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Andreea R Lupu[1,2#*], Lidia Cremer[2] and Traian Popescu[3#]

[1]Immunobiology Laboratory, "Victor Babes" National Institute of Pathology, Bucharest, Romania
[2]Immunomodulation Group, "Cantacuzino" National Institute for Research/Development in Microbiology and Immunology, Bucharest, Romania
[3]LASDAM Laboratory, National Institute of Materials Physics, Bucharest, Romania
[#]These authors contributed equally to this work

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***Corresponding author:** Andreea-Roxana Lupu, Immunobiology Laboratory, "Victor Babes" National Institute of Pathology, Bucharest, Romania; Tel: +40213192732; E-mail: andreea.lupu@ivb.ro

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Research Article

Combined *In vitro* Effects of TiO_2 Nanoparticles and Dimethyl Sulfoxide (DMSO) on $HepG_2$ Hepatocytes

Abstract

Introduction: Professional workers that manufacture or use titanium dioxide (TiO_2)-based paints are exposed to potentially toxic TiO_2 nanomaterials as well as to different paint solvents such as dimethyl sulfoxide (DMSO). In this context, we evaluate the combined cytotoxic effects of TiO_2 nanoparticles and DMSO on $HepG_2$ human hepatocytes.

Methods: Three types of TiO_2 nanoparticles were used: commercial Degussa P25 and two samples synthesized by a hydrothermal procedure – undoped and Fe^{3+}-doped TiO_2. The effects of TiO_2 nanoparticles on HepG2 cells exposed to DMSO before, after or together with the TiO_2 treatment were investigated by viability and intracellular reactive oxygen species (ROS) determinations, performed using the MTT and DCFH-DA(2',7'-dichlorfluorescein-diacetate) methods respectively.

Results: Results indicated that DMSO made HepG2 cells more susceptible to toxic effects induced by nanosized TiO_2. 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 striggered by the low and moderate amounts of produced ROS. The combined action of TiO_2 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 TiO_2 and afterwards exposed to DMSO. The hydrothermal materials exhibited reduced *in vitro* reactivity on $HepG_2$hepatocytes.

Conclusion: The study reveals the enhancement of nanosized TiO_2 toxicity induced by DMSO exposure, its findings having potential to help in the evaluation of professional health risks associated to the combined action of TiO_2 nanomaterials and paint solvents.

Abbreviations

BET: Brunauer-Emmett-Teller; DCFH-DA: 2'-7'-dichlorofluorescein-diacetate; DLS: Dynamic Light Scattering; DMEM-F12: Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12;DMSO: dimethyl sulfoxide; DRIFT: Diffuse Reflectance Infrared Fourier Transform Spectroscopy; EDX: Energy-dispersive X-ray Spectroscopy; FBS: Fetal Bovine Serum; FeHT: Fe^{3+}-doped anatase TiO_2 nanoparticles synthesized under hydrothermal conditions; HT: undoped anatase TiO_2 nanoparticles synthesized under hydrothermal conditions; LD50: lethal dose 50%;MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; P25: Degussa P25 TiO_2 nanoparticles; PBS: Phosphate-buffered Saline; ROS: Reactive Oxygen Species; TEM: Transmission Electron Microscopy; XRD: X-ray Diffraction

Introduction

Recent studies regarding the widespread use of paints that contain titanium dioxide (TiO_2, 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 TiO_2 from painted surfaces [1-4]. If the amount of released TiO_2 was shown to be relatively small under the studied conditions [5], significant professional health risks may be associated to workers that manufacture or use TiO_2-based paints. These workers are exposed to potentially toxic TiO_2 nanomaterials 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 TiO_2 nanoparticles.

To evaluate the implications and hazards involved by the potential exposure to both, TiO_2 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 exogenous 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 TiO_2 nanoparticles and DMSO (seen as a generic paint solvent).

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
- retainment 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-TiO_2 and DMSO.

We have used three types of TiO_2 nanoparticles: the commercial Degussa P25 TiO_2 (P25) and other two samples synthesized under hydrothermal conditions in our laboratory – undoped (HT) and Fe^{3+}-doped (FeHT) anatase TiO_2. Degussa P25 TiO_2 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 TiO_2 samples have similar structural characteristics (crystal structures, shapes, sizes and specific surface areas [20] – the relevance of these factors being frequently considered in TiO_2 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 TiO_2 nanoparticles were prepared starting from $TiCl_3$ (solution 15 % in HCl 10 %, from Merck) and Fe_2O_3 (RITVERC 95.44 % ^{57}Fe Isotopic Enrichment) [20].

To obtain the Fe^{3+} (1 at. %)-doped TiO_2, the titanium and iron precursors were processed as follows: $TiCl_3$ was oxidized (by air barbotage) to $TiCl_4$ and Fe_2O_3 was reacted to hydrochloric acid (4N) to form $FeCl_3$. The resulted solutions were involved in a coprecipitation process, NH_4OH 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 TiO_2 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 (DLS) 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 TiO_2 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 TiO_2 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'-dichlorfluorescein-diacetate) test respectively.

The experiments were such designed to elucidate the following aspects:

- effects of the studied TiO_2 nanoparticles on hepatocytes that were "already damaged" – the cells were first treated with DMSO and two hours later exposed to the action of nano-TiO_2;

- viability and intracellular ROS production in case of HepG2 cells simultaneously treated with DMSO and nano-TiO_2;

- response of hepatocytes to the action of DMSO, administered two hours after the cells were exposed to nano-TiO_2;

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-TiO_2 alone (without DMSO) on the studied cells;

Cell treatment

The HepG2 cells were seeded in 24-well culture plates at a density of 10^5 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 (TiO_2 (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 TiO_2 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 TiO_2 alone was used) or cells treated with DMSO (in case of experiments involving both TiO_2 and DMSO). Cell viability was 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.

The results are represented as average values +/- standard deviations (error bars).

Determination of intracellular ROS production

To quantify the intracellular ROS production, the cells were incubated for 24 hours with the treating agents (TiO_2 (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.

The results are represented as average values +/- standard deviations (error bars).

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 TiO_2 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 TiO_2 is a mixture of anatase and rutile polymorphs with anatase/rutile weight ratio of 85:15(%). BET specific surface areas are 49 m^2/g for Degussa P25, 130.62 m^2/g for HT and 114.81 m^2/g for FeHT. The band gap energies were approximately

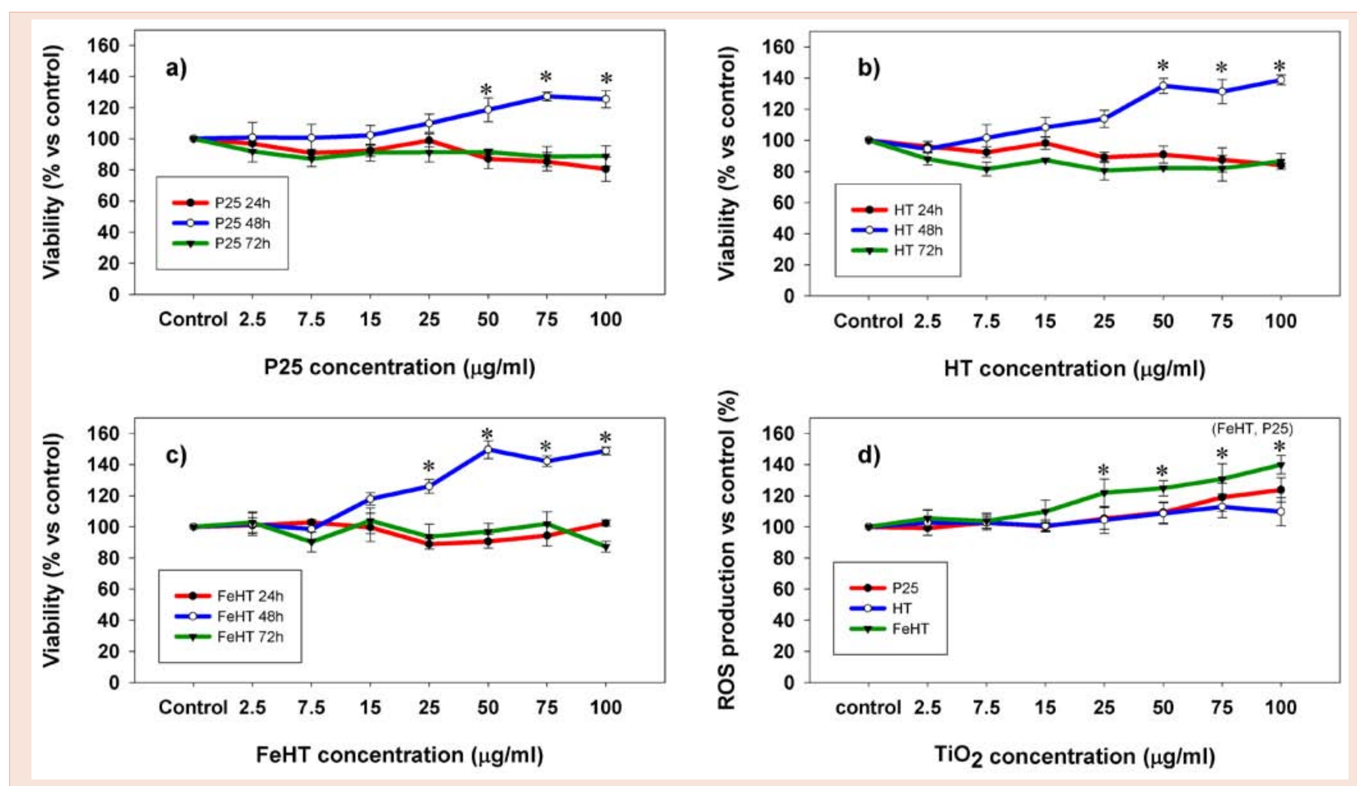

Figure 1: Cell viability (a-c) and intracellular ROS production (d) for HepG2 treated with P25, HT and FeHT; (*) - significant differences with respect to control (*p < 0.05* - calculated based on three biological replicates).

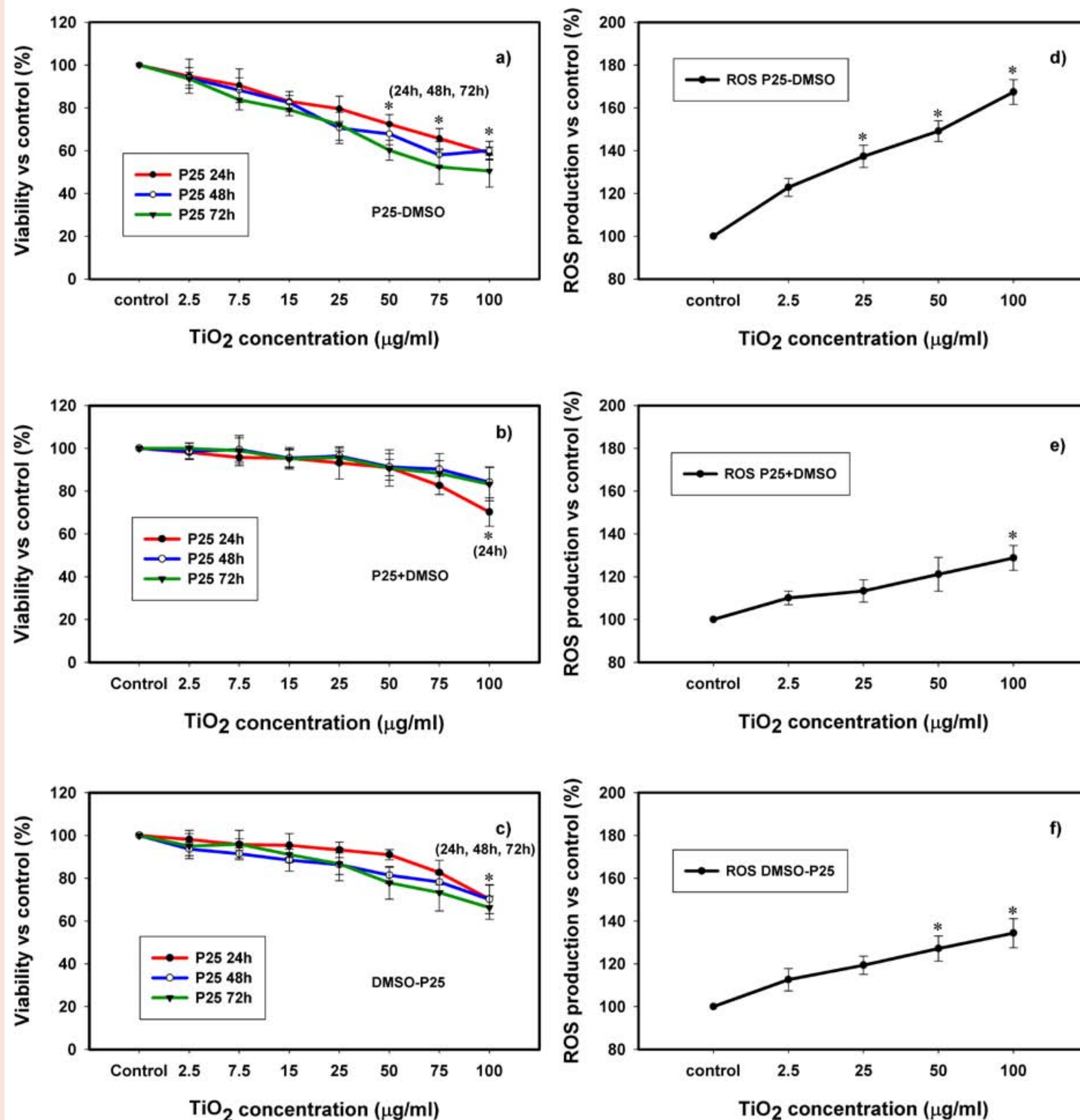

Figure 2: Cell viability (a-c) and intracellular ROS production (d-f) for HepG2 cells treated with Degussa P25 nanoparticles and DMSO; (*) - significant differences with respect to control (*$p < 0.05$* - calculated based on three biological replicates). The (*) symbols placed above or below particular data points refer to all cases (treatment times) specified in the brackets.

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 Fe^{3+}-doped TiO_2 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

TiO_2 alone: Cell viabilities obtained in experiments involving TiO_2 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 TiO_2 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 TiO_2 in the studied samples.

To better illustrate the specific features of each experimental case

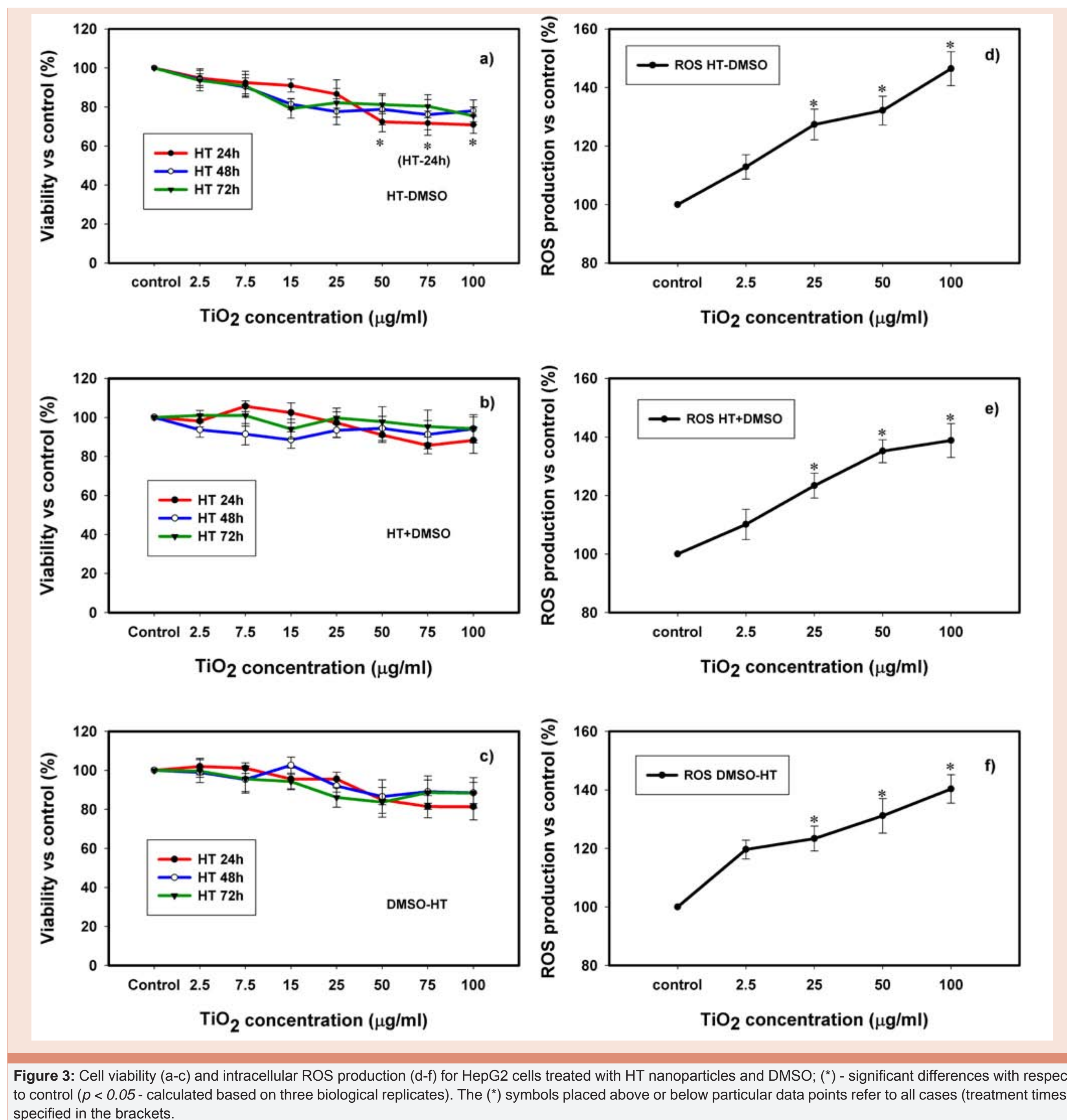

Figure 3: Cell viability (a-c) and intracellular ROS production (d-f) for HepG2 cells treated with HT nanoparticles and DMSO; (*) - significant differences with respect to control ($p < 0.05$ - calculated based on three biological replicates). The (*) symbols placed above or below particular data points refer to all cases (treatment times) specified in the brackets.

(pre-, co- or post-treatment with DMSO) and each type of tested TiO_2, the results are described and discussed comparatively bellow.

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

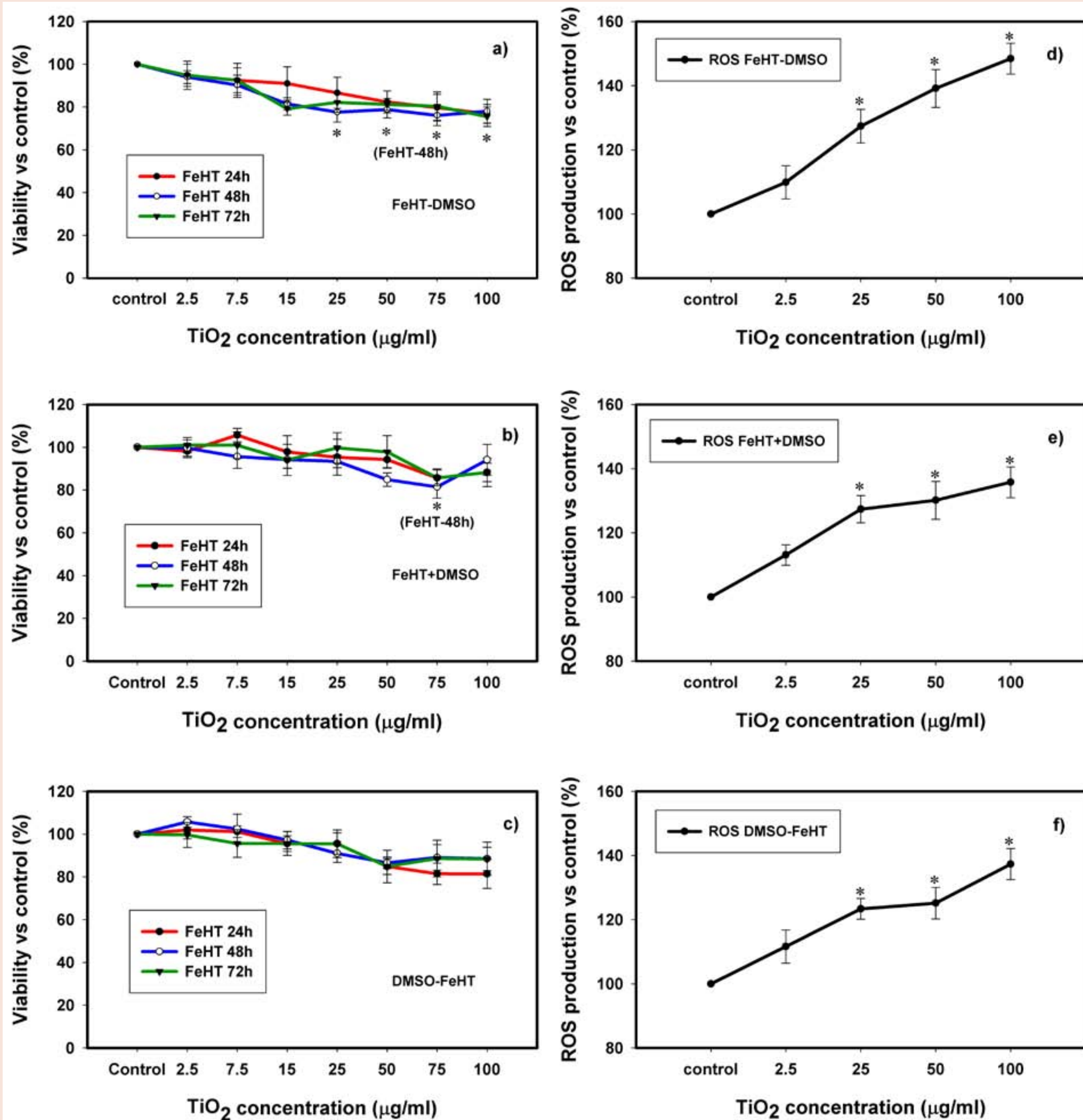

Figure 4: Cell viability (a-c) and intracellular ROS production (d-f) for HepG2 cells treated with FeHT nanoparticles and DMSO; (*) - significant differences with respect to control ($p < 0.05$ - calculated based on three biological replicates). The (*) symbols placed above or below particular data points refer to all cases (treatment times) specified in the brackets.

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_2O_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_2O_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 al 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.

In this view, cells exposed to DMSO show no proliferation effects induced by the tested TiO_2. Although a general tendency towards viability reductions can be observed, only Degussa P25 produces consistent (for all treatment times), concentration-dependent toxic effects, mainly visible when cells are first exposed to 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-iron mediated oxidative stress) [54].

The attenuated cellular effects observed either in the case of co-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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