Palmer BC, et al., J Toxicol Cur Res 2020, 4: 019
DOI: 10.24966/TCR-3735/100019

HSOA Journal of Toxicology: Current Research

Review Article

Morphology-Dependent Titanium Dioxide Nanoparticle-Induced Keratinocyte Toxicity and Exacerbation of Allergic Contact Dermatitis

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Abstract

Titanium Dioxide (TiO_2) nanoparticles are commonly found in consumer products, such as sunscreens, and human dermal exposures are relatively high. Research suggests potential differences in the toxicity of anatase and rutile crystalline forms of TiO_2. Additionally, transition metal dopants are frequently used to enhance physicochemical properties of TiO_2 and the toxicity of these nanoparticles is not extensively studied. Therefore, this work examined the keratinocyte toxicity and *in vivo* skin allergy responses after treatment with 30 nm anatase, 30 nm rutile, or <100 nm Mn-doped TiO_2 nanoparticles. After a 24-hour exposure, there were no differences in keratinocyte cytotoxicity; however, Mn-doped TiO_2 nanoparticles induced significant *in vitro* ROS generation and *in vivo* skin swelling responses in a model of allergic contact dermatitis.

Introduction

Titanium dioxide crystalline particles have been used as food and consumer product colorants for decades [1]. The unique photochemical properties of TiO_2 particles are also utilized in solar cells, water/air remediation technologies, and sunscreens [2-6]. The two most common crystalline forms of TiO_2 are anatase and rutile [7]. Furthermore, transition metal dopants, such as manganese, are commonly added during the TiO_2 manufacturing process to modify physicochemical properties [8-13]. These occupationally relevant, metal-doped nanomaterials are not typically encountered outside of industrial settings. Alternatively, consumer dermal exposures to anatase and rutile TiO_2 in sunscreens are relatively high [1]. The use of TiO_2-based sunscreens was approved by the United States Food and Drug Administration (US FDA) in 1999 [14]. Modern inorganic ultraviolet light filters are nano-sized to provide a more attractive cosmetic appearance, compared to the opaque look of micron-sized particles. Due to increasing human exposures to TiO_2 nanoparticles (NPs), research into the potential dermal toxicity of TiO_2 NPs has expanded.

Variations in the crystal structure of anatase and rutile TiO_2 NPs lead to differences in photochemical properties and cytotoxicity [7]. Multiple studies have observed increased cytotoxicity of anatase TiO_2 NPs, compared to the rutile morphology [15,16]. Specifically, Sayes et al. [17], exposed human dermal fibroblasts to 3-5 nm anatase or rutile TiO_2 NPs, and observed increased cytotoxicity in anatase treated samples [17]. These findings are often associated with increases in intracellular reactive oxygen species (ROS) production. Furthermore, the interaction of TiO_2 NPs with ultraviolet radiation increases ROS generation and leads to decreases in keratinocyte viability, which is often markedly higher in anatase TiO_2-induced phototoxicity reactions [18-20]. While these observations are common in *in vitro* models, *in vivo* TiO_2 dermal toxicity is less common, due to limited NP skin penetration [21-23]. Therefore, the uses of inorganic ultraviolet light filters are still recommended by most physicians and government agencies, despite the toxicological concerns surrounding TiO_2 NPs [24].

There are relatively few studies examining TiO_2 toxicity in both healthy skin and models of dermatitis. The skin barrier defect induced by irritant or allergic contact dermatitis could enhance NP interaction with viable skin cells and lead to dermal toxicity. Additionally, there are few toxicological studies of topically applied metal-doped TiO_2 NPs, which may be relevant for occupational exposures. Previously, our lab has observed exacerbation of 2, 4-dinitrofluorobenzene induced ear swelling responses after topical application of Mn-doped TiO_2 NPs [25]. The work presented here builds on those previous findings, and compares the keratinocyte toxicity and ear swelling responses of pure anatase, pure rutile, or Mn-doped TiO_2 NPs in both *in vitro* and *in vivo* models.

Results

TiO_2 physical characterization

The toxicities of anatase, rutile, or anatase dominant transition metal-doped TiO_2 NPs were compared in multiple *in vitro* and *in vivo* assays. The anatase and rutile TiO_2 NPs both had vendor reported physical diameters of 30 nm. However, the hydrodynamic diameters of the anatase and rutile NPs were

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Citation: Palmer BC, De Louise LA (2020) Morphology-Dependent Titanium Dioxide Nanoparticle-Induced Keratinocyte Toxicity and Exacerbation of Allergic Contact Dermatitis. J Toxicol Cur Res 4: 019.

Received: June 30, 2020; **Accepted:** July 10, 2020; **Published:** July 17, 2020

1170 ± 130.4 and 474.5 ± 8.2 nm, respectively (Table 1). Hydrodynamic diameters were markedly higher than the reported physical dimensions, and the relatively high polydispersity indices suggest a high degree of agglomeration in the water dispersant. Similarly, evidence of possible agglomeration was observed for the <100 nm manganese-doped TiO_2 NPs as previously reported [26]. All of the TiO_2 NPs had negative zeta potentials in the water dispersant, and the measurements ranged from -9.05 to -22.3 mV (Table 1). While particle interactions visualized via TEM imaging after NP deposition and drying may not accurately reflect particle agglomeration in a dispersant, a high degree of particle co-localization was observed in TEM images (Figure 1). Furthermore, the primary NPs displayed variations in morphology (Figure 1 and Figure S1), but the primary sizes appeared close to vendor reported dimensions.

Nanoparticle	Hydrodynamic Diameter (nm)	Polydispersity Index	Zeta Potential (mV)
Mn Doped (A/R)	556.40 ± 33.36	0.296	-9.05 ± 1.16
Anatase (A)	1170 ± 130.40	0.95 ± 0.06	-14.2 ± 0.75
Rutile (R)	474.47 ± 8.18	0.40 ± 0.03	-22.27 ± 0.78

Table 1: TiO_2 physical characterization by dynamic light scattering

Dilute dispersions of TiO_2 nanoparticles in water were analyzed in a Malvern Zetasizer. The hydrodynamic diameter, polydispersity indices, and zeta potentials are all reported (mean ± SD, n = 3).

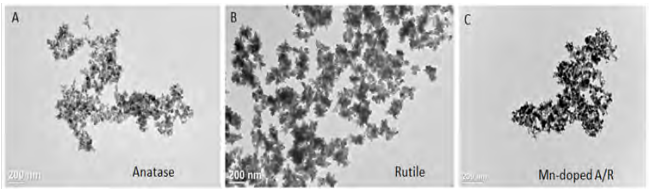

Figure 1: TiO_2 physical characterization by TEM.

TiO_2 nanoparticles were dispersed in acetone/water prior to deposition on grids for TEM analysis. The images of anatase (A), rutile (B), and 1% Mn-doped (C) particles show evidence for agglomeration. Scale bar 200 nm.

TiO_2 induced keratinocyte cytotoxicity and ROS generation

HaCaTs are an immortalized human keratinocyte cell line that is commonly used for *in vitro* studies of skin toxicity or irritation. In all TiO_2 NP experiments, the particles were dispersed in water prior to addition to fetal bovine serum supplemented cell culture media. The cells were exposed to 0, 5, 10, 50, 100, or 500 µg/mL TiO_2 NPs for 24 hours, after which an ATP indicator was used to assess cell viability. Results show that a 24 hour exposure (Figure 2) the TiO_2 NPs caused dose-dependent decreases in viability. The increased cytotoxicity became significant at 50 µg/mL, and there was no observed difference in the cytotoxicity induced by anatase, rutile, or Mn-doped TiO_2 NPs. Conversely, there were compositional-dependent differences in ROS generation, measured with a fluorescent ROS indicator, after 24 hours. While both pure anatase and rutile particles induced mild oxidative stress, the Mn-doped TiO_2 NPs markedly increased ROS generation at high-concentration exposures (Figure 3). To examine whether the increased ROS production observed at the 24-hour time point would induce a delayed cytotoxic response, a 48-hour cell viability assay was conducted. However, no differences in cytotoxicity were observed for any of the three TiO_2 NPs tested (Figure S2). As mentioned previously, *in vitro* keratinocyte cytotoxicity could be indicative of potential dermal toxicity. Therefore, these TiO_2 NPs were exposed to murine skin in healthy or allergic contact dermatitis models.

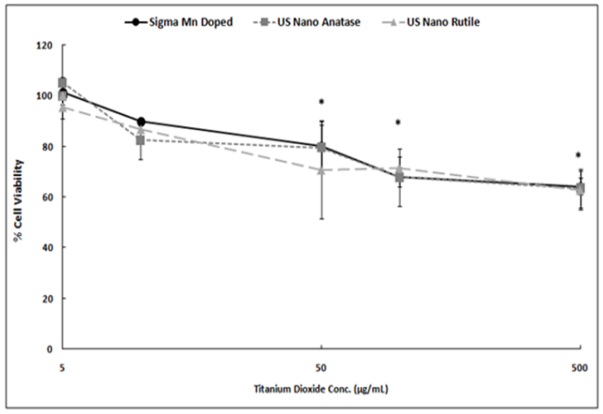

Figure 2: TiO_2 induced keratinocyte cytotoxicity after 24 hours.

HaCaTs were exposed to 0, 5, 10, 50, 100, or 500 µg/mL concentrations of anatase, rutile, or 1% Mn-doped TiO_2 nanoparticles for 24 hours. Dose-dependent decreases in cell viability were observed for all TiO_2 particles tested (mean ± SD, n = 3).

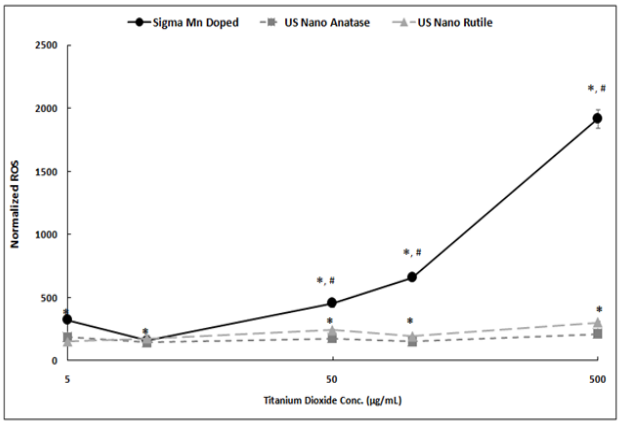

Figure 3: TiO_2 induced keratinocyte ROS generation after 24 hours.

HaCaTs were exposed to 0, 5, 10, 50, 100, or 500 µg/mL concentrations of anatase, rutile, or 1 % Mn-doped TiO2 nanoparticles for 24 hours. While there were significant increases in ROS for all TiO_2 nanoparticles, Sigma 1 % Mn-doped TiO_2-induced ROS generation was markedly higher than either pure TiO2 NP (mean ± SD, n = 3).

TiO_2 exacerbation of DNFB-induced allergic contact dermatitis

Our lab has previously observed the exacerbation of 2, 4-dinitrofluorobenzene (DNFB)-induced ear swelling in allergic contact hypersensitivity (CHS) mouse models after topical exposure to Mn-doped TiO_2 NPs [25]. To confirm that this effect occurs at concentrations that are relevant to human exposures, the ear swelling responses of all three TiO_2 NPs were examined *in vivo*. The allergic contact dermatitis mouse model consisted of topical DNFB exposures to hairless

mice backcrossed onto C57BL/6 mice for 6 generations. The mice were sensitized with 0.05% DNFB on the dorsum five days prior to challenge with 0.2% DNFB on the ears. The right ears of each mouse were exposed to only DNFB, and the left ears were coexposed to DNFB plus 200 µg of TiO_2 NPs. Additionally, a set of mice received only vehicle to examine the effects of TiO_2 NPs alone. The 200 µg dose was selected based on current FDA recommendations for sunscreen application (2 mg/cm^2) and a typical concentration of TiO_2 in UVR filters in commercial sunscreens (10% by weight). Results showed there was no effect of the TiO_2 NPs on ear swelling without DNFB, which indicates that these particles are not skin irritants. However, the Mn-doped TiO_2 NPs significantly increased the level of DNFB-induced ear swelling (Figure 4) as previously observed, albeit as a different dose [25].

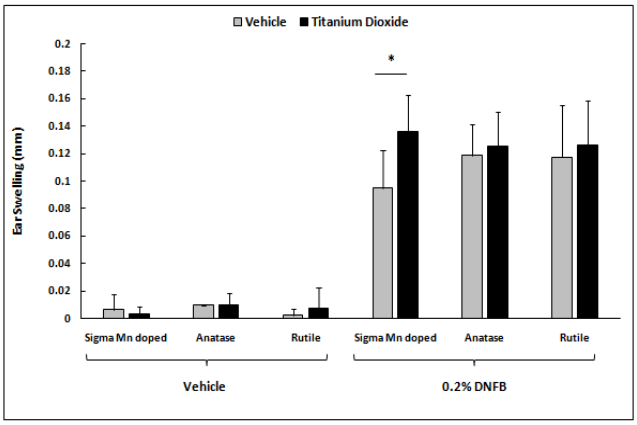

Figure 4: TiO_2 exacerbation of DNFB-induced allergic contact dermatitis.

Mice were sensitized with 0.05% DNFB five days prior to challenge with either vehicle or 0.2% DNFB. For each mouse, the right ear was exposed to vehicle (gray bars) and the left ear was exposed to 200 µg of TiO_2 nanoparticles (black bars). After 24 hours, the TiO_2 nanoparticles had no effect on skin swelling without DNFB challenge. However, Sigma Mn-doped TiO_2 nanoparticles significantly increased DNFB-induced ear swelling (mean ± SD, n=3-6).

Discussion

In vitro exposures of TiO_2 NPs to a keratinocyte cell line led to dose-dependent decreases in cell viability that were not dependent on NP composition. Interestingly, the Mn-doped TiO_2 NPs induced significantly more intracellular ROS generation than either pure TiO_2 NP variants. These observations were indicative of potential dermal toxicity, but *in vivo* studies displayed no signs of TiO_2 NP skin irritation in the CHS model. However, Mn-doped TiO_2 NPs did exacerbate skin swelling responses in a model of allergic contact dermatitis.

The allergic contact dermatitis response involves both innate and adaptive immune responses. Briefly, chemical haptens (such as DNFB) generate ROS and bind to proteins [27]. The ROS production is an important step in the activation of antigen presenting cells that express immune cytokines and present hapten-protein adducts [28,29]. This initial response is followed by mast cell degranulation and neutrophil influx to the skin [30,31]. Lastly, skin swelling and keratinocyte cell death occur in a cytotoxic and helper T cell-mediated manner [32-34]. While additional studies are required to elucidate the mechanism of action of Mn-doped TiO_2 NP exacerbation of allergic contact dermatitis, it is plausible that increases in intracellular ROS are involved. It is well established that Mn-induced neurotoxicity, commonly observed after occupational exposures, involves ROS generation [35]. Moreover, there is evidence of Mn hypersensitivity and irritation responses in skin patch tests [36,37]. Therefore, Mn ion dissolution and skin penetration is a plausible mechanism of action.

There was no TiO_2 NP-induced skin irritation identified in this study, which is a finding similar to other reports of pure TiO_2 NP skin administration [38]. Additionally, there is no evidence in the literature of TiO_2 NP-induced sensitization after topical administration [39], which is expected given the widespread use of TiO_2 NPs in consumer products. However, TiO_2-related effects on allergic skin models have been reported. One set of studies in 2, 4-dinitrochlorobenzene-induced allergic contact dermatitis models displayed significant increases in sensitization potential, measured by local lymph node assays, after TiO_2 subcutaneous or topical administration [40,41]. A second research group observed increases in skin lesion severity after intradermal injection of TiO_2 NPs (15, 50, or 100 nm) in a mite allergen-induced mouse model of atopic dermatitis [42]. While the results presented here do not suggest allergic contact dermatitis exacerbation by pure TiO_2 NPs, sensitization potentials were not studied. Additionally, discrepancies between these results could be explained by differences in particle morphology, dose, or animal model.

It remains unclear whether the *in vivo* effects of Mn-doped TiO_2 NPs are dependent on skin penetration. Some studies in mouse models report minimal TiO_2 NP skin penetration [43] and provided the skin barrier disruption that occurs during allergic contact dermatitis reactions [44], skin penetration is plausible. However, differences between mouse and human skin physiology, including thickness and hair follicle density, make interspecies comparisons difficult [45,46]. Future studies will include determination of both Mn ion release from the metal-doped TiO_2 NPs and TiO_2 NP penetration in mouse and human skin models.

Overall, the lack of observed *in vivo* dermal toxicity for pure anatase or pure rutile TiO_2 NPs is consistent with previously reported data. However, the TiO_2 NPs tested here may not accurately reflect those found in consumer products and sunscreens. Therefore, additional studies examining TiO_2 NPs extracted from consumer products are warranted. Moreover, this work suggests that occupationally relevant, metal-doped TiO_2 NPs represent a greater health hazard and future research will examine not only mechanisms of action but also toxicities associated with TiO_2 NPs doped with other commonly used transition metals.

Methods

Particle Characterization

Pure anatase (30 nm, >99.9% purity, Cat# US3498) or rutile (30 nm, >99.9% purity, Cat# US3520) titanium dioxide nanoparticles were purchased from US Research Nanomaterials, and an anatase dominant manganese-doped titanium dioxide (<100 nm, 1% Mn dopant, Cat# 677469) nanoparticle was purchased from Sigma-Aldrich. The nanoparticles were dispersed in ultrapure water prior to physical characterization. The physical size and shape were qualitatively examined via TEM, and a Malvern Zetasizer was used to measure hydrodynamic diameters and zeta potentials.

DNFB and TiO_2 *in vitro* exposure

An immortalized keratinocyte cell line (HaCaTs) was grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco Cat# 11965-092) supplemented with 10% fetal bovine serum (Gibco Cat# 10082-147)/1% penicillin/streptomycin (Gibco Cat# 15140-122). The HaCaTs were seeded into 96-well plates and incubated in a 37° C and 5% CO_2 atmosphere. All analyses were conducted in cells in a logarithmic growth phase (70% confluence).

All cells were exposed to 0, 5, 10, 50, 100, or 500 μg/mL TiO_2 for 24 or 48 hours. A Cell Titer-Glo assay (Promega Cat# G7571), which includes a luminescent ATP indicator, was used to measure cell viability. Briefly, 50 μL of CellTiter-Glo reagent was added to each well, and after 15 minutes the luminescence was measured with a Turner Biosystems Modulus microplate reader. For ROS measurements, the cells were incubated for 30 minutes in a 10 μM solution of 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Thermo Fisher Scientific Cat# D399) in sterile 1x phosphate buffered saline. After this pre-conditioning period, the cells were exposed to either vehicle or TiO_2 for 24 hours. Conversion of H_2DCFDA to the fluorescent 2',7'-Dichlorofluorescein (DCF), an indicator of intracellular ROS, was measured on a fluorescence plate reader.

DNFB and TiO_2 *in vivo* exposure

All *in vivo* experiments utilized an immunocompetent, hairless C57BL/6 mouse stain. These mice have a gene mutation that causes alopecia after the first follicular maturation. Therefore, these mice do not require potentially barrier disrupting depilation prior to topical exposures. Male mice between 5-6 months old were kept on a 12 hour light/dark cycle, and they were provided with food and water ad libitum throughout the study period. We have previously reported that DNFB reactions are age, but not sex-dependent [25,47]. On day 0, mice were sensitized on the dorsum with 30 μL of 0.05% DNFB (Sigma-Aldrich Cat# D1529) in a 4:1 acetone/olive oil vehicle. Five days later, 20 μL of 0.2% DNFB (with or without 200 μg TiO_2) was applied to each ear, and increases in ear thickness were measured 24 hours post-challenge. A separate group of mice were challenged with vehicle (with or without 200 μg TiO_2) to examine potential skin swelling effects of TiO_2 alone. *In vivo* experiments were approved by the University Committee on Animal Resources (UCAR#2010-024/100360) at the University of Rochester Medical Center.

Statistics

All statistics were analyzed with JMP Pro version 13.2.1 (SAS Institute Inc., Cary, NC). All *in vitro* data were analyzed by a two-way analysis of variance with post-hoc Tukey tests. Alternatively, *in vivo* data were analyzed by a Student's T-test, since the ear swelling data compared only the right (DNFB challenged) and left (DNFB + TiO_2 challenged) ears of each mouse. P-values ≤0.05 were considered significant and represented by an * (significance compared to control) or # (significance within a group). All data were presented as means ± standard deviation.

Acknowledgement

We would like to thank Karen Bentley of the URMC electron microscope research core facility for help with NP TEM imaging. Additionally, we thank Ian Krout, Joseph Lucas, Jakob Gunderson, and Ashley Peppriell for technical assistance with the *in vitro* experiments. The work was supported by both the NIH NIEHS training grant ES07026 and the NIH NIEHS research grant RO1 ES021492.

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Supplementary Figures

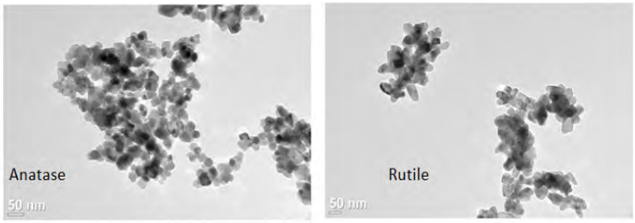

Figure S1: TiO_2 physical characterization by TEM.

TiO_2 nanoparticles were dispersed in acetone/water prior to deposition on grids for TEM analysis. The images of anatase (A) and rutile (B) particles show evidence for agglomeration and also difference in primary particle shape with anatase being square to spherical and rutile is showing more rectangular shape. Scale bar 50 nm.

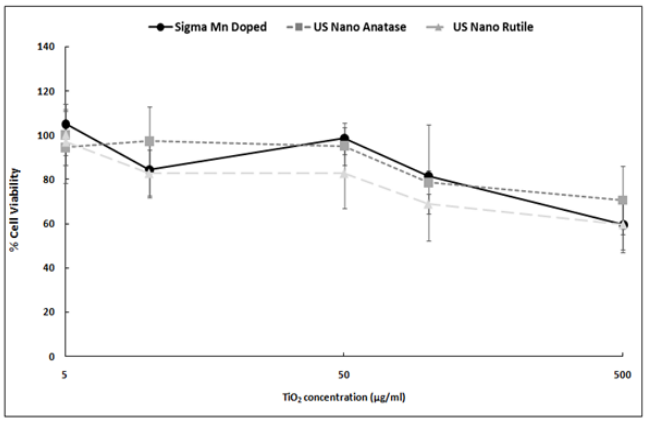

Figure S2: TiO_2 induced keratinocyte cytotoxicity after 48 hours.

HaCaTs were exposed to 0, 5, 10, 50, 100, or 500 µg/mL concentrations of anatase, rutile, or Sigma Mn-doped TiO_2 nanoparticles for 48 hours. Mild, dose-dependent decreases in cell viability were observed for all TiO_2 particles tested (mean ± SD, n = 3).

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