Physico-chemical characteristics and cyto-genotoxic potential of ZnO and TiO$_2$ nanoparticles on human colon carcinoma cells

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Abstract The aim of the present study is to investigate the role of the physico-chemical properties of ZnO and TiO$_2$ NPs in the potential cytotoxicity, genotoxicity and oxidative DNA damage induction on Caco-2 cell line. As negative control, fine TiO$_2$ particles were used. The characterization of particles was carried out by electron microscopy (SEM, TEM) using a Soft Imaging System. To evaluate the effects of ZnO and TiO$_2$ NPs induced on Caco-2 viability, Neutral Red assay was performed after treatment with different particle concentrations. Our results showed a significant dose and time dependent effect after treatment with ZnO NPs. On the contrary, no effect was observed on Caco-2 cells exposed to TiO$_2$ particles either in micro- and in nano-size. The role of surface in the cytotoxicity induced on Caco-2 was also considered. The levels of DNA 8-oxodG, as the main marker of oxidative DNA damage, were measured by high-performance liquid chromatography with electrochemical detection (HPLC/EC). A significant increase in the 8-oxodG levels was observed after 6 h exposure for both NPs. The estimation of the potential genotoxicity of the two NPs is ongoing by the cytokinesis-block micronucleus assay. Our preliminary results showed that a slight micronucleus increase in binucleated cells was detected in the dose range applied only for ZnO.

1. Introduction

The unique nature of nanoparticles (NPs) expressed in their size, shape and surface characteristics is expected to play an important role in their possible toxicological effects [1,2].

Moreover, in the last years several studies indicated that NPs may interfere with cellular system by the interaction with proteins, DNA, lipids, membranes, organelles and biological fluids. Although the mechanisms underlying the NPs toxicity are not yet to be elucidated, it has been suggested that oxidative stress and lipid peroxidation play an important role in DNA damage, cell membrane disruption and cell death [3-5].
Zinc oxide (ZnO) and titanium dioxide (TiO$_2$) nanoparticles are widely used in sunscreens because of their less scattering visible light ability. Many studies have been performed on cellular lines representative of dermal and pulmonary exposure [6,7], while few data are available on gastrointestinal exposure [8].

The aim of the present study is to investigate the ZnO and TiO$_2$ NP potential cytotoxicity, genotoxicity and oxidative DNA damage induction on Caco-2 cell line, a well known in vitro intestinal model. As negative control, fine TiO$_2$ particles were used. Moreover, the role of the physico-chemical characteristics of these particles on the observed toxic effects has been considered.

2. Materials and methods

2.1. Particles
ZnO NPs, TiO$_2$ anatase NPs e TiO$_2$ fine were obtained from Sigma Aldrich Company LTD. The nominal sizes of particles as purchased are 50-70 nm for ZnO, < 25 nm for TiO$_2$ NPs and about 1 µm for TiO$_2$ fine. The manufacturer makes no statement on purity of ZnO NPs and TiO$_2$ fine, while a purity of 99.7% for TiO$_2$ anatase NPs was reported.

2.2. Sample preparation for electron microscopy characterization
Few milligrams of particles were weighted with a Mettler H54 AR electric balance (precision 0.01 mg). The particles were suspended in cell culture medium in order to evaluate the state of agglomeration of particles. Stock suspension of particles was probe sonicated (Vibracell, Sonics & Materials Inc., USA) and immediately after sonication few millilitres of suspensions were filtered through 0.05 µm pore polycarbonate membranes.

2.3. Electron microscopy for single-particle characterization
Scanning electron microscopy and Transmission electron microscopy were used to characterize the single NPs. Morphological analysis of single particles was performed by a SEM FEI XL30 (FEI Company, The Netherlands) equipped with Soft Imaging System [9]. More than 1000 particles were analyzed. Known the volume of suspensions deposited on the filters, the area of exposed filters and the portion of them analysed by SEM, the number of particles in the suspensions was determined. From size distribution obtained by morphological analysis, the percentage of particles in granulometric size range of 100 nm and the average diameter of particles belonging to these intervals were determined. Therefore the surface area of particles was estimated, assuming them spherical. Morphology and primary size of the particles were also determined by a TEM (FEI Company, The Netherlands).

2.4. Cell culture
Caco-2 cells (ATCC collection) were routinely cultured in an atmosphere of 5% carbon dioxide in air at 37°C in Dulbecco’s modified Eagle’s medium with high glucose (4.5 g/L), supplemented with 100 IU/mL penicillin and 100 µg/mL streptomycin, 4 mmol/L glutamine, 1% non-essential amino acids, 10 mM HEPES and 10% heat inactivated foetal calf serum (FCS) (GIBCO BRL, Gaithersburg, MD).

2.5. Exposure conditions
TiO$_2$ fine, TiO$_2$ NPs and ZnO NPs were suspended in culture medium (with or without FCS) and sonicated. For cytotoxicity assay, Caco-2 cells were plated on 24-multiwell plates at a density of 8 x 10$^4$ cells/well and treated after 4 days culture with 1, 2.5, 5, 10 and 20 µg/cm$^2$ particles for 6 and 24 h.

2.6. Viability assay
Viability of Caco-2 cells was measured as neutral red uptake (NRU). Treated cells were processed for NRU determination according to Borenfreund and Puerner [10].
2.7. 8-oxoguanine determination
To evaluate the oxidative DNA damage, 1 x 10⁶ Caco-2 cells were exposed to 2.5 μg/cm² particles for 2-4-6 and 24 h. 8-oxodG was measured by high-performance liquid chromatography with electrochemical detection as previously described [11]. After RNase treatment and enzymatic hydrolysis, DNA hydrolysate were filtered through 0.22 μm cellulose acetate (Millipore) Centricon filter units and dispensed in autosampling vials. Separation of 8-oxodG was performed in a System Gold HPLC instrument (Beckman Coulter Inc., Brea, CA, USA) equipped with a diode array UV detector. The LC-18-DB (Supelco, 75 x 4.6 mm) column, equipped with a LC-18-DB guard column cartridge, was eluted with an isocratic mixture of 50 mM potassium phosphahte, pH 5.5, and methanol (90%/10%) at 0.5 ml/min flow rate. Electrochemical analysis was carried out by a Coulochem II detector (ESA Inc., Chelmsford, MA, USA). Deoxyguanosine was measured in the same run of corresponding 8-oxodG.

2.8. Genotoxicity assay
About 1.5 x 10⁵ Caco-2 cells were seeded in six well plates and cultured for at least 48 h before treatment in complete culture medium supplemented with 20% FCS. Cells were treated with 1, 2, 5, 10, 20 μg/cm² concentrations of ZnO and TiO₂ NPs and 20 μg/cm² TiO₂ fine for 24 h in culture medium without FCS. Cells were washed twice with PBS and cultured in complete medium with 10% FCS and 4.5 μg/mL of Cytochalasin B for further 24 h then rinsed with PBS and harvested after 1.5 h of recovery time in complete medium with 20% FCS.

Slides were prepared according to Bazin et al. [12] and 1000 binucleated cells for each point of treatment were scored; mean values and standard deviations were calculated on the basis of three repeated experiments. Based on the frequency of mono-, bi- and multinucleated cells a Nuclear Division Index was calculated as a measure of cell proliferation.

3. Results and discussion

3.1. Particle characterization
Size distribution of ZnO NPs obtained by SEM equipped with Soft Imaging System showed an average diameter equal to 128 nm. Only 34% of particles possessed dimensions below 100 nm, the remaining particles were agglomerates ranging from 100 to 800 nm.

Size distribution showed an average diameter equal to 220 nm for TiO₂ NPs. Only 16% of particles possessed dimensions below 100 nm. Most of particles were agglomerates ranging from 100 nm to 1.4 μm. The size distribution of TiO₂ fine showed an average diameter equal to 335 nm and a percentage of particles (10%) with dimension below 100 nm.

In table 1, the particle characteristics obtained by electron microscopy analysis are displayed.

| Particles       | Primary size (nm)ᵃ | Average diameter (nm)ᵇ | Particle number (part/g)ᵇ | Surface area (m²/g)ᵇ |
|-----------------|--------------------|------------------------|---------------------------|----------------------|
| ZnO             | 45-170             | 128                    | 2.5 x 10¹³                  | 3.0                  |
| TiO₂            | 20-60              | 220                    | 1.3 x 10¹³                  | 3.3                  |
| TiO₂ fine       | 80-2000            | 335                    | 1.9 x 10¹³                  | 6.7                  |

ᵃ determined by transmission electron microscopy
ᵇ determined by scanning electron microscopy
3.2. Cytotoxicity induced by ZnO and TiO$_2$ NP treatment on Caco-2 cells

Cell viability was measured by Neutral Red assay. No effect on Caco-2 viability was reported after treatment with TiO$_2$ NPs at all the concentrations tested either in presence or in absence of FCS (figure 1, top panel).

On the contrary, a dose-dependent decrease of cell viability was observed in presence of ZnO NPs already after 6 h of treatment. The presence of FCS strongly reduced zinc toxic effects, probably through its interaction with the NP surface (figure 1, bottom panel).

TiO$_2$ fine was always used as negative control and it never induces toxic effects until 40 µg/cm$^2$ (data not shown).

Figure 1. Effects of different concentrations of TiO$_2$ and ZnO NPs on cellular viability of Caco-2 cells determined by NRU assay, after 6 and 24 h of treatment with or without FCS. Data are expressed as mean ± SD of three independent experiments, performed in triplicate.

To evaluate the role of surface in the cytotoxicity induced on Caco-2 cells, Neutral Red assay was performed at several surface doses of the three types of particles examined. Our preliminary results suggest that the chemical composition played primary role in the different cytotoxic effect of ZnO and TiO$_2$ NPs (data not shown).

3.3. Analysis of oxidative DNA damage induced by particle exposure

8-oxodG is a highly pro-mutagenic lesion and is considered the main marker of oxidative damage to DNA. The induction of oxidative DNA damage was evaluated after ZnO and TiO$_2$ NP exposure (2.5 µg/cm$^2$ for 2-4-6 and 24 h) by measuring the levels of DNA 8-oxodG.

A significant increase in the 8-oxodG levels was observed after 6 h exposure for both NPs. Moreover, an increase in oxidation level was obtained with fine TiO$_2$ particles too, suggesting a probable damage induction at molecular level regardless of TiO$_2$ particle size. Interestingly, we noticed a decrease in the oxidation after 24 h exposure probably due to the action of the repair mechanisms involved in this type of damage (figure 2).
3.4. Analysis of the genotoxic effect induced by particle exposure by cytokinesis-block micronucleus (MN) assay

The percentage of Micronucleated Binucleated cells (BinMned) was determined in Caco-2 cells treated with different concentrations of ZnO and TiO$_2$ NPs and TiO$_2$ fine.

The % BinMned background was 9.03%. A slight increase of % BinMned was observed after treatment with 2 and 5 µg/cm$^2$ of ZnO (12.1 and 12.2% BinMned, respectively) (figure 3a). Conversely no effect was detected after treatment with TiO$_2$ both NP and fine (figure 3b).

The % of BinMned was not determined at the highest NP concentrations (10, 20 µg/cm$^2$) because of both high cytotoxicity exerts by ZnO and impossibility to perform a reliable score of cells treated with TiO$_2$, due to the great accumulation of NPs on cell surface.

A dose dependent decrease of Nuclear Division Index (NDI) was observed in cells treated with ZnO (figure 3c) whereas no reductions of NDI values were detected after treatment with TiO$_2$ NPs (figure 3d), according to Neutral Red assay results.

**Figure 2.** Levels of DNA 8-oxodG measured by HPLC/EC. Caco-2 cells were exposed to 2.5 µg/cm$^2$ TiO$_2$ fine, TiO$_2$ NP and ZnO NP for 2-4-6 and 24 h. Deoxyguanosine was measured in the same run of corresponding 8-oxodG and the data are mean ± SD from three experiments.
Figure 3. a and b panels show the percentage of Micronucleated Binucleated cells (BinMned) induced by 1.0, 2.0 and 5.0 \( \mu \text{g/cm}^2 \) of Zinc oxide (ZnO), Titanium dioxide (TiO\(_2\)) and 20 \( \mu \text{g/cm}^2 \) of Titanium dioxide Fine (TiF) respectively, after 24 h of treatment. c and d panels show the Nuclear Division Index (NDI) observed after 24 h treatment with 1.0, 2.0 and 5.0 \( \mu \text{g/cm}^2 \) of ZnO and TiO\(_2\) respectively, and 20\( \mu \text{g/cm}^2 \) of TiF. Data represent the mean of three experiments ± SD except for 5.0 \( \mu \text{g/cm}^2 \) of ZnO.

4. Conclusion
In this study we show that ZnO nanoparticles induced a significant cytotoxic effect on Caco-2 cells and this effect was strongly reduced in presence of FCS in culture medium. On the contrary, cell viability was not affected by the exposure to TiO\(_2\) particles and it was independent on the particle size. Preliminary data, obtained by cell exposure to the same particle surface dose, agree with neutral red results indicating a primary role of the NP chemical composition in the different cytotoxicity induced on Caco-2 cells. Further studies will be performed in order to analyse the involvement of ion release from ZnO NPs on the reported cytotoxic effect.

Oxidative damage to genomic DNA was observed after 6 h exposure to ZnO and TiO\(_2\) particles, suggesting a damage induction at molecular level. This damage was not dependent on particle size and chemical composition. The role of ZnO and TiO\(_2\) NPs surface reactivity on the oxidative damage to DNA will be further investigated. Besides, a decrease in the 8-oxodG levels was observed after 24 h exposure and this is probably related to the cellular mechanisms of repair.

In order to better understand our results, the production of reactive oxygen species (ROS) will be measured in the Caco-2 cells exposed to the three different particles. Moreover, it could be interesting to study both oxidative damage to mitochondrial DNA and the expression levels of the main protein involved in the repair mechanisms.

Chromosome damage analysis, evaluated by the cytokinesis blocked micronucleus assay 24 h after NP treatment, highlighted a slight increase of micronuclei induced only by ZnO. In order to elucidate the kinetic of chromosome damage in this cell line and the possible association to oxidative damage that peaked at 6 h, a different protocol for the treatment will be considered in future experiments.
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