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Engineered nanoparticles of titanium dioxide (TIO_2): Uptake and biological effects in a sea bass cell line

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ABSTRACT

With the rapid development of nanotechnology there has been a corresponding increase in the application of titanium dioxide nanoparticles (TiO_2-NPs) in various consumer and industrial products, consequently their potential health hazards and environmental effects are considered an aspect of great concern.

In the present study, in order to assess the impact of TiO_2-NPs in the marine environment, the biological effects of TiO_2-NPs on a sea bass cell line (DLEC) were investigated. Cells were exposed for 24 h to different concentrations of TiO_2-NPs (1, 8, 40, 200 and 1000 μg/ml) or co-exposed with $CdCl_2$ (Cd). The effects of UV light irradiation were also investigated in cells treated with TiO_2-NPs and/or Cd. The internalization of TiO_2-NPs and the morphological cell modifications induced by the treatments were examined by transmission and scanning electron microscopy, this latter coupled with energy dispersive X-ray spectroscopy (EDS) for particle element detection. In addition, the effects of controlled exposures were studied evaluating the cytotoxicity, the DNA damage and the expression of inflammatory genes.

Our study indicates that TiO_2-NPs were localized on the cell surface mainly as agglomerates revealed by EDS analysis and that they were uptaken by the cells inducing morphological changes. Photoactivation of TiO_2-NPs and/or co-exposure with Cd affects ATP levels and it contributes to induce acute cellular toxicity in DLEC cells dependent on Ti concentration. The inflammatory potential and the DNA damage, this latter displayed through a caspase-3 independent apoptotic process, were also demonstrated.

Overall our data suggest that the interaction of TiO_2-NPs with marine water contaminants, such as cadmium, and the UV irradiation, may be an additional threat to marine organisms.

1. Introduction

The production of nanomaterials is increasing worldwide. Engineered nanoparticles (ENPs) are used in diverse industrial fields [26] and new applications are constantly arising [9,71,90,106,109,124]. This means that the environmental contamination with ENPs is becoming a major issue [2,27]. In fact, there is a general concern about the potential hazards posed by released ENPs not only toward humans but also with respect to other organisms present in the environment [8,21,45,48,55,58,87].

Between these materials, the metal oxide nanoparticulate, and in particular the nanoparticles of titanium dioxide (TiO_2-NPs) are among those produced at the highest volume. TiO_2 is a versatile

List of abbreviations: ENPs, Engineered nanoparticles; TiO_2-NPs, titanium dioxide nanoparticles; TiO_2, Titanium dioxide; DLEC, sea bass continuous embryonic cell line; Cd, Cadmium Chlorid; EDS, energy-dispersive X-ray; ROS, reactive oxygen species; TEM, Transmission Electron Microscopy; FDA, fluorescein di-acetate; SEM, Scanning Electron Microscopy; HO, Hoechst; Ti, titanium; ATP, intracellular adenosine triphosphate.

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compound that is used in nano-form in a variety of consumer products, such as sunscreens and other cosmetics [122], specialist coatings and paints [39,56], food-processing technology [90] and in industrial photocatalytic processes [40,132]. Thus it has the great potential to be released into the aquatic environment, including surface waters that receive industrial and municipal effluents [60,93]. Moreover, we have to take into account that ENPs' behavior depends on the composition of water constituents; in fact particles may agglomerate and interact with the organic material and natural colloids present in these systems, which in turn will likely affect the ENPs potential ecotoxicity and their bioavailability to aquatic organisms [6,42]. However, relatively little is known about the magnitude, the fate and behavior of nanoparticles entering into the bodies of aquatic organisms and their subsequent biological effects [19,22,25,84,85,119] and regard the possible bioaccumulation in species used for human food.

Therefore, considering that the interaction of nanomaterials with cells can be regarded as a first step in the induction of possible health problems, some *in vitro* studies have focused on elucidating the uptake and biological effects of TiO_2-NPs in cell lines, being the *in vitro* systems the best experimental model for studying toxic mechanisms at the molecular and cellular levels in a controlled environment [11]. Common findings include general cytotoxicity [107,110], induction of an inflammatory response [103], as well as generation of free radicals [24], reactive oxygen species (ROS) and oxidative damage [74,103]. The studies have also shown the ability of TiO_2-NPs to cross cell membranes [34] and induce micronuclei formation and apoptosis [95].

In this context, in order to study the intrinsic hazard potential of TiO_2-NPs that may enter into fish from the aquatic environment, different biological effects of NP-TiO_2 on a sea bass continuous embryonic cell line (DLEC) [10] were investigated. Moreover, as the uptake and localization of nanoparticles are relevant for general cytotoxicity and induction of inflammatory responses, we examined the distribution and internalization of TiO_2-NPs in DLEC cells both by Scanning Electron Microscopy (SEM), coupled with an integrated energy-dispersive X-ray analyzer (EDS) for particle element detection, and by Transmission Electron Microscopy (TEM). In addition, as it is well known that the TiO_2-NPs may bind dangerous substances present in traces in marine water such as cadmium [46,130,133], and can absorb UV light [131], catalyzing the generation of ROS, such as superoxide anion radicals, hydrogen peroxide, free hydroxyl radicals, and singlet oxygen in aqueous media [49,63,64,107], the effects of controlled TiO_2-NPs exposure and combined treatment with UV light and/or $CdCl_2$ (Cd) were analyzed in term of quantitative parameters related to metabolic functions, morphological modifications, DNA damage and expression of some inflammatory related genes.

2. Materials and methods

2.1. Suspension of TiO_2-NPs

The suspension of the nanosized Titanium Dioxide (TiO_2-NPs), namely Aeroxide® (provided by Eigenmann & Veronelli, Milan, Italy; declared purity: 99.9%), was obtained according to the protocol described by Ref. [1]. Briefly, a stock suspension of 10 mg/ml of TiO_2-NPs, previously characterized by analytical [22] and morphological techniques [84], was added in FBS-free medium and sonicated for 1 min (VCX130, Vibra-Cell, 130 W, Sonics & Materials Inc., USA). The end-point concentrations of TiO_2-NPs for exposure were 1, 8, 40, 200 and 1000 μg/ml.

2.2. Cell culture and treatments

The DLEC cells, a continuous embryonic cell line established from sea bass (*Dicentrarchus labrax* L.) [10], were cultured in flasks (BD Falcon, Tissue culture treated, seal cap) at 22 °C in Leibovitz L-15 medium (Sigma-Aldrich) supplemented with 1% L-glutamine, 100 U/ml penicillin-streptomycin and 10% FBS. The cells were treated according to the experiment schedule which is designated in Table 1. $CdCl_2$ (Cd) 99% (Sigma-Aldrich) (0.1 μg/ml) nominal concentrations were chosen in accordance with a previous pilot study [84]. Differently, the intensity and the time of the UVA light exposition (30,000 $\mu W/cm^2$ for total 24 min) from five fluorescent 8-Watt UV-A lamps (365 nm) (Spectrolinker™ XL-1000A) were chosen in accordance with results shown in supplemental data section. The samples were analyzed by *ATPlite*™ assay, SEM, TEM, SCGE analysis and real time PCR as reported in Table 1. In particular, the study focused on 1 μg/ml TiO_2-NPs: this dose was chosen as it is far below the LC50 reported for fish species but still able to induce significant biological responses [127].

2.3. ATPlite assay

The intracellular adenosine triphosphate (ATP), widely accepted as a valid marker of viable cells, was measured by the *ATPlite*™ assay system (Perkin-Elmer), according to the manufacturer's instructions. DLEC cells were transferred (~10,000/well) to polystyrene 96-microwell plates (Perkin-Elmer) and cultured overnight at 22 °C in FBS-free L-15 medium, then the treatments were performed for 24 h as reported in Table 1. Controls were operated by changing the medium every two days to cultivate the cells at 22 °C (negative control) or by addition of 0.2% NaN_3 (positive control) for 24 h.

The ATP lite assay is based on the production of light caused by the chemical reaction of ATP with added luciferase and D-luciferin. The amount of emitted light, linearly correlated with ATP concentration [20], was measured with a luminometer (Victor II Perkin-Elmer) for 10 min in the dark. Five independent experiments and three replicates per treatment were performed.

2.4. Scanning electron microscopy (SEM) and microanalysis

For SEM analysis, cells (70,000) were seeded on sterile glass coverslips inserted in 24-well cell culture plates (IWAKI, Scitech Div. Asahi Techno Glass). The cells were cultured overnight at 22 °C in FBS-free L-15 medium and then exposed for 24 h to different treatments (Table 1). The control was obtained adding fresh FBS-free medium for 24 h.

After treatments, the samples were fixed overnight at 4 °C with 2.5% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 (all reagents from Sigma-Aldrich). Samples were washed four times with cacodylate buffer, then post-fixed with 1% osmium tetroxide (Sigma-Aldrich) and 0.15% ruthenium red (Sigma-Aldrich) in 0.1 M cacodylate buffer, pH 7.2, for 1 h at 4 °C. After different washings in distilled water, the samples were dehydrated with a graded acetone series (from 30% to 100%) and then dried with the critical point method, using CO_2 in a Balzers Union CPD 020. Dried coverslips were gold-coated in a sputtering unit equipped with an argon inlet (Balzer Union MD 010) for the observations by SEM (Jeol JSM 6010LA) (Tokyo, Japan). The identity of putative TiO_2-NPs was confirmed using the SEM (Jeol JSM 6010LA) (Tokyo, Japan) in combination with an integrated energy-dispersive X-ray analyzer (EDS) for particle element detection.