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Comparative cellular toxicity of titanium dioxide nanoparticles on human astrocyte and neuronal cells after acute and prolonged exposure

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ABSTRACT

Although in the last few decades, titanium dioxide nanoparticles (TiO_2NPs) have attracted extensive interest due to their use in wide range of applications, their influences on human health are still quite uncertain and less known. Evidence exists indicating TiO_2NPs ability to enter the brain, thus representing a realistic risk factor for both chronic and accidental exposure with the consequent needs for more detailed investigation on CNS.

A rapid and effective *in vitro* test strategy has been applied to determine the effects of TiO_2NPs anatase isoform, on human glial (D384) and neuronal (SH-SY5Y) cell lines. Toxicity was assessed at different levels: mitochondrial function (by MTT), membrane integrity and cell morphology (by calcein AM/PI staining) after acute exposure (4–24–48 h) at doses from 1.5 to 250 μg/ml as well as growth and cell proliferation (by clonogenic test) after prolonged exposure (7–10 days) at sub-toxic concentrations (from 0.05 to 31 μg/ml). The cytotoxic effects of TiO_2NPs were compared with those caused by TiO_2 bulk counterpart treatment.

Acute TiO_2NP exposure produced (i) dose- and time-dependent alterations of the mitochondrial function on D384 and SH-SY5Y cells starting at 31 and 15 μg/ml doses, respectively, after 24 h exposure. SH-SY5Y were slightly more sensitive than D384 cells; and (ii) cell membrane damage occurring at 125 μg/ml after 24 h exposure in both cerebral cells. Comparatively, the effects of TiO_2 bulk were less pronounced than those induced by nanoparticles in both cerebral cell lines.

Prolonged exposure indicated that the proliferative capacity (colony size) was compromised at the extremely low TiO_2NP doses namely 1.5 μg/ml and 0.1 μg/ml for D384 and SH-SY5Y, respectively; cell sensitivity was still higher for SH-SY5Y compared to D384. Colony number decrease (15%) was also evidenced at $\geq$0.2 μg/ml TiO_2NP dose. Whereas, TiO_2 bulk treatment affected cell morphology only.

TiO_2 internalization in SH-SY5Y and D384 cells was appreciated using light microscopy.

These findings indicated, that (i) human cerebral SH-SY5Y and D384 cell lines exposed to TiO_2NPs were affected not only after acute but even after prolonged exposure at particularly low doses ($\geq$ 0.1 μg/ml), (ii) these *in vitro* critical doses were comparable to literature brain Ti levels detected in lab animal intranasally administered with TiO_2NP and associated to neurotoxic effects.

In summary, the applied cell-based screening platform seems to provide effective means to initial evaluation of TiO_2NP toxicity on CNS.

1. Introduction

Titanium dioxide (TiO_2) is a versatile compound that has broadly been used in nanoparticles form, titanium dioxide nanoparticles (TiO_2NPs). According to the U.S. National Nanotechnology Initiative, they are one the most highly manufactured in the world due to their high physical stability, anticorrosion and photocatalytic activity (Baan et al., 2006; Shi et al., 2013). TiO_2NPs are in the top five NPs used in consumer products (Shukla et al., 2011), widely used in paints, printing ink, rubber, paper, car materials, cleaning air products, sterilization (Montazer and Seifollahzadeh, 2011), industrial photocatalytic processes (Douglas et al., 2000), decomposing organic matters in wastewater

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(Sun et al., 2004) and as an additive in cosmetics (Kaida et al., 2004; Wolf et al., 2003), pharmaceuticals, and food colorants (Jin et al., 2008; Vamanu et al., 2008a).

Human exposure to TiO_2NPs may occur during both manufacturing and use. TiO_2NPs can be encountered as aerosols, suspensions or emulsions. The major route of TiO_2NP exposure that have toxicological relevance in the workplace is inhalation.

The United States National Institute for Occupational Safety and Health (NIOSH, 2011) proposed a recommended exposure limit (REL) for TiO_2NPs at 0.3 mg/m^3, which was 10 times lower than the REL for TiO_2FPs (FP: fine particles). Despite TiO_2NPs have been regulated, there are still many concerns related to their small size and their potential toxic effects after inhalation.

Human data related to absorption through inhalation of TiO_2NPs are currently not available. Numerous pulmonary rodent studies were carried out to clarify the role played of TiO_2NPs in determining lung injury after acute or chronic exposure (Bermudez et al., 2004; Grassian et al., 2007; Kobayashi et al., 2009; Li et al., 2010; Ma-Hock et al., 2009; Osier and Oberdörster, 1997; Sun et al., 2012; Tang et al., 2011; Warheit et al., 2007a,b; Ze et al., 2014a,b). Several studies have also reported that, irrespective of the route of exposure, TiO_2NPs enter systemic circulation and migrate to various organs and tissues including brain where they could accumulate and cause damage (*e.g.*, structural changes in the neuronal architecture, changes in the release and metabolism of neurotransmitters) (Hu et al., 2010; Kreyling et al., 2002; Li et al., 2010; Ma et al., 2010; Oberdörster et al., 2009; Takenaka et al., 2001; Wang et al., 2007, 2008a,b; Ze et al., 2014a,b).

Currently, *in vitro* cellular studies regarding the molecular mechanism of the neurotoxicity and the effects of TiO_2NPs (pure anatase isoform or anatase/rutile mixture) on the nervous system are scarce and limited to rat neuronal (PC12, N27), murine glial cell lines (C6, BV-2, N9), primary neural cells obtained from rodents (Huerta-García et al., 2014; Liu et al., 2010; Long et al., 2006, 2007; Márquez-Ramírez et al., 2012; XiaoBo et al., 2009; Wu et al., 2010), or *in vitro* cell-based rat blood–brain model (Brun et al., 2012) after acute TiO_2NPs exposure. Few studies have been focused on neurotoxicity of TiO_2NPs in human nervous system cells (*e.g.* SH-SY5Y, U373, U87) and in any way after acute exposure only (Huerta-García et al., 2014; Lai et al., 2008; Valdiglesias et al., 2013).

Understanding the health impact of TiO_2NPs has become a priority both for ensuring health protection and for regulating the safe development of nanotechnologies. The ability of TiO_2NPs to enter the brain represents a realistic risk factor both in the case of chronic and accidental exposure, which needs to be investigated in more detail.

In vitro human cell models may represent a valid instrument to investigate TiO_2NP effects on CNS and to determine their underlying mechanistic processes, providing information about doses of exposure. Thus, to fulfill the goal of facilitating predictions of toxicological profiles and improve human risk assessment by using *in vitro* data (Westerink, 2013), a rapid and effective *in vitro* test strategy has been applied allowing to meet some basic requirements, including a relevant exposure paradigm (*e.g.*, prolonged exposure to low concentrations), relevant endpoint(s) and cell models (*e.g.*, both neuronal and glial cells) to match the neurotoxic evaluation question.

In this respect, the present study aimed at determining the effects of TiO_2NPs anatase isoform, on human glial cells (D384 cell line) and neuronal cells (SH-SY5Y cell line). Toxicity was assessed at different levels: mitochondrial function, membrane integrity and cell morphology after acute exposure (4–48 h) at doses ranging from 1.5 to 250 μg/ml as well as growth and cell proliferation after prolonged exposure (7–10 days) at doses ranging from 0.05 to 31 μg/ml. The cytotoxic effects of TiO_2NPs were compared with those caused by treatment with TiO_2 bulk counterpart.

2. Materials and methods

2.1. Chemicals

All reagents and chemicals for cell cultures, Titanium (IV) oxide (TiO_2), and chemicals for light microscopy analyses were purchased from Sigma–Aldrich (Milan, Italy).

2.2. Physico-chemical characterization of TiO_2NP

Titanium oxide nanoparticles (# 5430MR) were purchased from Nanostructured & Amorphous Materials, Inc. (Houston, USA). The presence of TiO_2 was confirmed by X-ray diffraction analysis (Fig. 1A, data provided by the Company). The TiO_2NP nanopowder, anatase isoform, presented spherical form, primary particle size of 15 nm, specific surface area of 240 m^2/g, density of 3.9 g/cm^3, purity of 99.6%. Metal impurities evaluated by inductively coupled plasma were: Na 0.01%, Fe 0.004%, Mg 0.006%, Al 0.01% and Cl 0.01%. Further evaluations by morpho-dimensional analysis of the raw nanomaterial in water by TEM and SEM (Fig. 1B1 and B2) and dynamic light scattering (DLS) indicated a diameter size of 69.3 ± 0.4 nm.

Moreover, the size of the nanoparticles and the zeta potential in the stock suspension and culture media were analyzed by dynamic light scattering (DLS) using the Malvern Zetasizer Nano ZS90. For these measurements, TiO_2NP were prepared using the dispersion protocol indicated below (Section 2.4) and diluted in the culture media for the two cell line treatments. Specifically, the evaluations were performed in stock solution (TiO_2NP: 2.5 mg/ml), and in completed DMEM and Ham's F12 media (as indicated in Section 2.3) (TiO_2NP: 31 μg/ml) after 24 and 48 h.

2.3. Cell lines

Human neuroblastoma (SH-SY5Y cell line purchased from ECACC, Sigma–Aldrich, Milan, Italy), and human astrocytoma cells (D384 clonal cell line was established from Balmforth et al., 1986), were used for *in vitro* studies of the TiO_2NP and TiO_2 bulk toxicity after acute (4–48 h) and prolonged (7–10 days) exposure.

SH-SY5Y cells were cultured in Eagle's minimum essential medium and Ham's F12 (1:1) with 15% fetal bovine serum (FBS), 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin. D384 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin and 1% sodium pyruvate. Cells were maintained at 37 °C in a humidified atmosphere (95%air/5% CO_2).

2.4. TiO_2NP and TiO_2 bulk stock suspension

For cell treatments in culture medium, we adopted an optimized dispersion protocol to yield stable stock suspensions of TiO_2NP at pH 7. Specifically, TiO_2NP suspension was prepared as reported by Guiot and Spalla (2013). Briefly, 34.1 mg of TiO_2 nanopowders were added to 10 ml of HNO_3 10^{-2} M, then the suspension was sonicated for 20 min at 40% amplitude (sonicator: Bandelin Sonopuls HD2070, Germany). To 7.5 ml of the sonicated suspension were added 2.5 ml of sterile-filtered solution of BSA (10.24 mg/ml), then the pH was adjusted to 7 with NaOH 10^{-1} M and NaOH 10^{-2} M. Subsequently, this stock suspension was used to make appropriate dilutions in culture medium for the cell treatments. Cells in culture media supplemented with vehicle