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Research Article

Combined In vitro Effects of TiO2 Nanoparticles and Dimethyl Sulfoxide (DMSO) on HepG2 Hepatocytes

Abstract

Introduction: Professional workers that manufacture or use titanium dioxide (TiO2)-based paints are exposed to potentially toxic TiO2 nanoparticles as well as to different paint solvents such as dimethyl sulfoxide (DMSO). In this context, we evaluate the combined cytotoxic effects of TiO2 nanoparticles and DMSO on HepG2 human hepatocytes.

Methods: Three types of TiO2 nanoparticles were used: commercial Degussa P25 and two samples synthesized by a hydrothermal procedure – undoped and Fe3+-doped TiO2. The effects of TiO2 nanoparticles on HepG2 cells exposed to DMSO before, after or together with the TiO2 treatment were investigated by viability and intracellular reactive oxygen species (ROS) determinations, performed using the MTT and DCFH-DA/2',7'-dichlorofluorescein-diacetate) methods respectively.

Results: Results indicated that DMSO made HepG2 cells more susceptible to toxic effects induced by nanosized TiO2. In the absence of DMSO, none of the tested nanoparticles exhibited significant cytotoxic effects. Viability increases were detected after 48 hours of treatment and attributed to possible redox-sensitive proliferation mechanism triggered by the low and moderate amounts of produced ROS. The combined action of TiO2 and DMSO led to a general viability decrease tendency. Significant effects (viability reductions and ROS generation) were observed in the case of cells first treated with Degussa P25 TiO2 and afterwards exposed to DMSO. The hydrothermal materials exhibited reduced in vitro reactivity on HepG2 hepatocytes.

Conclusion: The study reveals the enhancement of nanosized TiO2 toxicity induced by DMSO exposure, its findings having potential to help in the evaluation of professional health risks associated to the combined action of TiO2 nanoparticles and paint solvents.

Introduction

Recent studies regarding the widespread use of paints that contain titanium dioxide (TiO2, titania) nanoparticles for bacterial decontamination and self-cleaning purposes revealed significant advantages of these new technologies, but also potential health risks induced by the release of nanosized TiO2 from painted surfaces [1-4]. If the amount of released TiO2 was shown to be relatively small under the studied conditions [5], significant professional health risks may be associated to workers that manufacture or use TiO2-based paints. These workers are exposed to potentially toxic TiO2 nanoparticles as well as different solvents used during the processing or application of such paints. Among the used solvents, dimethyl sulfoxide (DMSO) was considered to be more convenient due to its relatively reduced toxicity [6,7]. However, DMSO is also known to easily penetrate human skin and, in some cases, to serve as carrier agent, promoting the percutaneous absorption of other compounds (including drugs and toxins) [8,9]. Under these circumstances, DMSO may facilitate the penetration of skin by TiO2 nanoparticles.

To evaluate the implications and hazards involved by the potential exposure to both, TiO2 nanoparticles and DMSO (or other paint solvent), it is essential to possess detailed knowledge regarding their combined effects on vital organs, such as brain, liver, heart, kidney, lung or spleen. Among these, the liver represents one of the most important organs involved in the processing of xenogenous compounds (including nanomaterials) and detoxification, a significant amount of published studies concerning the hepatotoxic effects of solvents (including DMSO in several cases) [10-13].

The present study gives an insight into the combined in vitro effects of TiO2 nanoparticles and DMSO (seen as a generic paint solvent).

Abbreviations


The experiments have been performed on HepG2 cells (human hepatocarcinoma cells-ATCC HB-8065™), a well characterized cell line, widely used in cytotoxicity studies due to its convenient specific characteristics, such as:

- biosynthetic capabilities similar to those of normal hepatocytes
- retention of cell surface receptors – response capacity similar to normal cells [14,15].

The studied effects concern the cytotoxicity and intracellular reactive oxygen species (ROS) production induced in HepG2 cells under different treatment schemes with nano-TiO₂ and DMSO.

We have used three types of TiO₂ nanoparticles: the commercial Degussa P25 TiO₂ (P25) and other two samples synthesized under hydrothermal conditions in our laboratory – undoped (HT) and Fe³⁺-doped (FeHT) anatase TiO₂. Degussa P25 TiO₂ was often tested (and utilized as reference material) in studies concerning intracellular ROS generation [16] and toxicity induced by titania nanomaterials [17-19]. The hydrothermal TiO₂ samples have similar structural characteristics (crystal structures, shapes, sizes and specific surface areas [20] – the relevance of these factors being frequently considered in TiO₂ nanotoxicology studies [21,22]), but different band gap energies (relative to each other) and colloidal behaviors (compared to Degussa P25) [20].

The obtained results are analyzed with respect to the structural and physicochemical properties of the tested nanomaterials [20], the characteristics of the used cells and the cell penetration and hydroxyl radical scavenger properties of DMSO [23].

Materials and Methods

Materials synthesis

The undoped and iron-doped TiO₂ nanoparticles were prepared starting from TiCl₄ (solution 15 % in HCl 10 %, from Merck) and FeO₂ (RITVERC 95.44 % ⁵⁷Fe Isotopic Enrichment) [20].

To obtain the Fe⁺⁺ (1 at. %)-doped TiO₂, the titanium and iron precursors were processed as follows: TiCl₄ was oxidized (by air barbotage) to TiCl₃, and FeO₂ was reacted to hydrochloric acid (4N) to form FeCl₂. The resulted solutions were involved in a coprecipitation process, NH₄OH being drop wise added to their mixture up to pH=8. The obtained precipitate was washed with deionised water, resuspended in double-distilled water and exposed to hydrothermal treatment in a 50 cm³ Teflon-lined autoclave at 200 °C for one hour. The undoped TiO₂ was synthesized using the same procedure involving the titanium precursor only.

Material characterization

The structural, morphological, optical and physicochemical characteristics of the used materials have been studied by X-ray diffraction (XRD), transmission electron microscopy (TEM), Energy-dispersive X-ray spectroscopy (EDX), Mössbauer spectroscopy, Brunauer-Emmett-Teller (BET) nitrogen adsorption, UV-Vis reflectance spectroscopy, Dynamic Light Scattering (DSL) and Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFT) and described in a previous work [20].

Cellular and noncellular experiments regarding the in vitro effects of TiO₂ nanoparticles on HepG2 hepatocytes pre-treated, co-treated or post-treated with DMSO

Cell viabilities and intracellular ROS productions in HepG2 hepatocyte cultures exposed to TiO₂ nanoparticles and DMSO have been performed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay and the DCFH-DA (2',7'-dichlorofluorescin-diacetate) test respectively.

The experiments were such designed to elucidate the following aspects:

- effects of the studied TiO₂ nanoparticles on hepatocytes that were “already damaged” – the cells were first treated with DMSO and two hours later exposed to the action of nano-TiO₂;
- viability and intracellular ROS production in case of HepG2 cells simultaneously treated with DMSO and nano-TiO₂;
- response of hepatocytes to the action of DMSO, administered two hours after the cells were exposed to nano-TiO₂;

1. The working protocol was established based on the following: LD50 for DMSO after 24 h of exposure – the DMSO dose capable of killing 50% of the cells after 24 h of exposure;
2. the effects of nano-TiO₂ alone (without DMSO) on the studied cells;

Cell treatment

The HepG2 cells were seeded in 24-well culture plates at a density of 10⁵ cells/cm² in volumes of 1 ml of DMEM-F12 culture medium containing 10% FBS. After 24 h required for cell adherence and growth, the culture medium was discarded and replaced with fresh medium (1 ml/well) containing the necessary treatment agents (TiO₂, 2.5, 7.5, 15, 25, 50, 75, 100 μg/ml), DMSO (LD50) or both) according to the stimulation scheme presented above. In case of pre-treatment or post-treatment with DMSO, either TiO₂ or DMSO, were added two hours after the initial stimulation.

Preliminary experiments have established the LD50 for DMSO to be 5 μl DMSO/1 ml of culture medium.

Cell viability assay

After 24, 48 and 72 h of treatment, the culture medium was removed and MTT solution (1 mg/ml MTT in phosphate buffered saline (PBS)) was added to each well (300μl/well). The obtained samples were incubated for 2 hours, the MTT solution being afterwards discarded. To dissolve the produced formazan crystals, DMSO (Sigma-Aldrich) was added to each well (300μl/well). The optical density of the purple formazan solution was determined at 540 nm using a Thermo Multiskan EX spectrophotometer.

The obtained results were quantified with respect to control samples consisting of untreated cells (in case of experiments in which TiO₂ alone was used) or cells treated with DMSO (in case of experiments involving both TiO₂ and DMSO). Cell viability was expressed as percents versus control. Standard deviations were
Determination of intracellular ROS production

To quantify the intracellular ROS production, the cells were incubated for 24 hours with the treating agents (TiO₂, 2.5, 25, 50, 100 μg/ml), DMSO (LD50) or both. After incubation, the culture medium was removed and replaced with fresh medium containing DCFH-DA (0.2 μl of 2’-7’-dichlorofluorescein-diacetate (DCFH-DA) stock solution (25 mg/ml DCFH-DA in TFS) in 1 ml of culture medium). The obtained samples were incubated again for 30 minutes. The DCFH-DA medium was afterwards removed; the cells were detached with trypsin (0.25% trypsin and 0.53 mM EDTA solution), suspended in PBS and centrifuged for 10 min at 1500 rpm and 4°C, the supernatant being discarded. The excess of fluorescein was removed by washing the cells twice in PBS and the cells were resuspended in 500 μl PBS. The homogenized suspensions were transferred to 96-well plates (100μl/well). A Fluoroskan FL (Thermo) equipment (excitation wavelength 485 nm/emission wavelength 530 nm) was used to perform the fluorimetric determinations.

The obtained results were quantified with respect to control samples (described above for cell viability experiments) and expressed as percents versus control. Standard deviations were computed based on three technical replicates corresponding to each sample and three independent biological replicates of each experiment.

Data analysis and representation

Data statistical analysis and representation were performed using the Sigma Plot-11 software package. Depending on data normality, either one-way ANOVA or one-way ANOVA on ranks tests were performed. The Student–Newman–Keuls (SNK) posthoc test was employed in order to complete the analysis. A value of p < 0.05 was considered significant. All samples statistically different from controls were marked on figures with a (*).

Results

Materials characterization

The detailed structural and physicochemical characterization of the three TiO₂ nanomaterials used in this study was published in a previous work [20]. Briefly, all three types of titania have similar shapes (no acicular shaped particles) and average particle sizes between 10-30 nm. The hydrothermal, HT and FeHT, samples have anatase structure and Degussa P25 TiO₂ is a mixture of anatase and rutile polymorphs with anatase/rutile weight ratio of 85:15(%). BET specific surface areas are 49 m²/g for Degussa P25, 130.62 m²/g for HT and 114.81 m²/g for FeHT. The band gap energies were approximately
3 eV for Degussa P25 and HT and 2.848 eV for the iron doped, FeHT, sample. Degussa P25 has considerably higher colloidal stability in aqueous suspensions compared to HT and FeHT. The colloidal stabilization effect of proteins from culture medium was revealed.

**In vitro effects of undoped and Fe³⁺-doped TiO₂ nanoparticles on HepG2 hepatocytes**

The results obtained in this study are displayed for each of the tested nanomaterials (P25, HT and FeHT) in Figures 1-4.

**Cell viability**

**TiO₂ alone:** Cell viabilities obtained in experiments involving TiO₂ alone (no DMSO) are represented in Figure 1a-1c. No significant viability variations were observed after 24h and 72h of treatment for none of the tested TiO₂ samples. At 48h after exposure, viability increases (up to 49 %) were detected for all nanomaterials, being more pronounced in case of FeHT. The observed increases were proportional to the concentration of TiO₂ in the studied samples.

To better illustrate the specific features of each experimental case
(pre-, co- or post-treatment with DMSO) and each type of tested TiO$_2$, the results are described and discussed comparatively below.

**TiO$_2$ and DMSO:** While for all the tested materials, the most prominent viability reductions were observed in the case of cells post-treated with DMSO (TiO$_2$-DMSO) (Figures 2a-4a), the highest cell killing effect was induced by Degussa P25 nanoparticles (Figure 2a). The hydrothermal materials (HT and FeHT) induced only weak or insignificant cytotoxic effects.

On the other hand, no significant cellular effects were detected in the case of cells simultaneously exposed to TiO$_2$ and DMSO (TiO$_2$+DMSO) (Figures 2b-4b).

The observed viability variations did not depend on the treatment time (24, 48, 72 hours), for none of the tested TiO$_2$ types.

**Intracellular ROS production**

**TiO$_2$ alone:** Regarding the intracellular ROS production,
significant increases (between 21-39%) were induced by the iron-doped sample (Figure 1d). The commercial P25 TiO$_2$ induced a significant increase only at its maximum concentration (100 µg/ml). The observed increases were concentration-dependent. No variation was detected in case of HT sample (Figure 1d).

**TiO$_2$ and DMSO:** Small or moderate increases in the intracellular ROS levels of the treated cells were observed in all experimental cases (Figures 2(d-f)-4(d-f)), being more pronounced in the case of DMSO post-treatment (TiO$_2$-DMSO) (Figures 2d-4d). Although the highest ROS production was determined for Degussa P25 TiO$_2$, corresponding to the TiO$_2$-DMSO case, no clear distinction can generally be made between the pro-oxidative effects of commercial and hydrothermal samples.

The determined ROS production increases were, in most cases, proportional to the concentration of TiO$_2$.

**Discussion**

To ensure the validity of the obtained results, one should...
consider the possible interferences that may occur between the studied nanomaterials and the biochemical methods used through the performed study. Being a known photocatalyst [24-29], TiO$_2$ may interfere with MTT and induce experimental artifacts, as described by Lupu and Popescu [30]. This hypothesis was tested in noncellular experiments for each of the studied TiO$_2$ samples. Only one experimental case (Degussa P25, no DMSO) revealed weak TiO$_2$ (P25)-MTT interferences (data not shown). This effect was considered in data analysis in a manner similar to that described by Lupu and Popescu [30].

The cell viability results obtained in experiments involving TiO$_2$ alone revealed the lack of toxicity of the three tested nanomaterials on HepG2 cells, under the experimental conditions of the present study. The proliferation effects observed after 48 hours of TiO$_2$ treatment suggest the possible involvement of redox-sensitive cell proliferation mechanisms [31-37], triggered by the low and moderate levels of intracellular ROS production induced by the action of nano-TiO$_2$. One such ROS species is H$_2$O$_2$, which is known to either promote cell proliferation or induce cell cycle arrest, as a function of its concentration in the studied system [35,38-45]. In this context, it is important to note that H$_2$O$_2$ is among the characteristic oxygen species detected by the DCFH-DA method [46-48], its formation and proliferative effects being thus likely to occur in our study. Moreover, the DCFH-DA test was performed after 24h of TiO$_2$ treatment, the detected ROS generation being a plausible cause for the cell proliferation effect observed after 48h of treatment. After longer times however, cell viability is reduced by the oxidative environment. The onset of ROS effects (either cell proliferation or toxic effects due to oxidative stress) requires more or less time to occur, depending on the type and amount of generated ROS. Under the experimental conditions of our study, the effects of ROS produced during the first 24h of TiO$_2$ treatment and later became significant after longer times, being determined after 48h and 72h respectively. The increased amounts of ROS generated at later times induced the viability decrease observed after 72h of treatment.

Although the mechanisms by which TiO$_2$ nanoparticles induce intracellular ROS formation are not well understood, in the case of HepG2 cells, ROS overproduction may be favored by their tumor nature [49,50] and/or their role in detoxification [51,52].

Regarding the combined effects of TiO$_2$ and DMSO on HepG2 cells, the results presented above (Figures 2-4) are displayed with respect to control samples containing DMSO (LD50). Thus, the observed variations in viability or intracellular ROS production describe only the effects of TiO$_2$ nanoparticles on cells exposed to LD50 of DMSO. To understand how DMSO exposure influences the action of TiO$_2$ on the studied cells, these results should be analyzed in comparison to those obtained on cells that were not exposed to DMSO (Figure 1). The comparison indicates that DMSO exposure makes HepG2 cells more susceptible to toxic effects induced by nanosized TiO$_2$ and afterwards treated with DMSO (the TiO$_2$-DMSO case (Figure 2a)). Not only the highest viability reduction but also the highest intracellular ROS production was associated to the TiO$_2$-DMSO experiment, this finding suggesting that the observed effects were dictated by the early action of TiO$_2$ and associated to oxidative stress.

A possible mechanism to account for the enhanced toxicity of TiO$_2$, in the case of DMSO treated cells involves autophagy, which may be induced by both TiO$_2$ [53,54] and DMSO [55]. TiO$_2$ was also reported to induce lysosome membrane permeabilization, which represents a well known cell death mechanism (including lysosomal-mediated oxidative stress) [54].

The attenuated cellular effects observed either in the case of pre-treatment (TiO$_2$+DMSO) or pre-treatment with DMSO (DMSO-TiO$_2$) may indicate a possible interaction between TiO$_2$ and DMSO, leading to the attenuation of the damaging effects of TiO$_2$ (especially of Degussa P25). This hypothesis is supported by two aspects: the known photocatalytic properties of TiO$_2$ and the known hydroxyl radical scavenger properties of DMSO [56-58]. In principle, by photocatalytic processes, TiO$_2$ nanoparticles may generate hydroxyl radicals on their surface and induce oxidation reactions. Also, in principle, DMSO may act as scavenger for these radicals and reduce their oxidative action. However, it is unfortunately not possible to accurately test, at least not in a straightforward manner, whether these processes can occur under relevant in vitro conditions.

All experiments performed in this study confirm the reduced toxicity of the hydrothermal TiO$_2$ samples (HT and FeHT) to HepG2 hepatocytes. Besides identifying nanomaterials with low toxicity, our study points towards the risks involved by the combined exposure to DMSO and nano-TiO$_2$. Results also reveal the importance of material properties (other than chemical composition) for their biological effects. Regarding the hydrothermal TiO$_2$ samples, although they exhibit larger surface specific area than Degussa P25, their in vitro reactivity appears to be reduced. This may be related to their different surface charge properties in culture media as well as ROS photogeneration capacities and hydrophilicity [20].

To clearly establish the relation between the in vitro reactivity of the tested TiO$_2$ nanomaterials and their complex physicochemical properties, further studies are required.

**Conclusion**

The described study gives an insight into the combined in vitro effects of TiO$_2$ nanoparticles and DMSO (seen as a generic paint solvent) on human hepatocytes. The observed effects were shown to depend on the properties of TiO$_2$ and the characteristics of the exposure. Results indicated that DMSO makes HepG2 cells more susceptible to toxic effects induced by nanosized TiO$_2$. These findings may help in the evaluation of professional health risks associated to workers that manufacture or use TiO$_2$-based paints.

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