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Negligible cytotoxicity induced by different titanium dioxide nanoparticles in fish cell lines

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ABSTRACT

Titanium dioxide nanoparticles (TiO_2-NPs) have a wide number of applications in cosmetic, solar and paint industries due to their photocatalyst and ultraviolet blocking properties. The continuous increase in the production of TiO_2-NPs enhances the risk for this manufactured nanomaterial to enter water bodies through treated effluents or agricultural amendments. TiO_2-NPs have shown very low toxicity in a number of aquatic organisms. However, there are no conclusive data about their deleterious effects and on their possible mechanisms of toxic action. At this level, *in vitro* cell culture systems are a useful tool to gain insight about processes underlying the toxicity of a wide variety of substances, including nanomaterials. Differences in the physiology of different taxa make advisable the use of cells coming from the taxon of interest, but collecting data from a variety of cellular types allows a better understanding of the studied processes. Taking all this into account, the aim of the present study was to assess the toxicity of three types of TiO_2-NP, rutile hydrophobic (NM-103), rutile hydrophilic (NM-104) and rutile-anatase (NM-105), obtained from the EU Joint Research Centre (JRC) repository, using various fish cell lines (RTG-2, PLHC-1, RTH-149, RTL-W1) and rainbow trout primary hepatocytes. For comparative purposes, the effect of different dispersion protocols, end-point assays and extended exposure time was studied in a fish cell line (RTG-2) and in the rat hepatoma cell line (H4IIE). TiO_2-NPs dispersions showed a variable degree of aggregation in cell culture media. Disruption of mitochondrial metabolic activity, plasma membrane integrity and lysosome function was not detected in any cell line after exposure to TiO_2-NPs at any time and concentration ranges tested. These results are indicative of a low toxicity of the TiO_2-NPs tested and show the usefulness of fish cells maintained *in vitro* as high throughput screening methods that can facilitate further testing in the framework of integrated testing strategies.

1. Introduction

Titanium dioxide nanoparticles (TiO_2-NPs) are widely used in a range of applications, so that they appear in for instance, cosmetic, domestic and painting products due to their photoactive or whitening properties, and are also applied in waste water treatments or in photoactive material (Weir et al., 2012). All these uses of TiO_2-NPs generate substantial hazard for human exposure and environmental release, which inevitably lead to a potential health risk to humans, livestock, and the eco-system.

Mammalian toxicology models employ airborne, injection and dermal routes of administration as a reflection of primary exposure scenarios for TiO_2-NPs (Shi et al., 2013). In the case of environmental toxicity testing TiO_2-NPs are applied suspended in water, spiked in sediments or soil, or through the diet. It has been observed that, depending on the route of administration, different processes could be affected at different throphic levels so that it has been difficult to arrive to solid conclusions about the potential deleterious effects of TiO_2 on ecosystems and about the mechanisms underlying toxicity. For instance, microalgae show low-to-moderate susceptibility to TiO_2-NPs exposure, with more pronounced toxic effects in the presence of UV irradiation (Hund-Rinke and Simon, 2006). Lethal effects of TiO_2-NPs to higher trophic levels are associated with aggregation of TiO_2-NPs to microalgae leading to diminished food availability and to a decrease of reproductive performance and fitness of zooplanktonic species as *Daphnia magna* (Campos et al., 2013). Probably due to the low toxicity

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detected in algae and aquatic invertebrates, there are very few studies on possible toxicity of TiO_2-NPs in aquatic vertebrates. In one of these works, it was observed that exposure of zebrafish (*Danio rerio*) embryos to TiO_2-NPs for eight days did not significantly affect survival, but caused a significant reduction of length at 1 mg/mL (Faria et al., 2014). In addition, TiO_2-NPs led to alterations in embryo oxidative stress and antioxidant responses. Low acute toxicity with half-lethal concentration (LC_{50}) > 100 mg/mL has been described for zebrafish (Xiong et al., 2011) and rainbow trout (*Oncorhynchus mykiss*) (Federici et al., 2007). However, prolonged exposure of fish to TiO_2-NPs induced biochemical and histopathological alterations in their gills, liver and intestines (Blaise et al., 2008; Boyle et al., 2013; Federici et al., 2007).

Studies with fish cell lines reported low cytotoxicity and genotoxicity of TiO_2-NPs for rainbow trout fibroblast-like gonadal cell line (RTG-2) (Vevers and Jha, 2008) and for goldfish skin cells (GFSk-S1) (Reeves et al., 2008), although this cytotoxicity and genotoxicity was enhanced in combination with direct ultraviolet radiation. However, although TiO_2-NPs are photoactive and produce reactive oxygen species (ROS) on illumination, this would only be applicable in environmentally-like exposure conditions. Thus, phototoxicity do not represent a relevant endpoint for *in vitro* testing approaches (Jovanovic, 2015). In short, some sub-lethal effects have been observed in diverse aquatic organisms after exposure to TiO_2-NPs, without reaching agreement on the mechanisms underlying this subtle toxic action. In addition, TiO_2-NPs risk assessment challenges are related to the lack of consistent toxicity data due to the high variety of materials tested and assay conditions.

In vitro cytotoxicity tests offer the advantage of allowing the rapid detection of potentially toxic concentrations of a given substance (chemical or nanomaterials) and studying mechanisms underlying toxic action. Taking all this into account, the main objective of the present work was to detect possible differences in the toxicity of a number of TiO_2-NPs towards rainbow trout hepatocytes and a variety of fish cell lines and to shed light on the possible cellular processes or structures that could be affected. With this approach, we wanted to avoid limitations, detected in previous works, related with the use of a single class of TiO_2-NPs or with a reduced number of cell types that could mask any possible effect of TiO_2-NPs. In the present study we used three types of TiO_2-NPs (all of them obtained from the EU Joint Research Centre, JRC, Reference Nanomaterials Repository) with different crystalline structures and properties: rutile hydrophobic, rutile hydrophilic and rutile-anatase TiO_2. Since the dispersion procedures can have a deep impact on the toxicity of any nanomaterial, due to variations in the final size of aggregates/ agglomerates and their stability (George et al., 2014; Lankoff et al., 2012; Prasad et al., 2014), dispersion protocols previously validated in European projects were applied. For the general cytotoxicity assays we used the NANOGENOTOX dispersion protocol (Jensen et al., 2011). Similarly, in order to increase the variability of our results, capturing any possible influence of cell type and origin on cytotoxicity, rainbow trout primary hepatocytes and various fish cell lines were utilized as *in vitro* models: the topminnow fish (*Poeciliopsis lucida*) hepatoma cell line (PLHC-1), the RTG-2 cell line, the rainbow trout hepatoma cell line (RTH-149) and the rainbow trout liver cell line derived from biliary preductural epithelial cells (RTL-W1). Cytotoxicity was evaluated with classical toxicity assays (alamarBlue, 5-Carboxyfluorescein diacetate-acetoxymethyl ester (CFDA-AM) and neutral red uptake, NRU) that inform about three different mechanisms of toxic action (alterations of cell metabolism, of plasma membrane integrity, and of lysosomal performance, respectively). To avoid variations related with the set of cells used, these assays were carried out on the same plate. For comparative purposes, further addressing the *in vitro* cytotoxic effects, we used rutile-anatase TiO_2 as reference nanomaterial, dispersed with a protocol established in a different EU project (NANOCARE dispersion protocol (Schulze et al., 2008)) and observed its cytotoxicity towards the mammalian rat hepatoma cell line (H4IIE) and the piscine RTG-2 cell line after 24 h and 72 h of exposure with two absorbance assays (MTT and NRU). Initial results showed high aggregation degree of the TiO_2-NPs and negligible cytotoxicity regardless type of cell line, exposure protocol and endpoint tested. Although cell lines are generally less sensitive than *in vivo* models, this information ultimately would be useful and pertinent in an integrated testing strategy generating information for TiO_2-NPs risk assessment.

2. Material and methods

2.1. Chemicals and reagents

All chemicals and reagents used were purchased from Sigma Aldrich (Madrid, Spain) unless otherwise stated. Fetal bovine serum (FBS), L-glutamine (200 mM), penicillin and streptomycin (P/S) (10,000 U/mL/ 10 mg/mL), phosphate-buffered saline (PBS), trypsin/ethylene diaminetetraacetic acid (EDTA) (17,000 U trypsin/L, 200 mg/l EDTA), non-essential amino acids (NEAA)100X, Eagle's minimal essential medium (EMEM), and Leibovitz's L-15 medium were purchased from Lonza (Barcelona, Spain). Serum-free/phenol red-free MEM (MEM(-)) was from PAN Biotech (Aidenbach, Germany). Ethanol was from Panreac (Barcelona, Spain). AlamarBlue® dye, GIBCO® Dulbeccos Phosphate Buffered Saline (DPBS) and the 5-Carboxyfluorescein diacetate-acetoxymethyl ester (CFDA-AM) probe were from Life Technologies (Madrid, Spain).

2.2. Cell culture and hepatocyte isolation

The hepatocellular carcinoma cell line PLHC-1 (derived from topminnow fish, *Poeciliopsis lucida*), the RTH-149 rainbow trout hepatoma cell line and the RTG-2 cell line, derived from rainbow trout (*Oncorhynchus mykiss*) gonadal tissue, were obtained from the American Type Culture Collection (ATTC) (Manassas, VA, USA). RTL-W1, a rainbow trout liver cell line that appears to be derived from biliary preductural epithelial cells (Malhão et al., 2013) was a generous gift from Dr. Bols (University of Waterloo) and Dr. Lee (University of Saskatchewan). H4IIE rat hepatoma cells were obtained from the European Collection of Animal Cell Cultures (ECACC) (Wiltshire, UK).

PLHC-1 were cultured in EMEM medium (Ref.:12–662, Lonza, Switzerland) containing 5% FBS, 1% P/S and 1% L-glutamine at 30 °C at a 5% CO_2 atmosphere (EMEM(5% FBS)). H4IIE, RTH-149 and RTG-2 were cultured in MEM Eagle (EMEM) media (Ref.:12–125, Lonza, Barcelona, Spain) with 1% L-Glutamine, 10% FBS, 1% P/S and additionally either 1% sodium pyruvate in the case of RTH-149 (EMEM(pyr)) or 1% NEAA for the H4IIE and RTG-2 cell lines (EMEM(NEAA)). The RTL-W1 cell line was cultured in L-15 medium supplemented with 1% L-glutamine and 1% P/S. The rat hepatoma cell line H4IIE was cultured in EMEM supplemented with 10% FBS, 1% P/S, 1% L-glutamine, and 1% NEAA at 37 °C in a humidified atmosphere of 5% CO_2. All rainbow trout derived cell lines were cultured at 20 °C and, in the case of RTH-149 and RTG-2 cell lines, under 5% CO_2 atmosphere. Cell cultures were maintained in 75 cm^2 culture flasks (Greiner Bio-one, CellStar, Frichenhausen, Germany) and routinely split one to two times per week using 0.5% trypsin/0.02% EDTA.

Hepatocyte isolation was done following a two-step perfusion method and collagenase digestion followed by mechanical dissociation of the liver tissue (Connolly et al., 2015; Navas and Segner, 2006; Segner, 1998). Briefly, primary hepatocytes were obtained from juvenile rainbow trout weighing on average 210 ± 5 g and measuring 22 ± 4 cm in length. Fish were sacrificed using ethyl 3-aminobenzoate methanesulfonate anaesthetic MS-222 (150 mg/L) and injected with liquid heparin (5000 U.I heparin /1 mL; Drossapharm AG/SA, Basel, Switzerland). Perfusion of the liver was attained through the portal vein and cannulated with a Venofix® A perfusion cannulae (15 × 0.5 mm length) (B. Braun Melsungen AG, Melsungen, Germany) and a peristaltic pump (ISMATEC® IPC High Precision Multichannel Dispenser, IDEX