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Comparative study on effects of two different types of titanium dioxide nanoparticles on human neuronal cells

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ABSTRACT

Titanium dioxide (TiO_2) are among most frequently used nanoparticles (NPs). They are present in a variety of consumer products, including food industry in which they are employed as an additive. The potential toxic effects of these NPs on mammal cells have been extensively studied. However, studies regarding neurotoxicity and specific effects on neuronal systems are very scarce and, to our knowledge, no studies on human neuronal cells have been reported so far. Therefore, the main objective of this work was to investigate the effects of two types of TiO_2 NPs, with different crystalline structure, on human SHSY5Y neuronal cells. After NPs characterization, a battery of assays was performed to evaluate the viability, cytotoxicity, genotoxicity and oxidative damage in TiO_2 NP-exposed SHSY5Y cells. Results obtained showed that the behaviour of both types of NPs resulted quite comparable. They did not reduce the viability of neuronal cells but were effectively internalized by the cells and induced dose-dependent cell cycle alterations, apoptosis by intrinsic pathway, and genotoxicity not related with double strand break production. Furthermore, all these effects were not associated with oxidative damage production and, consequently, further investigations on the specific mechanisms underlying the effects observed in this study are required.

1. Introduction

Nanotechnology is currently one of the fastest-growing industries on the planet, and its development is accompanied by promises of substantial benefits that will have significant economic and scientific impacts (Singh et al., 2009). Nanoparticles (NPs) – particles with size between 1 and 100 nm – present new physicochemical properties compared to coarse particles of the same material, which make them a highly suitable raw material for a number of medical, electronic and industrial products. At present, over 1000 consumer products containing nanomaterials are already available in the market (PEN, 2009). With the rapid expansion of the nanotechnology industry, there are increasing concerns as to the potential adverse human health and environmental effects that the production and subsequent exposure to NPs might pose (Clift et al., 2011). The small size of the NPs facilitates their penetrance into cells as well as their transfer across epithelial cells into bloodstream to reach sensitive target sites, including the neuronal cells (Gheshlaghi et al., 2008). This property has lead NPs to be increasingly employed on the drug therapy of the central nervous system diseases such as Alzheimer's (Wilson, 2011), Parkinson's (Brynskikh et al., 2010) or other brain disorders (Muthu and Singh, 2009). Thus, these compounds are able to gain entry into the body via inhalation, dermal or oral routes, and subsequently could exert potential toxic effects at molecular, cellular and/or genetic levels.

Metal oxide nanoparticles have become one of the most frequently used nanomaterials in consumer products. Specifically, titanium dioxide (TiO_2) NPs have the most numerous purposes compared to any other metal NPs. They are increasingly used in a variety of industrial and medical applications including cosmetics, sunscreens, paints, plastics, paper products, drugs, and medical orthopaedic implants. Furthermore, the US Food and Drug

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Administration (FDA) established a regulation for TiO_2 NPs as a colour additive for food (FDA, 2002), since they are currently used in different consumer products such as toothpaste, capsules and breath freshener. Therefore, the effects of these NPs on human health are an issue of great concern that has led to extensive study of their potential toxicity during the last years. In these studies, they were already demonstrated to induce oxidative damage, genotoxicity and cytotoxicity in different cell lines, mostly keratinocytes and bronchial and lung cells (Wang et al., 2007; Bhattacharya et al., 2009; Ghosh et al., 2010; Jin et al., 2011; Jugan et al., 2011; Shukla et al., 2011). Consequently, TiO_2 nanoparticles have been recently classified as possible human carcinogen (group 2B) by the International Agency for Research on Cancer (IARC, 2010).

Nevertheless, little is known on the effects of TiO_2 NPs on nervous system. Nowadays, there is an imperative need of studying and characterizing those effects, especially since incontrovertible evidence that some engineered NPs can reach the brain of small animals directly through axonal transport from nose (Wang et al., 2008; Zhang et al., 2012), or indirectly by crossing the blood brain barrier after different routes of administration (reviewed in Hu and Gao, 2010) was obtained. Specifically, TiO_2 NPs have been proven to reach the brain after intranasal (Wang et al., 2008; Zhang et al., 2011), dermal (Wu et al., 2009), intraperitoneal (Ma et al., 2010), and oral administration (Wang et al., 2007) in rodents. There are also growing concerns regarding the possible influence of TiO_2 NPs on nervous system after prenatal or early childhood exposures, since these NPs were reported to cross the placental tissue of mice after intravenous administration, reaching several foetal organs, including the brain, and causing pregnancy complications (Yamashita et al., 2011). TiO_2 NPs were also demonstrated to reach and affect the cranial nerve system (Takeda et al., 2009) and cause moderate neurobehavioral alterations (Hougaard et al., 2010) in offspring after subcutaneous or inhalation administration, respectively, to the pregnant mice. Despite these findings, studies regarding the potential neurotoxicity and the effects of TiO_2 NPs on the nervous system are scarce and limited to animal cells or organisms, excepting one report on human glial cells (U87 astrocytes) (Lai et al., 2008). Recent reports described apoptosis, cell cycle alterations, and oxidative damage in rat PC12 neuronal cells (Liu et al., 2010; Wu et al., 2010) and in U87 astrocytes (Lai et al., 2008). Besides, brain dysfunction, oxidative stress, lipid peroxidation, disruption of blood–brain barrier, and neuroinflammation related to TiO_2 NPs exposure in were also reported *in vivo* studies using rodents or fish (Wang et al., 2008; Ramsden et al., 2009; Ma et al., 2010). To our knowledge, no studies on human neuronal cells have been reported so far.

On this basis, the main objective of this work was to investigate the effects of TiO_2 NPs on human SHSY5Y neuronal cells, commonly used as a model in neurotoxicological studies. Toxicity was assessed at different levels, from viability and cytotoxicity to genotoxicity and oxidative damage. Two types of TiO_2 NPs (anatase and anatase + rutile) were tested in order to compare their effects and check if the controversy in the results regarding these NPs may come from their different crystalline phase.

2. Materials and methods

2.1. Chemicals

Titanium dioxide (TiO_2-S) (CAS No. 1317-70-0), mytomycin C (MMC) (CAS No. 50-07-7), bleomycin (BLM) (CAS No. 9041-93-4), camptothecin (Campt) (CAS No. 7689-03-4), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (CAS No. 298-93-1), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1) (CAS No. 3520-43-2), neutral red dye (CAS No. 553-24-2), 5-sulfosalicylic acid dihydrate (SSA), 1-Methyl-2-vinylpyridinium triflate (M2VP), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), and propidium iodide (PI) were purchased from Sigma–Aldrich Co. (Madrid, Spain). MMC, BLM, and Campt were dissolved in sterile distilled water. Titanium dioxide (TiO_2-D) (CAS No. 13463-67-7) was purchased from Degussa-Evonik (Germany), and 5,5′-dithiobis-(2-nitrobenzoic acid (DTNB) was purchased from Merck (Germany).

2.2. Nanoparticle suspension: preparation and characterization

TiO_2 NPs were suspended in either deionized water or complete cell culture medium (with FBS) at a final concentration of 150 μg/ml and ultrasonicated (Branson Sonifier, USA) at 30 W for 5 min (1.5 min on and 1 min off twice, and 2 min on). Average hydrodynamic size, size distribution and zeta potential of particles in suspension were determined by Dynamic Light Scattering (DLS) using a Zetasizer Nano-ZS equipped with 4.0 mW, 633 nm laser (Model ZEN 3600, Malvern Instruments Ltd., Malvern, UK).

2.3. Cell culture

Human neuroblastoma SHSY5Y cell line was obtained from the European Collection of Cell Cultures and cultured in nutrient mixture EMEM/F12 (1:1) medium with 1% non-essential aminoacids, 1% antibiotic and antimycotic solution, and supplemented with 10% heat-inactivated foetal bovine serum (FBS), all from Invitrogen (Barcelona, Spain). The cells were incubated in a humidified atmosphere with 5% CO_2 at 37 °C. To carry out the experiments, cells were seeded in 96-well plates (flat bottom) and allowed to adhere for 24 h at 37 °C. Cell densities were approximately in the range of 2–5 $\times$ 10^4 cells/well at the beginning of cell culture. For cell treatment, these were incubated at 37 °C for different periods in the presence of a variable number of NPs concentrations, depending on the assay performed, or the control solutions. Complete medium was used as negative control in all cases, whereas Campt (10 μM), MMC (1.5 μM), BLM (1 μg/ml), or H_2O_2 (2 μM for 3 h treatments, and 1 μM for 6 h treatments) were used as positive controls in cytotoxicity, genotoxicity, or oxidative damage assays respectively.

2.4. Cellular viability

The potential effects of both types of TiO_2 NPs on viability of neuronal SHSY5Y cells were tested employing the MTT and the neutral red uptake (NRU) assays, according to Mosmann (1983) and Borenfreund and Puerner (1985), respectively. A total of seven concentrations (20–150 μg/ml) and three exposure times (3, 6 and 24 h) were evaluated in each case. Absorbance was measured at 595 nm (MTT) or 540 nm (NRU) using a Cambrex ELx808 microplate reader equipped with kinetic analysis software (Biotek, KC4). The potential interaction of the NPs with the dyes used in MTT and NRU assays was excluded by a parallel set of experiments conducted without cells. Data obtained demonstrated no interaction between the TiO_2 NPs tested and the dyes used for cytotoxicity assessment. From the results obtained in the viability experiments, two times of exposure (3 and 6 h) and three different concentrations of each TiO_2 NPs (80, 120 and 150 μg/ml) were selected to perform the subsequent experiments.

2.5. Cellular uptake

The potential of the TiO_2 NPs to enter the cells was evaluated by means of flow cytometry using a FACSCalibur Flow Cytometer (Becton Dickinson, Madrid, Spain). The analysis was carried out on the basis of the size and the complexity of the cells by measuring the forward scatter (FSC) and the side scatter (SSC) following the protocol described by Suzuki et al. (2007).

2.6. Cell cycle

After treatments with each TiO_2 NPs, the relative cellular DNA content was evaluated by means of flow cytometry as previously described (Valdiglesias et al., 2011a), in order to examine the cell distribution along the different phases of the cell cycle. The analysis was performed using a FACSCalibur Flow Cytometer (Becton Dickinson, Madrid, Spain). A minimum of 10^4 events were acquired, and the DNA content was assessed from the PI signal detected by the FL2 detector. Cell cycle histograms were evaluated using Cell Quest Pro software (Becton Dickinson, Madrid, Spain), to obtain information on the percentage of occupancy of G0/G1, S and G2/M regions.

2.7. Apoptosis

Apoptosis rate was determined by means of annexin V/PI double staining, using the BD Pharmingen™ Annexin V-FITC apoptosis detection kit I (Becton Dickinson, Madrid, Spain), following the manufacturer's recommendations. At least 10^4 events were acquired with a FASCalibur Flow Cytometry (Becton Dickinson, Madrid, Spain). Data from annexin V-fluorescein isothiocyante (FITC) (FL1) and PI (FL2) were analysed using Cell Quest Pro software (Becton Dickinson, Madrid, Spain).