Genotoxicity and cytotoxicity of zinc oxide and titanium dioxide in HEp-2 cells

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Abstract

Aim: The rapidly growing industrial and medical use of nanomaterials, especially zinc oxide and titanium dioxide, has led to growing concern about their toxicity. Accordingly, the intrinsic genotoxic and cytotoxic potential of these nanoparticles were evaluated. **Materials & Methods:** Using a HEp-2 cell line, cytotoxicity was tested with the mitochondrial activity and the neutral red uptake assays. The genotoxic potential was determined employing the Comet and the cytokinesis-blocked micronucleus assays. Additionally, tyrosine phosphorylation events were investigated. **Results & Conclusion:** We found concentration- and time-dependent cytotoxicity and an increase in DNA and cytogenetic damage with increasing nanoparticle concentrations. Mainly for zinc oxide, genotoxicity was clearly associated with an increase in tyrosine phosphorylation. Our results suggest that both types of nanoparticles can be genotoxic over a range of concentrations without being cytotoxic.

Keywords: Nanoparticles, zinc oxide, titanium dioxide, cytotoxicity, genotoxicity, HEp-2 cells

Introduction

Nanoparticles have become very attractive for commercial and medical products in the last decade because of their various properties. Nanoparticles have been widely used for manufacturing many consumer products such as electronics, computers, food colorants or clothes, arriving on the market at a rate of 3-4 per day [1]. In medical fields such as oncology, cardiovascular medicine, molecular diagnostics, drug discovery and drug delivery nanoparticles are also increasingly used [2-4]. One of the future intended usages of titanium dioxide (TiO_2) is for artificial orthopedic implants [5]. Most recently, titanium dioxide nanoparticles chemically linked to antibodies via bivalent dihydroxybenzene, so-called phototoxic "nanobio hybrids", were used to selectively kill glioblastoma multiform brain cancer cells [6]. By conventional definition one dimension of nanoparticles has to be < 100 nm, however, due to their biological uniqueness not being constrained by this definition the pharmaceutical industry uses sizes of 1-1000 nm to describe nanomaterials [7, 8]. Nanoparticle properties are rather different from bulk materials and these differences include their small size in the nanometer range, a large surface area to volume ratio [9] and unique physico-chemical properties. Consequently, nanoparticles can easily pass through cell membranes and may show unpredictable toxic effects. The major toxicological concern arising from this is that many nanoparticles which cross cell membranes become lodged in mitochondria [10] with some of the engineered nanomaterials also being redox active [11]. Hence, the increasing applications of nanoparticles in various fields may lead to an increase in human exposure which in turn may increase direct toxicological effects. Despite apparent advantages of certain nanoparticles and their applications, many undesirable side effects of nano-sized particles have been documented in previous studies over the last decade [12, 13].

Nanoparticles such as zinc oxide (ZnO) and TiO_2 are increasingly used in a variety of industrial and medical applications including high-tech materials, plastics, paints and production of paper, and also very frequently in cosmetics and sun screens [14]. Zinc oxide and TiO_2 nanoparticles with sizes between 50–500 nm are employed to increase the sun protection factor (SPF) of e.g. sunscreens due to their scattering properties, which enables them to act as nano-mirrors reflecting UV light and as UV filters against sun radiation. In addition to UV reflection properties, their small size is also used as an advantage to improve homogeneity of distribution in cosmetics. However, TiO_2, in spite of its reflection and scattering properties to UVB and UVA, also absorbs about 70% of the total irradiated UV light [15].

The cytotoxic effects of TiO_2 nanoparticles on human A549 lung epithelial cells were accompanied by the active up-take of TiO_2 nanoparticles by endocytosis [16]. In animals, *in vivo* studies have also shown that exposure and inhalation of TiO_2 particles can induce cytotoxicity in lung cells with subsequent inflammatory responses in lung tissue being directly related to the particle size [17-21]. Garabrant and colleagues noted that 17% of the workers exposed to TiO_2 were affected by pleural diseases, which were proportionate to the duration of work in TiO_2 manufacturing [22]. *In vitro* studies on human sperm and lymphocytes also showed that both nano-sized ZnO and TiO_2 (10-100 µg/ml) were genotoxic to both cell types within the Comet assay in the absence of toxicity [23], the same was true for bacteria and vertebrates [24, 25]. In addition, *in vitro* studies of ZnO in Chinese hamster ovary (CHO) cells and in human epidermal cell lines have revealed clastogenic properties [26, 27]. Studies on lymphocytes treated with TiO_2 or ZnO nanoparticles showed significantly increased DNA breakage and micronucleus formation [23, 28]. Effects of TiO_2 nanoparticles on non-human mammalian cells were also reported, e.g.

exposure of rat liver cells to TiO_2 nanoparticles resulted in cytotoxicity [29]. Titanium dioxide also induced apoptosis and micronuclei in Syrian hamster embryo fibroblasts [30]. Various nanoparticles have shown some cytotoxicity and/or genotoxicity [14, 31-34], and different mechanisms of damage induction have been suggested including radical oxygen species (ROS) and lipid peroxidation, as well as alteration of signal transduction [35], but the exact mechanism(s) has (have) yet to be found.

Signal transduction pathways are important mechanisms through which the cell coordinates particular functions. Trans-membrane receptors receive and then deliver signals from the extracellular lumen via different mechanisms into the nucleus [36]. Tyrosine phosphorylation is the major signaling pathway through which receptor tyrosine kinases catalyze the transfer of a phosphate from ATP to the hydroxyl group on tyrosine residues of protein substrates in turn mediating cell growth, differentiation, host defense, and metabolic regulations [37]. The importance of tyrosine phosphorylation events in the presence of toxic nanoparticles was previously reported in a study characterizing the anti-bacterial effects of novel silver nanoparticles, which were able to penetrate the bacteria and subsequently modulate the phosphotyrosine profile [35].

In this study, we investigated the cytotoxicity and genotoxicity of ZnO and TiO_2 nanoparticles using the human epithelial HEp-2 cell line. We also tried to determine the tyrosine phosphorylation events caused by ZnO and TiO_2 occurring simultaneously independently of cytotoxicity and at the same time as genotoxicity.

Material and Methods

Cell culture of the HEp-2 cell line (HeLa derivative)

HEp-2 cells deriving from human negroid cervix carcinoma were obtained from the European collection of cell cultures (ECACC, catalogue No 86030501). Such cells have been the mainstay of cancer research for determining the cytotoxic effects of different chemicals [38-40]. HEp-2 cells were maintained in continuous culture in pre-warmed Eagle's minimal essential medium in Earle's balanced salt solution (EMEM-EBSS) supplemented with 1% non-essential amino acids (NEAA), 2 mM glutamine, 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin (P/S) from Gibco, Paisley, UK. The medium was changed every 3-4 days and the cells were maintained in a humidified atmosphere of 5% CO_2 at 37 ºC. Cells were passaged upon confluence at a ratio of 1:3. Stocks of cells were routinely frozen and stored in liquid nitrogen.

Chemicals

Zinc oxide nano-powder (ZnO; CAS 1314-13-2), and anatase titanium (IV) oxide nano-powder (TiO_2, 99.7%, CAS 1317-70-0), low melting-point agarose (LMA), normal melting-point agarose (NMA), thiazolyl blue tetrazolium bromide [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, CAS 298-93-1] (MTT) dye; neutral red (NRU) dye (CAS 553-24-2); ethidium bromide (EtBr) and Triton X-100 were purchased from Sigma Aldrich, Poole, Dorset, UK. Phosphate buffered saline (Ca^{2+}, Mg^{2+}-free; PBS), Trypan blue dye solution and 10,000 U/ml of trypsin–EDTA were purchased from Gibco. Acrylamide / bis-acrylamide solution (30%), Bio-Rad protein assay dye reagent concentrate (CAS 500-0006), buffers, membranes and

films were purchased from Bio-Rad, Hertfordshire, UK. Phosphotyrosine mouse monoclonal antibody (P-Tyr-100) and anti-mouse IgG-HRP linked antibody were purchased from Cell Signalling Technology, Hitchin, Hertfordshire, UK.

Particle preparation and characterization

Nanoparticles were suspended in 10 ml EMEM-EBBS medium at concentrations of 10, 20, 50, and 100 μg/ml. Suspensions were probe-sonicated at 30 W for 5 minutes on and off, and then allowed to equilibrate for different times: 0, 2, 4, 24, and 48 hours. The first two times were chosen to look at more immediate effects, the latter two time points to evaluated long-term effects. The resulting ZnO and TiO_2 suspensions were measured as a function of incubation time. Prior to evaluation of the toxic potential, the mean size (hydrodynamic diameter) of the nanoparticles was determined using a high performance particle sizer (Malvern Instruments Ltd., Worcestershire, U.K.). Before analysis suspensions were briefly shaken to resemble the cell culture incubation conditions. Suspensions were placed in disposable cuvettes and three consecutive measurements at 25 ºC each consisting of three runs were undertaken.

Mitochondrial activity (MTT) assay

The MTT assay to evaluate the mitochondrial activity was done according to the originally described method [41]. HEp-2 cells were plated in 96-well plates and the cell concentration (~$1.5x10^4$) was chosen as previously successfully used [42, 43]. The cells were incubated for 24 hours at 37° C and 5 % CO_2, then incubated for different time periods (2, 4, 24 and 48 hours) with ZnO or TiO_2 nanoparticles. The MTT dye was added 4 h prior to completion of incubation periods. The medium from

each well was discarded and resulting formazan crystals were solubilized by adding 200 µl of dimethylsulphoxide (DMSO) and quantified by measuring absorbance at 570 nm.

Neutral red uptake (NRU) assay

The NRU assay was done to determine the accumulation of the neutral red dye in the lysosomes of viable, uninjured cells [44].The medium was discarded and 100 µl of neutral red dye (50 µg/ml) dissolved in serum free medium was added to each well. After incubation at 37 °C for 3 h, cells were washed with PBS and the dye taken up by cells was then dissolved in 200 µl of a fixative solution (50% ethanol, 49% ddH2O and 1% acetic acid) and added to each well. Absorbance was then taken at 570 nm in MRX 11 micro-plate reader (Dynex Technologies, USA), using the software Revelation version 4.02.

Cell viability

Prior to the Comet assay, cells were incubated with ZnO or TiO_2 nanoparticles for 4 h at different concentrations (10, 20, 50, 100 µg/ml) and assayed for viability using the Trypan blue dye exclusion test. The cut-off point was 75% as suggested by Henderson and colleagues [45].

Comet assay

The sub-confluent monolayer was exposed to four different concentrations of ZnO and TiO_2, and 2.72 µg/ml (= 8 0 µM) of hydrogen peroxide (H_2O_2) as a positive control. There was also a negative control using untreated cells. After an incubation of 4 hours, cells were washed with cold PBS and harvested with trypsin-EDTA and

followed by a centrifugation at ~180 g for 9 minutes. The pellet was finally resuspended in PBS. The Comet slides were prepared by the method as previously described [46]. The cell suspension (100 µl) was mixed with 100 µl of 1% LMA, of which 100 µl was spread onto microscope slides pre-coated with 1% NMA. The slides were kept overnight at 4 °C in freshly prepared, chilled lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, with 1% Triton X-100; pH 10). The slides were subjected to freshly prepared cold alkaline electrophoresis buffer (1 mM EDTA sodium salt and 300 mM NaOH; pH≥13) for D NA unwinding, then electrophorized for 30 minutes each at 4 °C in electrophoresis buffer. The electrophoresis buffer was neutralized with Tris buffer (400 mM, pH 7.4) and stained with 20 µg/ml ethidium bromide. Fifty cells from each concentration were scored 'blindly' at a final magnification of 400x using an image analysis system (Komet 4.0 attached to a fluorescence microscope equipped with a CCD camera). The Olive tail moment and % tail DNA were used as parameters to measure the DNA damage in each cell. Three repeat experiments were carried out over the dose ranges used.

In vitro *cytokinesis-blocked micronucleus (CBMN) assay*

The CBMN assay was performed as described previously [47]. HEp-2 cells were grown in 6-well cell culture plates suspended in 2 ml EMEM medium at a concentration of $1x10^5$. After 24 hours of culture, either the TiO_2 nanoparticles (10, 20 and 50 µg/ml) or ZnO nanoparticles (10, 20, 50 and 100 µg/ml) or mitomycin C (positive control; 0.4 µM = 0.134 µg/ml) were added to the cultures. An untreated culture served as the negative control. Following 2 hours of incubation, the culture medium was removed and the cells were washed with PBS. After the treatment, 7.5 µl cytochalasin B (final concentration 0.01 mg/ml) were added to fresh culture medium

and incubated overnight. HEp-2 cells were trypsinized and centrifuged at ~180 g for 8 minutes. The cell pellet was treated with a cold hypotonic solution (70 mM KC1) for 15 minutes and centrifuged at ~180 g rpm for 8 minutes; then fixed in methanol / acetic acid (3:1), and air-dried. Slides were stained with 5% Giemsa for 10 min for the detection of micronuclei in binucleated cells. Micronuclei were scored and frequencies were evaluated per 1000 binucleated cells.

Detection of tyrosine phosphorylation in HEp-2 cells

A sub-confluent HEp-2 monolayer was treated with different concentrations (0, 10, 20, 50 and 100 µg/ml) of ZnO or TiO_2 nanoparticles and incubated for 4 hours. Untreated cells served as a negative control. After the treatment, the cells were trypsinized and suspended in 10 ml PBS and centrifuged at ~180 *g* for 9 minutes. The HEp-2 cell pellet was resuspended in 200 µl PBS and assessed (10 µl) for viability and membrane integrity using the Trypan blue dye exclusion test. Ninety microliters of cell suspension were added to 10 ml PBS for Western Blotting, while the rest (100 µl) was used for the Comet assay. After centrifugation (~180 *g* for 9 minutes), lysing buffer with protease and phosphatase inhibitors was added to the pellet and the resulting cell suspension was briefly sonicated (~5 seconds) at 30 W. Protein concentrations were determined in each sample using the Bio-Rad assay [48]. The cell lysate was suspended in Laemli sample buffer and 35 µg protein from each sample was loaded on SDS gel and detected by the immunoblotting technique using a phosphotyrosine mouse monoclonal primary antibody (P-Tyr-100) and an anti-mouse IgG-HRP linked secondary antibody.

Statistical analysis

The means from MTT and NRU data were compared to the means of the negative control data using the one-way ANOVA test. A Mann-Whitney U-test was used for the Comet assay. For the CBMN assay, the Chi square test and Fishers exact test were used to compare the number of MN in the treated samples to the negative control of untreated HEp-2 samples. The threshold of significance was $p < 0.05$. Values at or below this were considered significant.

Results

Particle characterization

Dynamic light scattering (DLS) measurements of ZnO and TiO_2 nanoparticle suspensions were obtained with a high performance particle sizer. Using DLS, particle sizes were observed to be widely distributed. The increase in size (aggregation) occurred with increasing dose but remained constant over a 48-hour period except at higher doses of TiO_2 (Table 1). Although the particle size considerably increased as a function of concentration was minimally increased as a function of time.

(Insert Table 1)

Cytotoxicity

The MTT assay and the NRU assay

The results demonstrated a concentration- and time-dependent cytotoxicity as measured by optical density (OD) at 570 nm after exposure to ZnO or TiO_2 nanoparticles which was evident for ZnO but not for TiO_2 due to precipitation after 24 h (Figure 1). The percentage (%) cell survival in the MTT assay compared to the untreated control was decreased dramatically in 24 and 48 hours in ZnO treated cells reaching ~16% at the highest ZnO concentration (100 µg/ml). However, the highest concentrations of TiO_2 (50 and 100 µg/ml) were precipitated within 24 hours as the results for the OD showed higher readings compared to the negative control (> 140%).

The NRU assay showed similar results as the MTT assay and demonstrated a concentration and time dependent cytotoxicity after exposure to ZnO or TiO_2 nanoparticles for more than 4 hours (Figure 1).

(Insert Figure 1)

Genotoxicity

Cell viability and membrane integrity

The cell viability in the Comet assay and immunoblotting assay using the Trypan blue dye exclusion assay was between 70-85% for all ZnO and TiO_2 samples, with the exception of the highest dose of TiO_2 (100 µg/ml), which showed 65% viability. All doses examined, therefore had high membrane integrity.

DNA damage

The parameters of Olive tail moment (OTM) and % tail DNA indicated significant ($p < 0.05$) dose-related DNA damage in HEp-2 cells. Exposure for 4 h to different concentrations of ZnO or TiO_2 nanoparticles showed a 3-fold increase for ZnO and a 2-fold increase for TiO_2 in DNA damage compared to the negative control of untreated HEp-2 cells. However, for the lowest ZnO dose the induced damage, measured in % tail DNA, did not reach significance levels, but did for the OTM (see Table 2 and Figure 2).

(Insert Figure 2 and Table 2)

In vitro *CBMN assay*

We observed the formation of MN after treatment with different ZnO (0, 10, 20, 50, and 100 μg/ml) and TiO_2 concentrations (0, 10, 20, and 50 μg/ml) for 2 hr (Table 3). The Chi-squared analysis of the MN frequencies from HEp-2 cells exposed to different ZnO and TiO_2 concentrations revealed a significant increase in induced MN treated with high concentrations of ZnO (50 and 100 μg/ml) or TiO_2 (50 μg/ml) compared to the untreated cell control ($p < 0.05$). However, lower concentrations (<50 μg/ml) of either ZnO or TiO_2 did not induce significant alterations in MN induction. The MN frequency increased with ZnO concentration in a specific dose-dependent manner ($p < 0.05$, Chi-squared test) from an average frequency of 11 MN per 1000 binucleated cells in the control to 28 MN per 1,000 binucleated cells at 100 μg/ml. For TiO_2, the MN frequencies increased in a similar way reaching 31 MN per 1000 binucleated cells at a dose of 50 μg/ml.

(Insert Table 3)

Tyrosine phosphorylation

HEp-2 cells were challenged with different concentrations of ZnO (0, 10, 20, 50, and 100 μg/ml) and TiO_2 (0, 10, 20, and 50 μg/ml) for 4 h, and phosphotyrosine expression was analyzed by Western blotting analysis. The level of tyrosine phosphorylation was up-regulated by ZnO nanoparticles, which induced an increase in phosphorylation of tyrosine residues on 20, 21-22, 25, 30, and ~80 kDa proteins (Figures 3). Less effect was seen with TiO_2 inducing a fainter effect at 20, 21-22, 25, and 30 kDa at the highest dose; however, no effect was observed at ~80 kDa.

(Insert Figure 3)

Discussion

Many toxicological studies over the past years have raised concerns regarding the safety of nanoparticles as they readily generate oxidative stress through reactive oxygen species (ROS). A strong link between nanoparticles and oxidative stress via ROS has been documented [49, 50]. Many investigators have shown that ROS play a major role in nanoparticle-induced DNA damage [51]. However, detailed molecular mechanisms involved in the cellular responses to genotoxic effects are not yet fully understood. Oxygen radicals can cause single and double strand breaks. Damaging the DNA sugar-phosphate backbone or the bases may then lead to acute DNA damage, which triggers cell cycle arrest and leads to prolonged repair time or cell death [52]. Accumulation of mutations which are caused by excessive or incomplete DNA repair is a known cause of oncogenesis [53]. Morz and colleagues found that nanoparticles produced an effect similar to irradiation by inducing carcinogenesis pathways through ROS-induced DNA damage, as well as activating p53 and proteins related to DNA repair [54]. The DNA damage may be either primary as a direct effect of the nanoparticles or a secondary effect due to generated ROS [55]. Accordingly, ROS may be one of the possible modes suggested for ZnO and TiO_2 induced DNA damage. Oxidative stress is now considered a major cellular signaling regulator and it was suggested that genotoxicity mediated by ROS could be further linked to other pathways [55]; therefore, we investigated the tyrosine phosphorylation pathway additionally to the induction of DNA damage.

In view of the importance of tyrosine kinase activity in the control of cell function and the importance of redox mechanisms in regulating the phosphorylation by tyrosine

kinases, it was of interest to determine whether the functional changes induced by altering the redox status of HEp-2 cells were associated with changes in the pattern of tyrosine phosphorylation. The immunoblotting analyses of HEp-2 cells cultured and treated with ZnO for 4 hours, then using a monoclonal anti-phosphotyrosine antibody, revealed heavily phosphorylated tyrosine residues on a protein with a molecular mass of 20 kDa (Figure 3). Also bands at 25 and 30 kDa showed phosphorylation of tyrosine residues. A diffuse band of immuno-reactive material could also frequently be observed migrating in advance of this protein with a molecular mass of 21-22 kDa. This could be highly significant as a correlation was previously found between cell transformation and the tyrosine phosphorylation of 22-kDa protein [56]. In addition, previous studies showed, that dysregulation of protein tyrosine phosphorylation is crucial for cell signaling maintenance with subsequent cell proliferation and the early stages of neoplasia [57, 58]. Stimulation of HEp-2 cells with ZnO was also associated with a dramatic enhancement in the intensity of phosphotyrosyl proteins. The increase in a 20 kDa protein tyrosine phosphorylation induced by ZnO was observed over the whole range of concentrations (10-100 µg/ml). Zinc oxide nanoparticles at a dose of 100 µg/ml additionally had a stimulatory effect on tyrosine phosphorylation of an ~80 kDa protein (Figure 3). The same dose gave a significant 3-fold increase in the Olive tail moment and % tail DNA in the Comet assay when compared to the untreated control (Table 2). On the other hand, TiO_2 produced fainter bands at 20 kDa and only for the 50 µg/ml treatment dose phosphorylation at 21-22, 25, and 30 kDa (Figure 3). No effect was seen at ~80 kDa for TiO_2. As a result, tyrosine phosphorylation may have contributed to the genotoxicity of ZnO but less to TiO_2 genotoxicity.

We examined the consequences of genotoxic injury to HEp-2 cells by ZnO and TiO2 to elaborate the signaling events that modulate cell survival after genotoxic exposure. Previous studies on HeLa cells showed activation of Cdk1 as a result of etoposide treatment lead to cell cycle checkpoint override with subsequent cell death [59]. In *in-vitro* studies on human U937 monocytes nano-cobalt increased the transcription of matrix metalloproteinases (MMP-2 and MMP-9) and protein tyrosine kinase (PTK) signaling pathways were involved through oxidative stress [60]. Previous studies also found that sodium vanadate induced protein tyrosine phosphatase (PTP) inhibition. This resulted in a bypass of growth arrest that occurred as the result of genotoxic exposure which indicates a role for tyrosine phosphorylation in the regulation of cell survival [61]. PTP inhibition was also previously reported in primary mammary epithelial cells which enhanced cell survival by decreasing apoptosis [62].

In our study, cytotoxic and genotoxic effects of ZnO and TiO_2 were tested in HEp-2 cells. Cytotoxicity was measured using the MTT and NRU assays; genotoxicity was assessed by the Comet and CBMN assays to measure the DNA damaging and genotoxic potential. Our data showed a cytotoxic effect of ZnO in mitochondrial activity (MTT assay) and the ability of lysosomes to retain neutral red stain (NRU assay) in HEp-2 cells after treatment for 24 and 48 hours with different ZnO concentrations with the exception of the lowest dose (10 µg/ml) which showed no cytotoxicity even after 48 hours treatment (Figure 1, Table 1 and 2). The treatment times used for cell cultures extended well beyond the 4 h treatment times used for the Comet assay. On the other hand, 100 µg/ml of TiO_2 proved to be significantly toxic after 2 hours on HEp-2 cells (Figure 1). For both doses, 50 and 100 µg/ml, TiO_2 precipitated in the wells after 24 hours. Hence, treated cells showed an OD which

was > 140% when compared to the untreated control in the MTT and NRU assays. The TiO_2 concentration of 20 µg/ml was cytotoxic to the cells in both assays to different extents in 24 h treatment assays.

Aggregation of nanoparticles observed in our study has been reported previously for TiO_2 [63] as well as for ZnO [64] and is considered as an inherent property of uncoated oxide nanoparticles which occur in aqueous conditions from distilled water to complex biological media. Although, previous studies have shown that the particle solubility of a range of metal oxide nanoparticles like ZnO with release of free hydrated zinc ions (Zn^{2+}) strongly influenced cytotoxicity compared to extremely less soluble metal oxides like TiO_2 [65-67]. In our study, as the particle size increased with the concentration, the aggregation did not change over a time period of up to 48 hours for ZnO and 24 hours for TiO_2. Despite increasing in size our particles stayed well below the pharmaceutical size definition for nanoparticles [7, 8]. For the phosphorylation studies, exposure was only 4 hours for both nanoparticle compounds. Therefore, we assumed that the rate and quantities of dissolution was either negligible or of little significance for our experiments.

Our results demonstrated the DNA damaging potential of different concentrations of ZnO or TiO_2 with significant increases in the tail moment and % tail DNA in the Comet assays (3-fold for ZnO) at the highest dose and (2-fold for TiO_2) at the highest dose compared to untreated control (Figure 2). The DNA damaging and mutagenic potential of ZnO or TiO_2 at various concentrations was also seen for the CBMN assays. Our result regarding the genotoxic potential of ZnO or TiO_2 is supported by a previous studies conducted by Gopalan and colleagues [23], which showed a

concentration-related increase in Olive tail moment in lymphocytes and sperm. Also, an increase in chromosome aberrations in CHO cells treated with ZnO has been previously reported [26]. PTP inhibition [61] or increased transcription of matrix metalloproteinases (MMP-2 and MMP-9) and PTK signaling pathways [60] could explain our findings of increased tyrosine phosphorylation as the result of genotoxic insult of ZnO and the highest concentration of TiO_2.

Conclusions

The present investigation conclusively provides further evidence for the genotoxic effect of ZnO and TiO_2 nanoparticles in both the Comet assay and the micronucleus assay with induction of chromosomal damage at high, but non-cytotoxic (according to Trypan blue exclusion) concentrations of ZnO (50 and 100 µg/ml) and TiO_2 (50 µg/ml). It also provides evidence of significantly increased chromosomal mutations at the highest concentrations which showed no cytotoxic effects in the NRU and MTT assays. Furthermore, ZnO increased tyrosine phosphorylation of five proteins at 20, 21-22, 25, 30, and ~80 kDa with less effect of TiO_2 which showed increased phosphorylation compared to the negative control at 20, 21-22, 25, and 30 kDa for the highest dose but not at ~80 kDa. Thus, tyrosine phosphorylation is modified by both ZnO and TiO_2 while causing genotoxic damage seen for two different end points in the Comet and CBMN assays in the absence of cytotoxicity.

Future perspectives

Nanotechnology holds great promises for medicine, manufacturing and for the environment including efficient water purification and removal of toxic waste. Currently, nanoparticles like ZnO and TiO_2 are already widely used in many consumer products. The variety of applications ranges from using nanoparticles within sunscreens, pigments, tooth pastes, antiseptic coatings, paints and the coating of spectacles to make them unbreakable and scratchproof. As a result, the evaluation of the toxic potential of nanoparticles within their substrate is imperative. However, in many cases detailed toxicological evaluation of nanoparticles including their genotoxic assessment is lagging behind the technological application of medical and consumer nano-products. Thus, in the very near future the focus will shift from nanotechnology towards nanotoxicology. Recent studies and our study have shown that under certain conditions nanoparticles can increase genotoxic damage within cells and potentially lead to adverse effects.

Executive summary

- Cytotoxicity of ZnO

The mitochondria and lysosome activity tests (MTT assay and NRU) indicated cytotoxicity of ZnO to HEp-2 cells treated beyond 4 h reaching cell survival of < 16% after 48 hours.

- Cytotoxicity of TiO_2

The highest TiO_2 concentration (100 µg/ml) was cytotoxic to HEp-2 cells in less than 2 hours.

- Genotoxicity of ZnO

Genotoxicity of ZnO in HEp-2 cells was seen within the Comet assay showing a 3-fold increase in DNA damage. For the CBMN assay, an increase in the formation of micronuclei was found after treatment with different concentrations ZnO.

- Genotoxicity of TiO_2

The genotoxic potential of TiO_2 in HEp-2 cells was confirmed in both cytogenetic assays, for the Comet assay showing a 2-fold increase in DNA damage after treatment and for the CBMN assay revealing a dose-dependent increase in MN formation.

- Tyrosine phosphorylation of HEp-2 cells treated with ZnO

The level of tyrosine phosphorylation was up-regulated after exposure to ZnO nanoparticles, significantly increasing phosphorylation events of tyrosine residues on 20 kDa, 21-22 kDa, 25 kDa, 30 kDa, and 80 kDa proteins.

- Tyrosine phosphorylation of HEp-2 cells treated with TiO_2

TiO_2 showed less effect on tyrosine phosphorylation at the higher doses. There was some effect on 21-22, 25, and 30 kDa proteins but not at ~80 kDa.

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Figure legends

Figure 1:

Cytotoxicity of ZnO (upper two graphs) and TiO_2 (lower two graphs) nanoparticles on human HEp-2 cells treated for different times for ZnO (2, 4, 24, and 48 hours) and for TiO_2 (2, 4, and 24 hours), was measured using the MTT and the NRU assays. The % cell survival compared to the negative control of untreated HEp-2 cells was evaluated for different concentrations of ZnO and TiO_2 nanoparticles. The values represent the mean of three separate experiments.

Figure 2:

The effect of TiO_2 or ZnO nanoparticle exposure is shown on DNA damage in HEp-2 cells as measured by the Comet assay. Cells were exposed to TiO_2 or ZnO at concentrations of 0, 10, 20, 50, and 100 μg/ml for 4 hours. The positive control was treated with 2.72 μg/ml H_2O_2 also for 4 hours (significance values are shown in Table 2). The values represent the mean of three separate experiments.

Figure 3:

Effects of ZnO and TiO_2 on HEp-2 receptor signaling are shown. An increase in phosphorylation of tyrosine residues can be seen in a Western blot of equal amounts of total proteins (Bio-Rad method) from HEp-2 cells treated for 4 h with different concentrations 10, 20, and 50 μg/ml as well as 100 μg/ml (only for ZnO). Untreated HEp-2 cells served as a negative control (Con). Panel A illustrates for ZnO that all bands at all doses are phosphorylated at 20 and 21-22 kDa, but also at 25 and 30 kDa and to some extent at ~80 kDa. For TiO_2, there is less of an effect at the three doses

examined at 20 and 21-22 kDa. Also at 25 kDa was some small increase above control values at 20 and 50 µg/ml and at 30 kDa at 50 µg/ml. There was no increase in phosphorylation found for TiO_2 at ~80 kDa. The values represent the mean of three separate experiments. Panel B shows as an example the effect of GAPDH as an internal control seen alongside ZnO at 50 and 100 µg/ml to confirm equal protein loading.