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***In vitro* cytotoxicity and genotoxicity studies of titanium dioxide (TiO₂)
nanoparticles in Chinese hamster lung fibroblast cells**

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Highlights: - Chinese hamster lung fibroblast cells exposed to nano-TiO₂ demonstrated cyto- and genotoxic responses. – There is a correlation between cyto- and geno-toxicological endpoints using different TiO₂ samples. – The treated cells demonstrated the intracellular ROS generation, a common toxicity mechanism. – The polyacrylate coated nano-TiO₂ decreased the cyto- and genotoxicity responses.

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Abstract

There are increasing safety concerns about the development and abundant use of nanoparticles. The unique physical and chemical characteristics of titanium dioxide (TiO₂) nanoparticles result in different chemical and biological activities compared to their larger micron-sized counterparts, and can subsequently play an important role in influencing toxicity. Therefore, our objective was to investigate the cytotoxicity and genotoxicity of commercially available TiO₂ nanoparticles with respect to their selected physicochemical properties, as well as the role of surface coating of these nanoparticles. While all types of tested TiO₂ samples decrease cell viability in a mass-based concentration- and size-dependent manner, the polyacrylate-coated nano-TiO₂ product was only cytotoxic at higher concentrations. A similar pattern of response was observed for induction of apoptosis/necrosis, and no DNA damage was detected in the polyacrylate-coated nano-TiO₂ model. Given the increasing production of TiO₂ nanoparticles, toxicological studies should take into account the physiochemical properties of these nanoparticles that may help researchers to develop new nanoparticles with minimum toxicity.

Keywords: *Nanotoxicology, surface modification, cell death, oxidative stress, DNA damage, comet assay, flow cytometry*

1. Introduction

In the past decade, there has been a drastic increase in the use of nanoparticles (NPs) in a diverse array of industrial and medical applications, ranging from biomedicine, nano-electronics to mechanical engineering (Mazzola, 2003; Paull et al., 2003). However, with the increase in large scale production of manufactured nanoparticles, and intentional (McCarthy and Weissleder, 2008; Pons et al., 2010) or unintentional (Lee et al., 2010) exposure to NPs, the potential toxicity of such materials due to their large surface area and the potential to accumulate in the body has raised concern (Lewinski et al., 2008; Meng et al., 2009; Oberdörster et al., 2005). Various studies *in vivo* and *in vitro* have shown the impact of unique properties of NPs on physical and chemical functional activities compared to the micron-sized counterparts of the same compound (Colvin, 2003; Maurer-Jones et al., 2010). Thus, a comprehensive study of the cyto- and genotoxicity effects of nanoparticles with respect to their different physicochemical characteristics may help improve the quality and performance of such materials with minimum toxicity. One of the key toxicity mechanisms of NPs is the generation of oxidative stress. This refers to a redox imbalance within cells usually as a result of increased intracellular reactive oxygen species (ROS) and decreased antioxidant concentrations. As NPs can be translocated from the lung into the blood, they can move to other organs and tissues (Donaldson et al., 2001; Nemmar et al., 2001), and may generate ROS at these sites. The ROS are highly reactive molecules that can disturb the homeostasis of the intracellular milieu and cause breakdown of membrane lipids, and cause DNA damage (Barillet et al., 2010; Oberdörster et al., 2005; Oberdörster et al., 2007; Papageorgiou et al., 2007). Similarly, recent *in vitro* studies demonstrated that NPs induce oxidative stress and inflammatory responses leading to

genotoxicity and cytotoxicity in cells (Ahmad et al., 2012; Asare et al., 2012; Kumar et al., 2011; Patil et al., 2012).

Among the manufactured NPs, nano-sized titanium dioxide (TiO_2) is one of the most widely produced nanoparticles. TiO_2 is a poorly soluble particulate with numerous applications such as food colorant or white pigments in a number of products including paints, plastics, paper, cosmetics, medicines, and pharmaceutical products, or use in sunscreens as an ultraviolet blocking agent (Baan et al., 2006; Hext et al., 2005; Lomer et al., 2002). Rutile and anatase are two crystalline forms of TiO_2 that have important industrial uses. The photocatalytic activity and cytotoxicity of the anatase nano- TiO_2 are higher than that of the rutile form (Kakinoki et al., 2004; Sayes et al., 2006). Similarly, several studies suggested that lung inflammation and consequently cancer in rats could be induced after inhalation and intratracheal instillation of TiO_2 nanoparticles, with stronger inflammogenic activity in comparison with its micron-sized counterpart (Donaldson et al., 2002; Falck et al., 2009; Renwick et al., 2004).

Commercial TiO_2 is usually coated with inorganic (such as silica) or organic substances to improve surface properties; however, the toxicity of coated nano- TiO_2 has not been well explored. Therefore, the aim of the present study was to examine the *in vitro* cytotoxicity and genotoxicity of TiO_2 particles having different sizes, crystal structure, shape, and one form of surface treated nanometric sized- TiO_2 (nano- TiO_2) using Chinese hamster lung fibroblast cells. The cytotoxicity and genotoxicity of nano- TiO_2 (anatase, rutile, and an anatase-rutile mixture), micrometer-sized- TiO_2 (anatase), and polyacrylate-coated nano- TiO_2 (rutile) particles were compared. Our results showed that while all different types of tested TiO_2 particles (coated and uncoated) can cause mass-based concentration-dependent cytotoxicity, only non-coated nano-

TiO₂ induces DNA damage. Furthermore, surface treatment can influence the toxicity of nanoparticles in cells.

2. Materials and methods

2.1 Chemicals

Dulbecco's modified eagle's medium (DMEM), L-glutamine, sodium pyruvate, trypan blue 0.4%, trypsin-EDTA, benzalkonium chloride (BNZ), dimethyl sulfoxide (DMSO), and 2',7'-dichlorofluorescein diacetate (H₂ DCFDA) were purchased from Sigma-Aldrich (Saint-Louis, MO). Earle's balanced salt solution, pen-strep antibiotics, fetal bovine serum (FBS) solution, and phosphate buffered saline (PBS) were obtained from Gibco Invitrogen (Grand Island, NY). The MTT and comet assay kits were supplied by Promega (Madison, WI) and Trevigen (Gaithersburg, MD), respectively. APC annexin V and propidium iodide were purchased from BD Pharmingen (Mississauga, ON) and Invitrogen, respectively.

Nano-TiO₂ MTI5 (anatase) was obtained from MTI Corporation (Richmond, CA), P25 (anatase/rutile) was supplied by Evonik Industries (Düsseldorf, Germany), and nanofilament rutile was supplied by Sigma-Aldrich (Saint-Louis, MO). Hombitan LW-S (H. Bulk anatase) was provided by Sachtleben Chemie (Duisberg, Germany), and Vive Nano Titania (-) (rutile) and Allosperse-A (polymer without the nano-TiO₂, control) were kindly provided by Vive Nano Inc. (Toronto, ON). Preliminary chemical analysis indicated that Vive Nano Titania contained about 78% (w/w) polymer and 22% nano-TiO₂ (D. Anderson, personal communication). According to the manufacturers, all TiO₂ samples tested were more than 99.5% pure and metal-based.

2.2 Cell culture

Chinese hamster lung fibroblast (V79) cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were seeded in DMEM without phenol red, and supplemented with 10% FBS, 1% sodium pyruvate, 2% L-glutamine, and 1% pen-strep antibiotics. Cultures were maintained and multiplied every 24 h at 37°C in a humidified atmosphere containing 5% CO₂.

2.3 Dispersion and characterization of the TiO₂ particles

All nanoparticles were suspended at 200 mg/L in a serum-containing culture media and were sonicated (Sonics & Materials Inc., Newtown, CT) for 60 sec at 30% amplitude and 20 KHz f. The dispersions were cooled during sonication with an ice/water bath in order to prevent heating of the dispersion. The suspension was immediately added to cultured cells seeded in multi-well plates. The size, polydispersity index (PDI), and zeta potential of the suspension were characterized by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instrument, Westborough, MA). The dispersions were also characterized by optical microscopy, and transmission electron microscopy (TEM) using a few drops of the material dispersed in double-distilled water on a carbon/formvar-coated grid and allowed to air dry. Nanoparticle crystallinity and phase structures were determined using an X-ray diffractometer (XRD) (Discover 8, Bruker AXS Inc., Madison, WI). The measurement of specific surface area of nanoparticles was carried out by Micromeritics Analytical Services (Norcross, GA) using the Brunauer Emmett Teller (BET) analysis (Brunauer et al., 1938).

2.4 Cell viability assay

The effects of nanoparticles on the viability of cells were assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on the reduction of the dye MTT by cellular dehydrogenases to crystalline formazan, an insoluble

intracellular blue product. Cells were seeded on 96-well plates (1×10^4 in 100 μl of medium/well) and left to settle. The freshly prepared nano-TiO₂ suspension was then diluted to different target concentrations (1, 10, and 100 mg/L), added to each well (100 μl), and left for 24 or 48 h. Concentrations were adopted from an earlier study (Sohaebuddin et al., 2010). Cultured medium without the TiO₂ particles served as a negative control in each experiment. Benzalkonium chloride was used as the reference toxicant (Borenfreund and Puerner, 1984) to ensure the consistent quality of the cells. At the end of incubation, 15 μl of dye solution was added to each well, and the plate was incubated at 37°C in a humidified CO₂ incubator for 4 h. Then, 100 μl of solubilization solution was added to each well, and mixed with the cells thoroughly until the formazan crystals were dissolved completely. The absorbance was measured at 570 nm using a microplate spectrophotometer (Bio-TEK Instruments, Inc., Winooski, Vermont). The cell viability was expressed as a percentage of the viability of the control cells.

2.5 Detection of dead cells by flow cytometry

Double staining for APC annexin V/propidium iodide (PI) was carried out to assess the apoptosis/necrosis induced by nano-TiO₂ according to the manufacturer's protocol with slight modifications. Briefly, 1×10^5 cells/well grown on 12-well plates were incubated without or with nano-TiO₂ at concentrations of 10 and 100 mg/L for 24 h. Incubation with 20 mM BNZ for 2 h was used as a reference toxicant and particles without cells were used to detect the interaction of particles with the assay reagents. At the end of incubation, cells were trypsinized and centrifuged, followed by washing twice with pre-chilled PBS. Cells were then resuspended in 50 μl of medium to which 2.5 μl CaCl₂ (100 mM) and 4 μl annexin V were added, then incubated for 15 min at room temperature in the dark. After incubation, cells were centrifuged and washed once with 3 ml medium containing CaCl₂ (2.5 mM), then resuspended in 500 μl medium

containing CaCl_2 (2.5 mM), and stained with 5 μl PI. The cell suspension was then analyzed by flow cytometry (Becton Dickinson LSRII, BD Biosciences), and the percentage of positive cells for annexin V and propidium iodide in each sample was determined. Viable cells are typically negative for both annexin V and PI, whereas cells undergoing apoptosis and necrosis display both annexin V and PI labeling. All samples in the present study were treated gently to reduce mechanical damage to the cells.

2.6 Detection of DNA damage by the comet assay

The alkaline comet assay (single-cell gel electrophoresis) was used to study DNA strand breaks in cells after exposure to the test particle according to the manufacturer's protocol. Briefly, cells were cultured in the absence or presence of nano- TiO_2 at a final concentration of 10, and 100 mg/L for 24 h. Hydrogen peroxide (H_2O_2) at 100 μM for 10 min was used as a positive control. Cells were trypsinized and centrifuged for 5 min, rinsed three times with pre-chilled PBS (Mg^{+2} - and Ca^{+2} -free) to remove unbound nanoparticles and medium, and were then resuspended in PBS. Cells at 1×10^5 cells/ml were combined with molten low melting point (LMP) agarose (at 37°C) at a ratio of 1:10 (v/v) and spread (50 μl) onto CometSlides™. The slides were sequentially immersed in cold lysis solution (2.5 M NaCl, 100 mM Na_2EDTA , 10 mM Tris) for 1 h, in an alkaline solution ($\text{pH} > 13$) for 30 min, and then placed in a horizontal gel electrophoresis tank filled with fresh electrophoresis solution (300 mM NaOH, 1 mM EDTA, $\text{pH} > 13$) in the dark. Electrophoresis was conducted at a low temperature for 20 min at 15 V. The slides were then rinsed with deionized water and immersed in 70% ethanol for 5 min, and then drained followed by addition of 70 μl of SYBR Green 1. Slides were observed with an inverted fluorescent microscope and photographed with a high resolution LUCA-S camera; at least 50 randomly selected images were analyzed from each sample using the KOMET 6.0 image

analysis software (Andor, Gamble Technologies Ltd, ON, Canada). The olive tail moment and the percentage of tail DNA were calculated according to the following formulae:

$$\text{Olive Tail Moment} = (\text{Tail.mean} - \text{Head.mean}) \times \% \text{Tail DNA} / 100$$

$$\% \text{ Tail DNA} = 100 - \% \text{ Head DNA}$$

$$\% \text{ Head DNA} = (\text{Head.OptInten} / (\text{Head.OptInten} + \text{Tail.OptInten})) \times 100$$

where tail parameters are computed from the tail intensity profile.

Tail.mean = profile centre of gravity, and Tail.OptInten = Tail.Optical Intensity.

Head parameters are computed from the head intensity profile.

Head.mean = profile centre of gravity, and Head.OptInten = Head.Optical Intensity.

2.7 Intracellular generation of ROS

Cellular generation of ROS was determined using the H₂ DCFDA oxidation method (Elbekai and El-Kadi, 2005; Wang and Joseph, 1999). Cells were cultured on 6-well plates (2×10⁵ cells/well) in the absence or presence of nano-TiO₂ at a final concentration of 100 mg/L for 24 h. Exposure to 100 μM hydrogen peroxide (H₂O₂) for 10 min was used as a positive control for ROS detection, and particles without cells were used to detect the interaction of particles with the assay reagents. Cells were then rinsed three times with PBS to remove unbound nanoparticles and incubated with 50 μM H₂ DCFDA for 30 min. At the end of the incubation, cells were washed twice with PBS. Fluorescence was visualized using a Leica DMIL inverted fluorescence microscope with an excitation of 485 nm and an emission of 530 nm (Leica Microsystems, Wetzlar, Germany), and images were photographed using a Retiga 2000R cooled monochrome CCD camera (Qimaging, Surrey, BC, Canada).

2.8 Statistical analysis

Data were presented as the mean $\pm$ SEM of at least three separate and independent experiments run in triplicate. Sigma Stat 3.1 (Systat Software, San Jose, CA) was used to generate statistical data. Statistical comparisons of cell viability and mortality were conducted using one-way analysis of variance (ANOVA), followed by a multiple comparison post-hoc test (Holm-Sidak method). Comparisons of the percentage of Tail DNA and olive tail moment of treated groups relative to the negative controls were calculated using ANOVA, followed by a multiple comparison post-hoc test (Holm-Sidak method) or Mann-Whitney Rank Sum test. Differences at $p \leq 0.05$ were considered statistically significant.

3. Results

3.1 Physical and chemical characterization of test TiO_2 particles

Differences in particle size, dispersion or agglomeration have been shown to play an important role in nanoparticle interaction with cell membranes and toxicity (Rivera Gil et al., 2010; Sohaebuddin et al., 2010). We therefore, determined selected physical and chemical properties (Nel et al., 2006) of the particles examined in their dry state, as well as in the physiological media containing the culture media and serum. A summary of the particles characterization is shown in Table 1. Data indicate that nano-sized particles have larger specific surface areas (using BET) than micron-sized particles in the dry powder form, and larger agglomerate sizes (DLS measurements) in solution. In the dispersed state, nano- TiO_2 consisted of several sizes and the observed average size ranged from 460 nm (MTI5) to 600 nm (Vive Nano Titania). The highest polydispersity index value is 0.5 for Vive Nano Titania and that means this sample has a very broad size distribution and may contain large aggregates that could slowly sediment. The crystal form (anatase versus rutile) as measured by XRD, had no apparent

impact on the agglomerate size of the particles, as MTI5-TiO₂ particles (nano-sized anatase) formed larger agglomerates than Hombitan LW-S TiO₂ (bulk anatase); P25 (a mixture of anatase and rutile) formed agglomerate sizes between those of the MTI5 and Hombitan LW-S particles (400 nm). Polyacrylate-coated nano-TiO₂ particles were less dispersed in media compared to other particles tested, and formed large agglomerates. Differences in the size and shape of particles are evident from the TEM micrograph (Fig. 1). The particles were mostly dispersed as agglomerates or aggregates, having an average size ranging from 0.36 µm (Hombitan LW-S TiO₂) to 0.6 µm (Vive Nano Titania). However, all dispersions also contained nano-sized particles as verified by TEM analysis.

Fig. 2 shows examples of particles dispersed in media as seen by light microscopy. It is interesting to note that MTI5 (Fig. 2b) and Hombitan LW-S (Fig. 2c) particles (i.e., both nano- and micrometer-sized particles, respectively) could penetrate inside the cells and change cellular morphology. In contrast, the Vive Nano Titania particles mostly formed large agglomerates and remained outside the cells (Fig. 2d).

3.2 Cytotoxicity of TiO₂ particles

The effects of different types of TiO₂ nanoparticles on cell viability were examined after 24 and 48 h exposure, using the MTT assay, as shown in Fig. 3. Results were confirmed later by the trypan blue staining assay. Cell viability was not changed at the lower concentration (1 mg/L) compared to the control group (data not shown), but decreased at higher TiO₂ concentrations (10 or 100 mg/L). Among the non-coated TiO₂ samples tested in the present study, the nanofilament (10 x 40 nm) samples caused the highest decrease in the number of living cells with 70% and 68% of controls after 24 and 48 h exposure, respectively. On the other hand, the Hombitan LW-S sample caused the lowest decrease, with only 83% and 79% of the control levels after 24 and 48

h, respectively. Using Vive Nano Titania, the cell viability remained unchanged at 1 and 10 mg/L, but was decreased to 85% of the control at 100 mg/L after 48 h treatment. No significant effect ($p > 0.05$) on the number of viable cells was observed even at the highest concentration of the Allosperse-A (the polyacrylate control to Vive Nano Titania). Statistical analysis revealed that all TiO₂ test samples significantly inhibited ($p \leq 0.05$) cell viability in a concentration-dependent manner compared to controls, and the lowest effect was shown in the negatively charged coated nano-TiO₂ (Vive Nano Titania). The non-coated micron-sized TiO₂ (Hombitan LW-S) significantly induced less cytotoxicity ($p \leq 0.05$) compared to non-coated nano-sized TiO₂ particles. In other experiments, the toxicological effects were only investigated at higher concentrations of TiO₂ (10 and 100 mg/L) because the cell viability was not significantly changed at 1 mg/L compared to the control.

3.3 Apoptosis/necrosis induced by TiO₂ particles

The percentage of cells undergoing apoptosis/necrosis was examined quantitatively by flow cytometry (Table 2 and Fig. 4). Annexin V/PI staining combined with flow cytometry is commonly used to differentiate between viable cells and dead cells (those that have already undergone an apoptotic cell death and those that have died as a result of necrosis pathway). The apoptosis/necrosis rate of cells exposed to 100 mg/L of TiO₂ for 24 h was increased from $0.9 \pm 0.2\%$ in the negative control group to $8.6 \pm 1.2\%$ in Vive Nano Titania, $12.3 \pm 1.1\%$ in Hombitan LW-S, $20.6 \pm 2.0\%$ in MTI5, $26.2 \pm 1.6\%$ in nanofilament, and $30.6 \pm 2.1\%$ in P25 treatment groups (Table 2). All of the tested TiO₂ samples induced more dead and apoptotic cells in a mass concentration-dependent manner compared to the negative controls ($p \leq 0.05$). At the highest concentration of TiO₂ particles tested (100 mg/L), the number of cells undergoing apoptosis/necrosis was significantly lower in Hombitan LW-S and Vive Nano Titania treatment

groups than in the nano-TiO₂ groups ($p \leq 0.05$) (Table 2). Fig. 4 illustrates the influence of particle composition and size on TiO₂ uptake by cells; control cells showed a size distribution population with minimal side scatter (SSC). The latter is related to cell granularity or internal complexity, in which either particles are in the cell or organelles in the cell are changed. All types of non-coated TiO₂ exposure produced a pronounced right shift in the cell population accompanied by a large increase in side scatter. This indicates an increase in TiO₂ uptake is associated with a reduction in cell size. Even at a high concentration (100 mg/L), Vive Nano Titania caused very little to no increase in side scatter compared to the negative controls.

3.4 DNA damage induced by the nano-TiO₂

The comet assay was carried out under alkaline conditions to investigate the effect of TiO₂ particles exposure on DNA strand breaks using V79 cells (Fig. 5). The results indicated that the TiO₂ particle size and surface coating may have a role in inducing DNA damage in exposed cells. Fig. 5 illustrates the migration of DNA from the nucleus of each cell in representative negative control and in TiO₂ exposed groups. The nuclei in control cells appeared round (Fig. 5a), while non-coated nano-TiO₂ caused an increase in DNA breakage in exposed cells (Fig. 5b-e) compared to the polymer-coated test sample (Fig. 5f). The percentage of DNA in the comet tail (% Tail DNA) and the olive tail moment (OTM) from 50 cells in each of two replicate samples were used to measure DNA damage; these two parameters are considered as the most informative and reliable measurements (Kumaravel and Jha, 2006; Olive and Durand, 2005). A large tail was detected in the nucleus of each cell treated with the 100 μ M hydrogen peroxide positive control group (data not shown). Cells viability was more than 40% after exposure to 100 mg/L of nano-TiO₂ after 24 h. Results in Fig. 6 summarizes the quantitative effects of nano-TiO₂ by the comet assay, and indicates an almost three-fold increase in % Tail DNA (Fig. 6a) and

four-fold increase in OTM (Fig. 6b), in cells exposed to 100 μ M hydrogen peroxide for 10 min, compared to the negative control. The % Tail DNA and the OTM were increased by two-fold ($p \leq 0.05$) in cells treated with 100 mg/L of non-coated nano-TiO₂ after 24 h, whereas cells exposed to Hombitan LW-S TiO₂ only showed a significantly greater OTM (i.e., no change in % Tail DNA), compared to the negative control group. These results reflect the lower genotoxic effect of micrometer-sized compared to nano-size TiO₂. No significant differences were detected in % Tail DNA or OTM between cells exposed to 10 mg/L of all test samples of TiO₂ or 100 mg/L of Vive Nano Titania (data not shown).

3.5 Correlation between cyto- and geno-toxicological endpoints

The correlation between cell viability and apoptotic/necrotic cells are shown in Fig. 7a. Correlations are also shown between apoptotic/necrotic cells and DNA damage (Fig. 7b), and between cell viability and DNA damage (Fig. 7c). These results showed that the highest DNA damage occurs as exposure to non-coated nano-TiO₂ and the lowest as exposure to Vive Nano Titania and subsequently Hombitan LW-S.

3.6 Detection of ROS generation

To investigate whether nano-TiO₂ particles stimulate ROS generation, the intracellular ROS level was observed using H₂ DCFDA (Fig. 8). The H₂ DCFDA can passively enter the cell and react with the ROS to produce the fluorescent compound, dichlorofluorescein (DCF) (Wang and Joseph, 1999). A high level of ROS generation was detected in cells treated with 100 μ M H₂O₂ for 10 min (positive control group) (Fig. 8a). ROS was also generated in cells treated for 24 h with a high concentration (100 mg/L) of non-coated nano-TiO₂ such as MTI5 (Fig. 8b), P25 (Fig. 8c), nanofilament TiO₂ (Fig. 8d), and to a lesser extent Hombitan LW-S (Fig. 8e) or Vive Nano Titania (Fig. 8f). These data indicate that micrometer-sized TiO₂ and polyacrylate-coated nano-

TiO₂ induce less ROS production than uncoated nano-TiO₂, although we were not able to detect this difference quantitatively (and statistically) because of the low levels of fluorescence. No fluorescent staining was observed in cells cultured in the absence of TiO₂ or H₂O₂ (data not shown).

4. Discussion

In spite of increasing research on potential health risks of manufactured nanoparticles during the last few years, our understanding is still behind the rapid increase in nanotechnology products and applications. The majority of earlier investigations concerning the toxicity of nanoparticles were based on *in vitro* studies, as testing a large variety of different NPs is costly and labor-intensive, and is only feasible by using *in vitro* toxicity systems.

Most of the toxicological results on NPs have been generated by comparing different types of NPs (Aruoja et al., 2009; Karlsson et al., 2008; Xu et al., 2009), or one nano-sized particle either alone or versus larger sized counterparts (Liu et al., 2010; Park et al., 2008; Sohaebuddin et al., 2010; Wang et al., 2007). In the present study, we focused on the effects of one type of NP, nano-TiO₂, with respect to its different physico-chemical properties, including particle surface modification. The physico-chemical characteristics of the TiO₂ test samples (shown in Table 1) were consistent with findings of others (Falck et al., 2009; Limbach et al., 2005). Particles with smaller sizes possess larger surface areas, have a stronger tendency to agglomerate in medium, and interact more with biomolecules such as proteins and DNA in biological environment. These characteristics may contribute to particle uptake and various intracellular responses (Allouni et al., 2012; Horie et al., 2010; Thevenot et al., 2008; Xia et al., 2008). Similarly, our results indicated that smaller TiO₂ particles (nano-TiO₂) were taken up by cells more than the larger-sized particles (micron-sized TiO₂), as detected by microscopy (Fig. 2) and flow cytometry (Fig.

4). It is presumed that NP is taken up by cells via passive diffusion or by endocytosis including caveolae, clathrin coated pits or receptor-mediated mechanisms (Oberdorster et al., 2007).

Among several routes of nano-TiO₂ exposure, inhalation is apparently more likely than others such as ingestion, and dermal penetration. Nano-TiO₂ can induce lung cancer in rats, and toxicity in human bronchial cells (Falck et al., 2009; Pott and Roller, 2005). In the present study, hamster lung fibroblast cells were chosen to investigate the toxicity of TiO₂ particles because the lung is the primary target organ of TiO₂ particle toxicity (Baan et al., 2006; Rothen-Rutishauser et al., 2006), using concentrations that were relevant to those that induced toxicity in earlier *in vivo* studies. For example, lung epithelial injury and toxicity were identified in rats after exposure to 1000 mg/L of ultrafine TiO₂ (29 nm), and DNA damage was detected in the blood cells of the mice treated with P25 (300 mg/L) for 5 d (Renwick et al., 2004; Trouiller et al., 2009; Warheit et al., 2007). It is noteworthy that the response of rats to insoluble-particle “lung overload” is stereotyped and unique to that species. When the lung-overload threshold is exceeded, rats develop lung tumors from ongoing inflammation as opposed to particle-specific toxicity (Oberdörster, 1995; Valberg et al., 2009).

All TiO₂ particles tested in this study induced cytotoxicity in a mass concentration-dependent manner (Fig. 3), using the MTT assay that measures the combined effects of cell proliferation and metabolic activity of cells. Our results were validated by directly counting the number of surviving cells using the trypan blue staining assay because the MTT assay is prone to artifacts under certain experimental conditions (L’Azou et al., 2008). The cell viability in cell culture medium containing non-coated TiO₂ was reduced to almost 50% of control values at the highest concentration examined (100 mg/L), which was in agreement with earlier studies of P25-TiO₂ (Liu et al., 2010; Park et al., 2008). The cell viability in the coated nano-TiO₂ (Vive Nano

Titania) treatment group was unchanged at 10 mg/L, with a small effect at 100 mg/L; even higher concentrations up to 400 mg/L did not reduce the percentage of viable cells less than 80% of control values (data not shown). Vive Nano Titania (-) is a negatively charged water-dispersible rutile nanoparticle powder stabilized by sodium polyacrylate. According to the manufacturer, less than 22% of its weight is TiO_2 . Therefore, our experiments were also carried out at concentrations greater than 100 mg/L, and gave negative effects. The coating helps to disperse particles; however, it also creates large agglomerates, as detected by DLS (Table 1) and in microscopic images (Fig. 2). Therefore, whether the polyacrylate surface coating makes the particles less bioavailable to cells because of its physico-chemical nature (e.g., either large size or negative charge) should be further investigated. It is also possible that the polymer coating may quench the activity of TiO_2 particles, i.e., decreased ROS generation (as seen in Fig. 8). It should be noted that in all experiments described in the present article, the polyacrylate surface coating without nano- TiO_2 (Allosperser-A) was solely used as a control for our coated nano- TiO_2 studies, and no toxic effects were observed. The role of surface coating was evidenced by the induction of greater toxicity of aluminum oxide and/or silica content on coated TiO_2 particles in lung compared to their uncoated counterparts (Warheit et al., 2005). A similar result was observed in TiO_2 particles having a silane coating (hydrophobic) compared to uncoated TiO_2 (hydrophilic) (Oberdörster, 2001). In contrast, the impact of surface methylation on TiO_2 toxicity was negligible (Hohr et al., 2002). Moreover, the duration of exposure had no effect on the cytotoxicity results, although it was previously reported that nanomaterials cause a time-dependent decrease in cell viability, an effect that it may also depend on the cell types tested (Sohaebuddin et al., 2010).

The flow cytometry results of ability of different TiO₂ particles to induce apoptosis/necrosis were shown for the first time in this study, in which the composition and size of particles can exert an impact on their cellular uptake (Fig. 4; Table 2). Vive Nano Titania, even at a high concentration (100 mg/L), had no effect on the side scatter, although some cells were positively labeled for annexin V and PI (Fig. 4). In future study, an apoptotic marker, e.g., caspase-3 will be used to distinguish between apoptotic and necrotic cells. Nano-TiO₂ induced apoptosis or necrosis in a human monoblastoid cell line (Vamanu et al., 2008). These authors suggested that the increased granularity in these cells could be due to internalized or membrane attached nano-TiO₂.

The present study shows the genetic effects of different nano-TiO₂ particles on V79 cells using the comet assay (Fig. 6). Recent studies have reported chromosomal damage induced by nano-TiO₂ in hamster embryo fibroblasts, blood lymphocytes, and in a human B-cell lymphoblastoid cell line using the micronucleus assay (Kang et al., 2008; Rahman et al., 2002; Wang et al., 2007), which was mostly associated with ROS-related DNA injury. Similarly, an *in vivo* study has shown the induction of DNA breakage in mice cells after exposure to P25-TiO₂ in drinking water (Trouiller et al., 2009). In human bronchial epithelial cells exposed to TiO₂ particles, the oxidative DNA damage was shown to be higher in nano-sized anatase compared to its larger counterparts (Gurr et al., 2005), and nano-sized anatase was more toxic than nano-sized rutile (Falck et al., 2009). Our genotoxicity findings agree with earlier studies in the induction of DNA damage in cells after exposure to nano-TiO₂, and a stronger induction was observed in nano-sized anatase compared to rutile. The olive tail moment and the percentage of DNA in the tail (% tail DNA), which are the most informative and reliable measurements (Kumaravel and Jha, 2006; Olive and Durand, 2005), were used as DNA damage indicators in our study. Neither

the % tail DNA nor the olive tail moment was shown to be significantly different in cells exposed to Vive Nano Titania compared to negative control groups (Fig. 6), even at concentration 200 mg/L (data not shown). The correlations (Fig. 7) observed between cell viability, cell death, and DNA damage suggests the presence of a common mechanism of toxicity of TiO₂ particles in treated cells. It seems reasonable that cell viability is inversely related to the apoptosis/necrosis and the DNA damage, and that apoptosis/necrosis is related to the DNA damage. These results are supported by recent reports that an increase in oxidative stress led to an increase in apoptosis formation and DNA damage, and subsequently a decrease in cell viability (Guichard et al., 2012; Jagan et al., 2011; Kumar et al., 2011). Another recent study demonstrated the correlation between DNA damage and micronucleus formation with ROS in human epidermal cells treated by anatase nano-TiO₂ (Shukla et al., 2011). Nanoparticles compared to their larger counterparts having the same mass weight possess a higher surface area to volume ratio, which enhance contact area with their surroundings and may induce more formation of ROS (Fig. 8), and this may be one of the common mechanisms of toxicity induced by different TiO₂ samples in these cells. However, more *in vivo* studies are required to fully understand the mechanism of TiO₂ nanoparticles toxicity. Likewise, more caution should be considered for toxicity assessment of nanoparticles because of the limitations and potential artifacts of the results (Doak, et al., 2012, Landsiedel et al., 2009). This study provides a better insight into the potential toxicity of nanoparticles with respect to their physiochemical characteristics. However, the number of NPs tested in present study was too limited to determine whether a definitive and systemic correlation exists between the biological effects and material properties.

5. Conclusions

The present study was conducted because of the inconsistency and controversial results of nanoparticles toxicity reported in the peer-reviewed literature. For the first time, the toxicity of different types of one NP, nano-TiO₂, differing in their physicochemical properties under certain conditions, was measured. Because experimental test conditions will play a critical role in determining the toxicity of nanoparticles, results from *in vitro* studies require *in vivo* validation. Our results showed that while there is a significant difference between nano-TiO₂ and micron-sized TiO₂ in causing cytotoxicity, there is also a significant difference between coated- and uncoated nano-TiO₂ in causing cyto- and genotoxicity. Further studies are needed to elucidate the underlying mechanisms and their correlation with the physico-chemical properties of nano-TiO₂.

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Declaration of interest:

The authors have nothing to declare.

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Figure Legends

- Figure 1.** Characterization of TiO₂ particles by transmission electron microscopy (TEM). The images show that the size of distributed particles varies from 1 to 200 nm. Figures (a) represent the TEM image of MTI5, (b) P25, (c) Hombitan LW-S, and (d) Vive Nano Titania.
- Figure 2.** Light micrographs of (a) V79 control cells, (b) cells treated with 100 mg/L MTI5, (c) Hombitan LW-S or (d) Vive Nano Titania for 24 h. Figures indicate morphological changes and irregular shaped cells exposed to non-coated TiO₂ particles, whereas cells in control and coated nano-TiO₂ groups remain regular in shape.
- Figure 3.** Effects of TiO₂ particles on the viability of cells after 24, and 48 h. Cell viability was assessed by the MTT assay, and results are presented as a percentage of control group viability. Cell viability was greatly reduced in a concentration-dependent manner by TiO₂ exposure. Data are expressed as the mean $\pm$ SEM of at least three independent experiments; asterisk indicates that treated group is statistically significant ($p \leq 0.05$) from the control group; and a, b, c, and d indicate that Hombitan LW-S (H. Bulk) is less cytotoxic compared to nano-TiO₂ particles.
- Figure 4.** Analysis of apoptotic/necrotic cell death induced by TiO₂ particles using flow cytometry. Top rows show the forward scatter (FSC; y-axis) area versus the side scatter (SSC; x-axis) area of total population of cells. The FSC correlates with the cell volume, and SSC depends on the inner complexity of the organelles. Bottom rows show the SSC area (x-axis) versus the number of cells (y-axis). Figure 4a represents results of control cells; 4b represents results of cells treated with 20 mM benzalkonium chloride (BNZ) for 2 h; 4c and 4d represent results of cells treated with 100 mg/L nanofilament (10 x 40 nm), and 100 mg/L Vive Nano Titania (-), respectively. Black dots indicate the total population, whereas blue dots and purple dots represent the single cell of total population and non-viable cells, respectively.

Figure 5. Induction of DNA damage by TiO₂ particles in cells as detected by the comet assay. Figure shows the microscopic images of nucleus of the cells under different conditions: untreated as negative control (a), treated with 100 mg/L MTI5 (b), P25 (c), nanofilament (d), Hombitan LW-S (e), and Vive Nano Titania (-) (f) after 24 h exposure to TiO₂ sample.

Figure 6. Analysis of DNA strand breaks using the comet assay as measured by the percentage of tail DNA (a), and olive tail moment (b). Data are expressed as the mean $\pm$ SEM of three separate experiments of 50 cells in each of two replicate samples; asterisk indicates that treated group is significantly different compared to the control group ($p \leq 0.05$).

Figure 7. Relationship between cyto- and geno-toxicological endpoints using different TiO₂ samples. There is a correlation between the percentage of cell viability and % of apoptotic/necrotic cells ($y = -0.40x + 41.71$; $R^2 = 0.66$) (a), DNA damage and % of apoptotic/necrotic cells ($y = 3.94x - 9.44$; $R^2 = 0.56$) (b), and % of cell viability and DNA damage ($y = -0.08x + 11.92$; $R^2 = 0.77$) (c) in cells treated with 100 mg/L of different TiO₂ samples for 24 h. Note that the x- and y-axes do not start from the origin.

Figure 8. Phase contrast-fluorescence microscopic images of ROS generation by TiO₂ particles using H₂ DCFDA staining. The ROS is generated at a high level after 10 min in cells treated with 100 μ M H₂O₂, as a positive control (a), and to a less extent after 24 h exposure to 100 mg/L MTI5 (b), P25 (c), nanofilament (d), Hombitan LW-S (e), and Vive Nano Titania (f).

Table 1. Physico-chemical characteristics of titanium dioxide (TiO₂) test samples

	Dry powder		Particles in media		
	Specific surface area (BET; m ² /g)	Nominal size (nm)	Agglomerate size* (DLS, nm)	Polydispersity Index*	Zeta potential of agglomerates* (pH = 8)
MTI5 (100% anatase)	280.9	5.9	460	0.26	-12
P25 (83% anatase & 17% rutile)	48.9	34.1	400	0.39	-12
Nanofilament (10 x 40; 100% rutile)	107.4	15.5	420	0.38	-12
Hombitan-Bulk (100% anatase)	9.8	169.4	365	0.14	-13
Vive Nano Titania (-) (coated nano-TiO ₂)	-	1-10	600	0.50	-19

BET: Brunauer Emmett Teller, DLS: Dynamic Light Scattering

* indicates the mean of three replicates, and the SEM of each value (not reported) is less than 1% of its mean.

Table 2. Summary of the flow cytometry results of the percentage of cells undergoing apoptosis and necrosis under different conditions.

Treatment	Concentration used	Single cells – % total Anx ⁺ /PI ⁺
Control		0.9 ± 0.2
Benzalkonium chloride	20 µM	65.5 ± 3.8*
MTI5	10 mg/L	10.5 ± 1.4*
	100 mg/L	20.6 ± 2.0*§
P25	10 mg/L	9.6 ± 0.3*
	100 mg/L	30.6 ± 2.1*§
Nanofilament (10 x 40 nm)	10 mg/L	9.6 ± 0.5*
	100 mg/L	26.2 ± 1.6*§
Hombitan - Bulk	10 mg/L	7.1 ± 0.8*
	100 mg/L	12.3 ± 1.1*§
Vive Nano Titania (-) (coated nano-titanium dioxide)	100 mg/L	8.6 ± 1.2*§
Alloperse-A	100 mg/L	0.9 ± 1.1

% total Anx⁺/PI⁺: Annexin V⁺/PI⁺, percentage of cells undergoing apoptosis and necrosis,

Results represent the mean ± SEM of three independent experiments; asterisk indicates that values in treated group are significantly different compared to the control group ($p \leq 0.05$), and § indicates that values in nano-TiO₂ group are significantly different compared to those in Hombitan LW-S and Vive Nano Titania treatment groups ($p \leq 0.05$).