dispersed in the suspension. For DLS measurements we used an Analysette 12 DynaSizer (Fritsch GmbH, Idar-Oberstein, Germany). The ζ-potentials of 737 μM ZnO NPs and MPs in deionized water and Dulbecco's Phosphate Buffered Saline (DPBS) were measured with ZetaPALS potential analyzer (Brookhaven Instruments Corp, Holtsville, NY, USA).

2.3. Cell culture

MDCK cells were a courtesy from Prof. Dr. Mateja Erdani-Kreft and Prof. Dr. Peter Veranič from the Institute of Cell Biology, Faculty of Medicine, University of Ljubljana, Slovenia. MDCK cells were cultured in 1:1 (v/v) mixture of Dulbecco's modified Eagle's medium (DMEM) and F-12K medium. Medium was supplemented with 4 mM L-glutamine and 5% (v/v) fetal bovine serum (FBS). Cells were grown at 37 °C in a humidified atmosphere with 5% CO_2 and were routinely passaged twice a week. All experiments were conducted in the dark in order to avoid any potential additional DNA-damage due to UV-radiation and phototoxic potential of ZnO NPs.

2.4. ZnO NP dissolution analyses in the cell culture medium

ZnO NP suspensions in the cell culture medium were prepared at different concentrations (0, 61, and 123 μM). The suspensions were kept in the cell culture incubator under the same conditions as in the cell experiments, *i.e.* in a controlled atmosphere (37 °C, 5% CO_2, 95% relative humidity) for 24 h. After the incubation, 8 mL of cell medium samples were ultracentrifuged at 100000 rcf for 30 min at 20 °C. The supernatant from each sample was divided into two aliquots. 750 μL from each of the first aliquots were acidified with 750 μL 65% HNO_3 (Fischer Scientific, Leicester, UK) and samples were subjected to closed-vessel digestion in the ETHOS One (Milestone, Bergamo, Italy) microwave lab station equipped with SK-10 high-pressure segmented rotor and 3 mL quartz microsampling inserts. Digestion was conducted at 200 °C and 700 W power, with step 1 (heating) lasting 15 min, step 2 (constant temperature) lasting 15 min, and 45 min cooling to 60 °C. Total Zn concentrations in the digests were measured by flame atomic absorption spectroscopy (Perkin-Elmer AAnalyst 100, Waltham, Massachusetts, USA). The second aliquots were left unacidified and were analysed by square-wave anodic stripping voltammetry following the protocol described in Romih et al. (2017). The measurements were performed using the modular voltammetric analyzer Autolab PGSTAT 10 (Eco Chemie, Utrecht, Netherlands) in combination with GPES 4.9 software (Eco Chemie B.V.). The usual three-electrode configuration was employed with the bismuth film electrode prepared *in situ* on a substrate glassy carbon disk electrode (d = 2 mm) as the working electrode, a platinum wire as the counter electrode, and a double-junction saturated Ag/AgCl/KCl (sadt.) reference electrode containing 0.1 M HNO_3 as the outer electrolyte. A computer-controlled magnetic stirrer rotating at approximately 300 rpm was employed during the accumulation and cleaning step. All experiments were carried out at room temperature of 23 ± 1 °C in a 20 mL one-compartment voltammetric cell. The standard solutions of bismuth(III) and zinc(II) were provided by Merck (Darmstadt, Germany) and further diluted as required. A mixture of 0.1 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; Avocado Research Chemicals Ltd., Lancaster, UK) and 0.1 M KNO_3 was used as the supporting electrolyte. The pH was adjusted to 6.5 with 0.5 M KOH (Kemika Zagreb, Croatia). Water used throughout the work was first deionized and then further purified using Elix 10/Milli-Q Gradient unit (Millipore). 100–300 μL of the samples were added to the