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**Understanding the Implications of Engineered Nanoparticle Induced Autophagy in Human
Epidermal Keratinocytes In Vitro**

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Abstract

Engineered nanoparticles (NPs) such as TiO₂ and ZnO are key UV-blocking ingredients in sunscreens. While toxicological risks of applying these materials are generally regarded as low due to minute levels of penetration across the skin, our understanding of the physiological influence of potential cell-nanoparticle interactions in the skin is limited. This study plugs the current knowledge gap by profiling TiO₂ and ZnO NP interaction with primary human epidermal keratinocytes, based on potential levels of NP penetration across the skin. Specific attention was given to profiling real-life relevant levels of exposure, and accurate dosimetry measurements *in vitro*. ZnO was expectedly more cytotoxic than TiO₂. Although both NPs generated Reactive Oxygen Species (ROS) and Mitochondrial Superoxide (MSO) within 4 hours exposure to sub-lethal concentrations, ZnO induction of these oxidative stress markers increased much more significantly after 24 hours exposure. Exposure to increasing NP concentrations increased autophagy induction along with activation of inflammatory responses in keratinocytes, primarily through the TRAF6-mediated pathway. Sustained induction of autophagy led to degradation of TRAF6 and, only in the case of TiO₂, reduced NF-κB activation. This pro-survival mode of autophagy induction provides further insights into the on-going debate on the use of these NPs in consumer products.

Keywords

Keratinocytes, Sunscreen, Nanoparticles, Oxidative stress, Inflammation, Autophagy

1. Introduction

Among the wide array of UV-blocking agents in sunscreens, titanium dioxide (TiO₂) and zinc oxide (ZnO), both of which are inorganic filters, have been used for over three decades and are generally regarded as safe (GRAS) materials according to US FDA. Their aesthetic shortcoming, being white and opaque, was addressed with advancements in nanotechnology by lowering particle sizes to 10-50 nm, which reduced their scattering of visible light, making them invisible to the naked eye without compromising, if not even further enhancing, their properties as UV filters (Lautenschlager et al. 2007). Thereafter, TiO₂ and ZnO NPs have become major active ingredients in cosmetics and personal care products, especially high sun protection factor (SPF) rating sunscreens. However, this has come with some apprehensive stigma regarding the potential health risks associated with exposure to engineered nanomaterials (Ng et al. 2014; George et al. 2015).

According to one exposure assessment analysis, an average (40 kg) female teenager could be exposed to as much as 1.61 mg·day⁻¹·kg⁻¹ or 23.5 g per year of NPs from sunscreen application alone (Lorenz et al. 2011). Numerous reports have shown that these NPs can penetrate the stratum corneum and reach the viable cell layers beneath, especially when the skin barrier is compromised such as via UV irradiation (Zhao and Ng 2014; Filon et al. 2015). Even though most studies report that a very low percentage of NPs penetrate (0.19% of the TiO₂ NPs applied on skin reach the epidermis and dermis combined (Miquel-Jeanjean et al. 2012)), given the large amounts which might be applied onto the skin, the absolute payload that could penetrate the skin might still be significant. Furthermore, given the small particle sizes, even low

amounts of penetration based on mass could mean a very large number of NPs, which further substantiates the concerns.

In light of this, many studies have been conducted to find out the potential influence of TiO₂ and ZnO NPs on skin cell physiology in the event that there is penetration across the skin to reach viable cells. Preceding knowledge, corroborated by our previous studies, have demonstrated that, independently and in combinations, TiO₂ and ZnO NPs are able to cause dose-dependent extents of toxicity ranging from DNA damage to causing apoptosis and cell death (Heng et al. 2011; Ng et al. 2011; Setyawati et al. 2013; Kathawala et al. 2015a). One of the major concerns is that these NPs can induce elevated levels of intracellular Reactive Oxygen Species (ROS) which have been shown to irreversibly oxidize DNA, mitochondria and other organelles and cause a variety of genotoxic effects in cells (Huang et al. 2009; Iavicoli et al. 2011; Ghosh et al. 2013). Increased oxidative stress caused by ROS induction has also been reported to bring about a series of toxic events such as inflammation, genotoxicity, fibrosis and carcinogenesis in various cell types (Roursgaard et al. 2011; Sun et al. 2011; Manke et al. 2013). Studies on human epidermal keratinocytes have revealed that TiO₂ and ZnO NPs impart adverse effects on cells due to the intracellular formation of free radicals causing changes in cellular morphology, mitochondrial dysfunction, DNA damage and cell cycle disruption (Kocbek et al. 2010). These effects depend on size, concentration and length of exposure to the NPs. Furthermore, ZnO NPs have been known to cause more pronounced effects than TiO₂ due to the solubility of the ZnO NPs in the cell culture medium which results in cells being exposed to aqueous Zn²⁺ (Sharma et al. 2009).

In addition to the toxic effects, keratinocytes also respond to TiO₂ and ZnO NPs by upregulation of autophagy (Xiong et al. 2013; Zhao et al. 2013; Roy et al. 2014). In our previous study, we have demonstrated that TiO₂ NPs are able to induce autophagy in keratinocytes at very low administered dosages (Xiong et al. 2013). Several other cell lines have demonstrated upregulation of autophagy through ZnO NP exposure as well (Kocbek et al. 2010; Bai et al. 2017; Zhang et al. 2017). Autophagy regulates the degradation of various components of the inflammatory pathway, and there is numerous evidence of the interplay between the two pathways. For instance, under the influence of Heat shock protein 90 (Hsp90) inhibitors, autophagy degrades the IκB kinases (IKK) – IKKα, IKKβ and IKKγ which can prevent activation of the p65 nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Qing et al. 2006; Yan et al. 2007). In another study, it was shown that p62, a cargo receptor for autophagic degradation, activates p65 NFκB in response to different stimuli like tumor necrosis factors (TNF) or interleukins (IL) (Moscat and Diaz-Meco 2009). The activation of NFκB, in turn, can also regulate p62 expression in certain tumor cell lines (Yang et al. 2017).

Despite the numerous studies available, a few gaps and unanswered questions remain which this current study is designed to address. Firstly, most, if not all, toxicity studies do not justify the choice of concentrations tested to real-life exposure or penetration. We address this shortcoming by analyzing data reported in two previous studies one of which performs detailed analysis on NP exposure from sunscreens while the other measures the penetration of similar NPs through the different layers of skin. Secondly, most *in vitro* studies suffer from a lack of dosimetry inaccuracy which comes from the sedimentation kinetics in 2D cell cultures. While concentrations are reported in volumetric parameters, the NPs do not interact with the cell

monolayer at the bottom of the culture plate until they deposit which happens over time. We address this problem by employing the *In vitro* Sedimentation, Deposition and Dosimetry (ISDD) model to our culture system to derive the time-weighted doses. Lastly, it is not yet uncharacterized how different dosages of TiO₂ or ZnO NPs influence autophagic response and whether these stimuli can affect the inflammatory pathway in keratinocytes, although it has been established that ZnO NPs can induce inflammation in keratinocytes through the ROS pathway (Jeong et al. 2013). In our current study, we, therefore, investigated the interaction of commercial TiO₂ and ZnO NPs with primary human epidermal keratinocytes, based on physiologically relevant amounts of NP exposure and anticipated penetration across the skin as supported by current literature.

2. Materials and method

2.1. NPs Characterization

Zinc oxide (ZnO) and Titanium dioxide (TiO₂) NPs were purchased from Evonik Degussa (Essen, Germany) and Melorium Technologies (USA) respectively. Transmission electron microscopy (TEM, Carl Zeiss Libra 120 Plus) with an accelerating voltage of 120 kV was used to determine their morphology and size. The samples were prepared by dispersing NPs in absolute ethanol followed by ultrasonication in a water bath at 50 Hz for 30 minutes (42 J/ml) and were drop cast onto carbon-coated copper grids. The average size was calculated using ImageJ (n=100). Crystal structure of the NPs was investigated using X-ray diffraction (XRD Bruker D8 Advance) using Cu-K α X-rays of wavelength (λ)= 1.540598 Å with a 2 θ scan from 10° to 70° with a step size of 0.19. Brunauer–Emmett–Teller (BET) surface area was determined by using ASAP Tri-star II 3020 surface area and porosity analyzer from Micromeritics. The NPs

were degassed under vacuum at 200°C for 2 hours before BET analysis. The behavior of NP in the liquid was characterized by dynamic light scattering (DLS) technique, using Malvern Zetasizer (DLS; Malvern Co., UK) with a He-Ne laser at a wavelength of 633 nm. The NPs were dispersed in ultrapure water and cell culture medium with ultra-sonication in a water bath at 50 Hz for 10 minutes (14 J/ml). Immediately after sonication, the dispersion was analyzed by the Zetasizer. The hydrodynamic size, zeta potential and polydispersity index (PDI) was measured. In order to measure the solubility of Zn^{2+} from ZnO NPs, ZnO suspension in medium and PBS were incubated for 24 hours at 37°C. The suspension was then centrifuged, and the supernatant was filtered with 0.22 μ filter and used for ICPOES measurement.

2.2. Cell culture

Primary Epidermal Keratinocytes; normal human adult foreskin (ATCC® PCS-200-010™) was cultured in serum-free EpiGRO™ Human Epidermal Keratinocyte medium (Chemicon, SCMZ-BM) supplemented with growth factors (Chemicon, SCMK001-S). The cells were maintained to grow in a humidified environment at 37°C and 5% CO_2 . Keratinocytes ≤ 4 were used for the experiments. The keratinocytes were seeded in well plates as density 10,000 cells·cm⁻².

2.3. Cell exposure to NPs

The stock solution (5 mg·ml⁻¹) of NPs was prepared in 1X PBS (PAN Biotech, Germany). The solution was kept in a petri dish and exposed to UV for 15 minutes for sterilization of NPs. The stock solution was then ultra-sonicated in a water bath for 10 minutes (14 J/ml) and diluted in the complete medium into required working concentrations. The working

solution of NP suspension at various concentration was again ultra-sonicated for 10 minutes prior to exposure to the cells. The NP suspension was always prepared immediately before exposure to the cells. To observe the toxic effects contributed by aqueous Zn^{2+} in ZnO NPs dispersion, $ZnCl_2$ was dissolved in DI water and was diluted in culture medium to obtain equimolar amounts of aqueous Zn^{2+} (measured by ICPOES).

2.4. Dosimetry

The dosimetry was performed using the ISDD model (Hinderliter et al. 2010). The effective density of the NPs aggregates in the medium was calculated using Volumetric centrifugation method (DeLoid et al. 2014). 1 ml of $1\text{ mg}\cdot\text{ml}^{-1}$ suspension of TiO_2 and ZnO NPs in the complete medium was kept in packed cell volume (PCV) tubes (Techno Plastic Products (TPP), Trasadingen, Switzerland). The tubes were centrifuged in a bench-top swing out rotor centrifuge at 2000g for 1 hour. The volume of the agglomerate pellet (V_{pellet}) was measured with the help of slide-rule like easy measure device which was also provided by the PCV tube manufacturers. Because of the partial solubility of ZnO NPs in the medium, the amount of aqueous Zn^{2+} in the supernatant was measured by ICPOES. The V_{pellet} was used to calculate the effective density (ρ_{EV}) of the agglomerate unit using the equation below.

$$\rho_{EV} = \rho_{media} + \left[\left(\frac{M_{ENM} - M_{ENMsol}}{V_{\text{pellet}} SF} \right) \left(1 - \frac{\rho_{media}}{\rho_{ENM}} \right) \right]$$

Where ρ_{media} is the density of media, M_{ENM} is the mass of NPs, M_{ENMsol} is the solubilized mass, V_{pellet} is the volume of the pellet, SF is the stacking factor and ρ_{ENM} is the density of NPs.

The effective density obtained was used to simulate the sedimentation of TiO₂ and ZnO NPs over time administered on cell culture well plate (48 well plate, 96 well plate, T75 flask) with the help of ISDD model. Media height, volume, temperature, viscosity, particle diameter, density, concentration, agglomerate diameter (hydrodynamic size of the particle) and effective density was input in ISDD model in the MATLAB program and the fraction of the particles sedimented over time was simulated. The fraction of the administered dose sedimented was plotted against time and the area under the curve obtained from the profile gives the Time Weighted Exposure (TWE) for the administered dose used. Since effective exposure is only the amount of time particles spend after being sedimented on the cells, the TWE is a parameter which summates the total time that every unit of the administered dose is effectively exposed to the cells.

2.5. Cell viability assay

The cell viability was determined by investigating the cellular metabolic activity and cell proliferation through DNA quantification. The cells were seeded in a 48 well plate and exposed to NPs for 4 and 24 hours. After incubation, Alamar blue metabolic assay (Invitrogen) was used to quantify cellular metabolic activity. The absorbance of the reagent was measured with a microplate reader (Infinite M200, TECAN Inc., Maennedorf, Switzerland) which was then correlated with cellular metabolic activity according to the manufacturer's protocol. PicoGreen, DNA quantification assay (Molecular Probes) was used to evaluate cell proliferation. After incubation of the cells with NPs, the culture media was removed, and the cells were washed thrice with PBS and chilled Cell lysis buffer (1X in DI water) (Cell Signaling Technology) was

added in each well. The cell lysates were mixed with PicoGreen reagent according to the manufacturer's protocol and fluorescent reading was measured with the microplate reader.

2.6. Intracellular ROS and MSO measurement

Cm-H₂DCFDA (Molecular Probes) probe was used as an indicator of intracellular ROS. The cells were seeded in a sterile black 96 well plates and incubated with NPs for 4 and 24 hours. After incubation, the medium was removed, and the cells were washed with PBS and incubated with 10 μ M of the indicator dye solution, in PBS, at 37°C, 5% CO₂ for 30 minutes and the fluorescence reading was taken according to manufacturer's protocol. Similarly, for MSO measurement, cells were incubated with 5 μ M MitoSOX^{TM} Red MSO indicator (Molecular Probes), diluted in PBS, for 15 minutes and the fluorescence reading was measured according to the manufacturer's protocol. The antioxidant N-acetyl cysteine (NAC) (Sigma Aldrich) 10 mM was added to the cells 1 hour before exposing the cells to NPs and was incubated for 24 hours along with NPs.

2.7. Western Blot analysis

To study the changes in total protein levels, total cellular proteins were extracted by lysing the cells in ice-cold RIPA lysis buffer (150mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris, pH 8). Protein concentration was determined by Bradford protein assay (Bio-Rad Laboratories, Inc., USA) using bovine serum albumin (BSA) as a standard. 30-70 μ g of proteins per sample were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto a nitrocellulose membrane (Whatman, Millipore, USA). Membranes were subjected to reversible Ponceau S

staining (0.5% (w/v) Ponceau S in 1.0% (v/v) acetic acid) to visualize total amounts of proteins resolved in each lane prior to blocking with 5% milk in TBST buffer (22.4 g^l⁻¹ Tris base, 80 g^l⁻¹ sodium chloride and 0.05% Tween 20 (BioRad, USA) pH 7.5). Proteins of interest were detected by specific antibodies and visualized using enhanced chemiluminescence Western Blot detection reagent (GE Healthcare, USA) and autoradiography with Kodak films (USA) or hyperfilms (GE Healthcare, USA). Densitometric analysis was performed by quantifying the intensity of the bands using ImageJ software (NIH, USA).

2.8. Antibodies and chemicals

The following primary antibodies were used for immunoblotting at the following concentrations: anti-Actin (1:10,000) (Abcam, UK); anti-LC3 (1:1000, Cell Signaling Technology and Novus Biologicals, USA), anti p62, NF- κ B and pNF- κ B (1:1000; Cell Signaling Technology, USA) and anti TRAF6 (1:2000; Cell Signaling Technology, USA). Secondary antibodies used for immunoblotting were goat anti-mouse and goat anti-rabbit horseradish peroxidase-conjugated IgG (1:10000, Sigma-Aldrich, USA). The following different chemical inhibitors were used – leupeptin (100 μ M working, Enzo Life Sciences) and Ammonium Chloride (20mM working, Sigma-Aldrich).

2.9. Calculation of LC3 II flux

To study the autophagy activity, LC3 II flux was calculated. During autophagy, LC3 II is degraded in the autophago-lysosome. As such the rate of turnover of LC3 II was studied under different conditions to calculate autophagic flux. Hence, the cells were treated with or without lysosomal inhibitors (NH₄Cl and leupeptin; NL) for 4 hours. As a positive control, cells were

treated with 4 μ M tamoxifen (TAM) to induce autophagy. The cells were harvested and lysed using RIPA buffer and subjected to immunoblotting. The LC3 II flux was calculated using the following formula;

$$LC3\ II\ FLUX = \frac{\text{Amount of LC3 II in NL treated condition}}{\text{Amount of LC3 II in untreated condition}}$$

2.10. Cytokine array

The levels of cytokines secreted by the keratinocytes exposed to different concentration of NPs were measured using Human Inflammation Antibody Array Membrane (Abcam 134003) according to the manufacturer's protocol. The array membranes were blocked with the blocking solution, provided in the array kit for 30 mins. The supernatant of the cells exposed to TiO₂ and ZnO NPs were added on the membranes and incubated overnight at 4°C. After incubation, the membranes were washed with Wash buffer I for three times and Wash buffer II for two times and were incubated for 2 hours at room temperature with Biotin-Conjugated Anti-Cytokines. The membranes were washed with Wash buffer I and II as mentioned earlier and incubated with HRP-Conjugated Streptavidin. Finally, the membranes were washed again and transferred onto a plastic sheet with Detection buffer for chemiluminescence detection. The detection was done in an imaging system with 2 minutes exposure to CCD camera. The background signals were subtracted, and each array was normalized with the corresponding DNA amount of the cells treated to NPs before comparing the cytokine expression levels among different groups.

2.11. Cellular Apoptosis

Apoptosis assay was carried out with Muse® Cell Analyzer. After exposure to NPs for 24 hours, the cells were collected by using trypsin and was stained with Annexin V & Dead Cell Assay Kit (Merk Millipore) for 20 minutes and analyzed according to manufacturer's protocol.

2.12. Statistical Analysis

All the results are shown as mean $\pm$ standard deviation. Unless stated otherwise, all experiments were carried out in triplicates (n=3). One way analysis of variance (ANOVA) was performed together with Tukey's post-hoc analysis to compare means. All the statistical analysis was performed using OriginPro software. $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. NPs Characterization

NPs characterization confirmed the expected physical properties of the commercial TiO₂ and ZnO NPs used in this study. Representative TEM micrographs of the NPs showed that TiO₂ NPs were either spherical or polygonal in shape (supplementary figure 1a) whereas ZnO NPs were mostly spherical (supplementary figure 1b), with mean particle diameters of 22 ± 4 nm and 20 ± 2 nm, respectively. XRD profiles of TiO₂ NPs (supplementary figure 1c) revealed sharp diffraction peaks at 25.31° , 37.90° and 47.96° corresponding to its anatase structure and sharp peaks at 27.46° , 36.19° , 41.32° and 62.7° corresponding to its rutile structure (White et al. 2013). Similarly, the peaks between 31° to 69° (supplementary figure 1d) confirmed the polycrystalline zincite structure of the ZnO NPs (Akhtar et al. 2012). The specific surface areas of TiO₂ and ZnO NPs were $52.33 \pm 1.70 \text{ m}^2 \cdot \text{g}^{-1}$ and $23.22 \pm 2.42 \text{ m}^2 \cdot \text{g}^{-1}$, respectively. The hydrodynamic sizes and zeta potential of the NPs in cell culture medium and water are shown in

Table

Table 1. The level of Zn^{2+} ions introduced into the media by the dissolution of ZnO NPs was measured using ICP-OES (Table 2).

3.2. Translating Real-Life Exposure to In Vitro Doses

Findings from two previously published studies were used as primary references to determine relevant in vitro doses (Lorenz et al. 2011; Miquel-Jeanjean et al. 2012). Figure 1 shows the schematic representation of the flow of calculating realistic doses for the in vitro assessments. The detailed calculations can be found in the supplementary notes. Here we present the key findings of the calculations. The study by Lorenz et al. grouped the sampled individuals into 3 categories based on the exposure levels experienced. These depended on the frequency and amount of product application, NP content in the product and their body weight (which had implications on the applied surface area of the skin). To cover the entire spectrum, we assessed both the extremes: (A) “High Exposure” - large product amounts with the highest frequency of application, and (B) “Low Exposure” - small product amounts with the lowest frequency of application (**Error! Reference source not found.**). We translated the data reported into some key numbers, some of which, presented as a range covering low to high exposure groups, are as follows:

a. Total yearly NP exposure: 1.43-23.5 g

b. Total yearly NP exposure per surface area of the skin: $0.12\text{-}2.85 \text{ mgcm}^{-2}$

c. Daily NP exposure per surface area of the skin: $13.4\text{-}96.9 \text{ }\mu\text{gcm}^{-2}$

To evaluate the portion of applied NPs that could penetrate through the topical barrier to the viable layers of the skin, data from a penetration study performed on human skin explants was utilized (Miquel-Jeanjean et al. 2012). The study suggests that 0.19 ± 0.15 % of TiO_2 NPs penetrated into the dermis and epidermis of healthy skin and in the case of damaged and/or UV irradiated skin explants, the penetrated amounts were higher, up to 0.55 ± 0.46 % (Miquel-Jeanjean et al. 2012). Based on these assumptions, the amount of NPs penetrated through the dermis and epidermis for the low and high exposure groups described earlier was calculated to be $0.026\text{-}0.184 \mu\text{gcm}^{-2}$ per day for intact skin and $0.074\text{-}0.533 \mu\text{gcm}^{-2}$ per day for damaged skin.

In order to translate these numbers to volumetric concentrations used to measure *in vitro* doses, we can use the cell culture plate surface area as the representative surface of the skin. Considering a typical experiment whereby a 48 well plate ($0.875 \text{ cm}^2/\text{well}$) is used with 0.25 ml of media in each well, the volumetric equivalent of the above-mentioned penetrated payloads would be approximated from $0.1\text{-}2 \mu\text{gml}^{-1}$. Therefore, in all our *in vitro* tests, we ensure that the concentration ranges are either similar to these or at least cover this range for realistic comparisons to be made.

3.3. Time Weighted Exposure (TWE)

In suspension, NPs experience two driving forces; isotropic diffusion caused by Brownian motion and anisotropic (downwards) sedimentation caused by gravity. The ISDD model simulates the particle kinetics using a key parameter called effective density (ρ_{EV}) of NP agglomerates (Hinderliter et al. 2010). The effective density (ρ_{EV}) was calculated by measuring the effective volume of NPs agglomerates using the *in vitro* dosimetry characterization (DeLoid

et al. 2014). The effective density (ρ_{EV}) of TiO_2 and ZnO agglomerates in the cell culture medium used was calculated to be $1.370 \text{ g}\cdot\text{cm}^{-3}$ and $1.731 \text{ g}\cdot\text{cm}^{-3}$, respectively. These values are much lower than the density of pristine TiO_2 and ZnO ($4.23 \text{ g}\cdot\text{cm}^{-3}$ and $5.61 \text{ g}\cdot\text{cm}^{-3}$, respectively). This is because the agglomerates are limited by packing factors, allowing lots of empty spaces between individual NPs. These spaces can be filled up with the medium, which is less dense than the particulates, thereby bringing down the average density of the agglomerates.

Supplementary figure 2 shows the plot of the fraction of NPs sedimented (f_D) as a function of time, simulated using the ISDD model. Due to the low effective density, the time taken for the NPs to sediment is very high in media suspensions. Comparing the two NP suspensions in 96 well plates (100 μl media volume), it was noted that ZnO NPs would sediment completely between 4 to 5 hours, whereas complete sedimentation of TiO_2 NPs takes place after 9 hours. Regardless of culture plate format (supplementary figure 2), ZnO sedimented faster than TiO_2 due to a higher effective density. In the context of *in vitro* testing, effective exposure is only the time each particle spends after sedimenting onto the cells. Considering that, using the TWE which measures the total time of effective exposure for every fraction of the administered dose would be more appropriate. The TWE will have units which include the unit in which administered dose is measured (e.g. mass, particle surface area, particle number, etc.) as well as the unit of time (in hours). For example, $1 \mu\text{g}\cdot\text{hrs}$ is equivalent to $1 \mu\text{g}$ of sedimented particles exposed for 1 hour. A higher administered dose and/or a longer incubation time would obviously increase the TWE but more importantly, sedimentation dynamics would also regularize the dosage. For particles which sediment slower, the TWE would be lower as the fraction of sedimented particles would be lower over time. Consider an experimental setup where different

particles are exposed to monolayer cultured cells at an administered dose of 1 μg of particles over a period of time. Assuming a hypothetical scenario that 100% of Particle 1 sediment after 6 hours of incubation, while 100% of Particle 2 sediment after only 1 hour, the TWE of Particle 1 will be 3 $\mu\text{g}\cdot\text{hrs}$ while that of Particle 2 will be 6 $\mu\text{g}\cdot\text{hrs}$ (further illustrated in supplementary figure 3). Therefore, the TWE has greater relevance than the conventional way of reporting administered dose and incubation time only because it additionally considers the time of contact between particles and cells. Taking this approach, we generated the TWE values for the different experimental conditions, as listed in supplementary notes table 4, along with a detailed explanation.

Another important consideration is that since TWE is built on the assumption that only sedimented NPs contribute to effective dosage, using volumetric concentrations to derive dosage is meaningless. Therefore, per unit cell growth surface area concentrations are used instead e.g. $\text{mg}\cdot\text{hrs}\cdot\text{cm}^{-2}$. Wherever applicable, both administered dose ($\mu\text{g}\cdot\text{ml}^{-1}$) and TWE ($\text{mg}\cdot\text{hr}\cdot\text{cm}^{-2}$) are reported to correlate cell response results herein. The term “administered dose” represents the concentration of NPs suspended in the cell culture medium. For example, a 48-well plate (0.875 cm^2 culture surface area per well) treated with an administered dose of 250 μl of 25 $\mu\text{g}\cdot\text{ml}^{-1}$ ZnO NPs has a per surface area dosage of 7.143 $\mu\text{g}\cdot\text{cm}^{-2}$. When incubated for 4 hours (TWE factor: 1.991 $\text{frac}\cdot\text{hr}$) and 24 hours (TWE factor: 21.997 $\text{frac}\cdot\text{hr}$), the equivalent TWE would be 0.0142 $\text{mg}\cdot\text{hr}\cdot\text{cm}^{-2}$ (7.143×1.991) and 0.157 $\text{mg}\cdot\text{hr}\cdot\text{cm}^{-2}$ (7.143×21.997) respectively.

3.4. Dose and Time-dependent toxicity of TiO_2 and ZnO NPs

For evaluation of cytotoxic effects of TiO₂ and ZnO NPs, the human epidermal keratinocytes were exposed to each NP for 4 (Figure 2a) and 24 (Figure 2b) hours and measured for cell viability and proliferation. Minimal effects on cell viability and proliferation were observed after 4 hours of exposure to TiO₂ NPs. A significant effect was observed at a very high administered dose of 1000 µg·ml⁻¹ (TWE 0.29 mg·hr·cm⁻²) at which there was 20% reduction in cell viability and 40% reduction in cell proliferation (*p*<0.05). On the other hand, ZnO NPs caused a significantly higher reduction in cell viability and proliferation: even at 25 µg·ml⁻¹ (TWE 0.0142 mg·hr·cm⁻²) there was 64% reduction in metabolic activity and 70% reduction in cellular proliferation compared to the untreated cells (*p*<0.05).

As incubation time was increased, both viability and proliferation of keratinocytes were reduced (Figure 2b) as expected. At TiO₂ administered dose of 100 µg·ml⁻¹ for 24 hours (TWE 0.57 mg·hr·cm⁻²), cell viability was reduced by 20%, compared to the untreated cells (*p*<0.05), and at a higher administered dose of 1000 µg·ml⁻¹ (TWE 5.72 mg·hr·cm⁻²), 62% reduction in cell viability was observed (*p*<0.05). Similarly, a significant reduction in cell proliferation was observed at the same doses with a maximum observed reduction of 60% at the highest administered dose of 1000 µg·ml⁻¹ (TWE 5.72 mg·hr·cm⁻²) tested (*p*<0.05). ZnO NPs after 24 hours exposure caused cell viability to be significantly reduced by 35% at 10 µg·ml⁻¹ (TWE 0.063 mg·hr·cm⁻²) and by 90% at the highest tested administered dose of 100 µg·ml⁻¹ (TWE 0.628 mg·hr·cm⁻²) (*p*<0.05). Likewise, there was a 93% reduction in cellular proliferation at higher administered dose 100 µg·ml⁻¹ (TWE 0.628 mg·hr·cm⁻²) of ZnO NPs (*p*<0.05).

The untreated cells showed healthy polygonal shapes and were well attached to the culture surface. No drastic changes were observed in the morphology of keratinocytes exposed to TiO₂ NPs for both 4 and 24 hours (supplementary figure 4a). However, drastic changes were observed in the morphological appearances of the keratinocytes exposed to ZnO NPs at both 4 and 24 hours (supplementary figure 4b): after 24 hours exposure to 25 µg·ml⁻¹ (TWE 0.157 mg·hr·cm⁻²) and above, the cells started to appear rounded and partially detached from the surface.

3.5. Effects of Zn²⁺ on cell viability

The amount of aqueous Zn²⁺ in the cell culture medium from the partial dissolution of ZnO NPs at various concentration was measured by ICPOES (Table 2). Based on this, keratinocytes were exposed to ZnCl₂ solutions of concentrations which would contain an equimolar concentration of Zn²⁺ compared to their counterpart ZnO NPs suspensions at the test concentrations. This was done to assess the contribution of aqueous Zn²⁺ generated by ZnO dissolution. Cellular proliferation and metabolic activity measured (supplementary figure 5a) after exposing the cells to Zn²⁺ and ZnO NPs for 24 hours demonstrated that Zn²⁺ alone had minimal effects on cells up to a concentration of 3.337 µg·ml⁻¹ (the number of ions generated by a 10 µg·ml⁻¹ ZnO NPs suspension). In comparison, cellular proliferation was significantly reduced when the cells were exposed to 10 µg·ml⁻¹ ZnO ($p<0.05$). Likewise, metabolic activity (supplementary figure 5b) also remained unaffected when the cells were exposed to Zn²⁺ alone up to a concentration of 3.337 µg·ml⁻¹ (amount generated by 10 µg·ml⁻¹ ZnO NPs), whereas when the cells were treated to 10 µg·ml⁻¹ ZnO NPs (which contained both NPs and soluble Zn²⁺), metabolic activity was significantly reduced ($p<0.05$). Zn²⁺ alone caused a significant toxic

response in cells only at concentrations above $7.144 \mu\text{g}\cdot\text{ml}^{-1}$ (dissolved from ZnO $50 \mu\text{g}\cdot\text{ml}^{-1}$) ($p<0.05$).

3.6. *TiO₂ and ZnO NPs induced Oxidative stress by the generation of ROS and MSO*

A significantly higher level of ROS and MSO signals was observed in the cells exposed to the NPs (Figure 2c and 2d). After 4 hours exposure to TiO₂ NPs, a significant increase in ROS was only observed at higher concentrations: about 2- and 3.5-fold increase at an administered dose of $100 \mu\text{g}\cdot\text{ml}^{-1}$ (TWE $0.029 \text{ mg}\cdot\text{hr}\cdot\text{cm}^{-2}$) and $1000 \mu\text{g}\cdot\text{ml}^{-1}$ (TWE $0.29 \text{ mg}\cdot\text{hr}\cdot\text{cm}^{-2}$) respectively ($p<0.05$). Likewise, ROS was also significantly increased after 4 hours exposure to ZnO NPs, but the onset was at much lower doses: $25 \mu\text{g}\cdot\text{ml}^{-1}$ (TWE $0.0142 \text{ mg}\cdot\text{hr}\cdot\text{cm}^{-2}$) and $100 \mu\text{g}\cdot\text{ml}^{-1}$ (TWE $0.057 \text{ mg}\cdot\text{hr}\cdot\text{cm}^{-2}$) caused a significant increase in the ROS levels by 1.27 times and almost 2 times respectively ($p<0.05$). The MSO levels in cells treated to TiO₂ NPs were lower than ROS levels at 4 hours exposure. Again, a significant increase in MSO levels was only observed at higher concentrations of TiO₂ NPs: $100 \mu\text{g}\cdot\text{ml}^{-1}$ (TWE $0.057 \text{ mg}\cdot\text{hr}\cdot\text{cm}^{-2}$) and $1000 \mu\text{g}\cdot\text{ml}^{-1}$ (TWE $0.57 \text{ mg}\cdot\text{hr}\cdot\text{cm}^{-2}$) causing 1.3 times and 2.6 times increase compared to untreated controls respectively ($p<0.05$). In contrast, ZnO NPs exhibit more increase in MSO generation than ROS. However, the onset of the significant increase was again at much lower concentrations than that of the TiO₂ NPs counterpart: $25 \mu\text{g}\cdot\text{ml}^{-1}$ (TWE $0.0412 \text{ mg}\cdot\text{hr}\cdot\text{cm}^{-2}$) and $100 \mu\text{g}\cdot\text{ml}^{-1}$ (TWE $0.057 \text{ mg}\cdot\text{hr}\cdot\text{cm}^{-2}$) causing 2.2 and 2.6 times increase in MSO respectively ($p<0.05$).

On increasing the exposure period to 24 hours, the ROS and MSO levels continued to increase in a strong dose-dependent manner for both NPs (Figure 2d). As expected, the onset of significant increase for both ROS and MOS levels was at much lower concentrations for ZnO

NPs as compared to TiO₂ NPs. After 24 hours exposure, TiO₂ NPs at 100 µg·ml⁻¹ (TWE 0.57 mg·hr·cm⁻²) and 1000 µg·ml⁻¹ (TWE 5.72 mg·hr·cm⁻²) caused significant increase in ROS levels by about 1.5 and 3 times respectively while ZnO NPs at 10 µg·ml⁻¹ (TWE 0.063 mg·hr·cm⁻²) and 100 µg·ml⁻¹ (TWE 0.628 mg·hr·cm⁻²) caused the ROS levels to increase by almost 1.5 and 4 times respectively ($p<0.05$). Interestingly, the MSO levels were higher than the ROS levels for both NPs exposure. TiO₂ NPs at 1000 µg·ml⁻¹ (TWE 5.72 mg·hr·cm⁻²) caused an increase by almost 5-fold and ZnO at 100 µg·ml⁻¹ (TWE 0.628 mg·hr·cm⁻²) caused a drastic increase by almost 7-fold in the MSO level ($p<0.05$).

3.7. Autophagy induction in response to TiO₂ and ZnO induced oxidative stress

Analysis of the autophagy markers: adaptor protein p62 and autophagosomal membrane protein LC3 II indicated a significant increase in the levels of both these proteins upon treatment with TiO₂ and ZnO NPs in a dose-dependent manner (Figure 3a, 3b and 3c). Furthermore, to confirm the autophagy activity, the autophagic flux was studied in the presence of lysosomal inhibitors (ammonium chloride and leupeptin; NL). There was a significant increase in autophagic flux, similar to tamoxifen response, upon treatment with TiO₂ (500 µg·ml⁻¹) and ZnO (5 µg·ml⁻¹) (Figure 3d, 3e and 3f) ($p<0.05$). Apart from the induction of autophagy, TiO₂ and ZnO NPs are also known to elicit an inflammatory response in different cell lines (Nel et al. 2006; Ma et al. 2009). TNF receptor-associated factor 6 (TRAF6), which is an adaptor protein in the Toll-like receptor (TLR) mediated inflammatory pathway also induces pro-inflammatory cytokines by an alternative pathway. The rate of autophagic turnover of TRAF6, under low and high dosage of both TiO₂ (100 fg·ml⁻¹ and 500 µg·ml⁻¹) and ZnO (50 pg·ml⁻¹ and 5 µg·ml⁻¹) NPs, was studied. It was observed that TRAF6 was not significantly degraded by autophagy under

basal (UT) or tamoxifen (TAM) treated conditions (Figure 3c and 3f). TRAF6 was also not degraded by autophagy under low administered doses of the NPs, however, under higher administered doses of the NPs, there was a significant increase in levels of TRAF6 degradation by autophagy (Figure 3d and 3g) ($p<0.05$). Autophagosomes were also visualized by monodansylcadaverine (MDC) staining of keratinocytes as shown in supplementary figure 6.

3.8. Inflammatory reaction in response to different administered dosages of TiO_2 and ZnO NPs

Western blot analysis revealed a significant increase in levels of p65 (Rel A) upon treatment with low administered doses of TiO_2 ($100 \text{ fg}\cdot\text{ml}^{-1}$) while treatment with a low dosage of ZnO ($50 \text{ pg}\cdot\text{ml}^{-1}$) did not affect the p65 levels significantly (Figure 4a and 4b) ($p<0.05$). Upon treatment with higher doses of TiO_2 ($500 \text{ }\mu\text{g}\cdot\text{ml}^{-1}$), the p65 (Rel A) levels reduced to basal levels while increasing ZnO dosage to $5 \text{ }\mu\text{g}\cdot\text{ml}^{-1}$ showed no further change in p65 levels (Figure 4a and 4b). p65 (Rel A) is activated by phosphorylation, and hence the effect of the TiO_2 and ZnO NPs on phosphorylation of p65 was also studied. Treatment of keratinocytes at low doses of TiO_2 ($100 \text{ fg}\cdot\text{ml}^{-1}$) and ZnO ($50 \text{ pg}\cdot\text{ml}^{-1}$) showed no change in p65 phosphorylation (Figure 4a and 4c). At higher doses of TiO_2 ($500 \text{ }\mu\text{g}\cdot\text{ml}^{-1}$), there was a reduction in p65 phosphorylation, but increasing ZnO concentration to $5 \text{ }\mu\text{g}\cdot\text{ml}^{-1}$ showed no change in phospho-p65 levels (Figure 4a and 4c) ($p<0.05$). Western blot analysis also revealed significantly increased levels of TRAF6 upon treatment with low doses of both TiO_2 and ZnO NPs while increasing dosage led to a drop in TRAF6 levels (Figure 4a and 4d) ($p<0.05$).

Both TiO_2 and ZnO NPs resulted in changes in various cytokine levels secreted by the keratinocytes when compared to untreated controls. Figure 4e and 4f show the level of cytokines

secreted by keratinocytes exposed to TiO₂ and ZnO NPs for 24 hours. At TiO₂ administered dose 500 µg·ml⁻¹ the cytokine levels were increased when compared to untreated control as seen in figure 4e. The most prominent increase was observed in IL-6 with almost 7 times increment. The other administered doses 10 µg·ml⁻¹ and 100 µg·ml⁻¹ did not show much increase. Whereas, ZnO NPs caused more increase in cytokines levels at all administered doses. IL-6 was increased by 11 times when treated with ZnO administered dose 10 µg·ml⁻¹. Similarly, IL-2 and IL-8 showed 7 times increase compared to untreated cells. A dose-dependent increase in cytokine level was observed in IL-1a, TNF-α, TNF-β and sTNFRII. The profile of various cytokines secreted by keratinocytes is shown in supplementary figure 7.

3.9. *N-acetyl cysteine (NAC) protected the cell from undergoing apoptosis by suppressing ROS generation*

A significant reduction, typically around 20-30%, in the ROS levels was observed in NPs-treated keratinocytes at almost all concentrations in the presence of antioxidant NAC after 24 hours exposure (Figure 5a) ($p < 0.05$). With the reduction in ROS, the percentage of viable or early apoptotic cells were increased (Figure 5b and 5c). The cell viability was increased from 71% to 84% at 1 µg·ml⁻¹ (TWE 0.0057 mg·hr·cm⁻²) TiO₂ NPs in the presence of NAC. Even at the highest TiO₂ administered dose of 500 µg·ml⁻¹ (TWE 2.86 mg·hr·cm⁻²) tested, the percentage of viable cells increased from 41% to 64%. Consequently, late apoptotic cells or dead cells were also reduced in the presence of NAC. Similar effects were observed in the cells treated with ZnO NPs (Figure 6a). Significant reduction in ROS levels was observed at all administered doses when treated with NAC (Figure 6a) ($p < 0.05$). At ZnO 1 to 25 µg·ml⁻¹ (TWE 0.0063 to 0.157 mg·hr·cm⁻²), ROS level was dropped by a quarter in the presence of NAC ($p < 0.05$). Likewise, an

increase in the percentage of viable cells or early apoptotic cells was observed in apoptosis profile (Figure 6b). The most prominent effect was observed at $25 \mu\text{g}\cdot\text{ml}^{-1}$ ZnO (TWE $0.157 \text{ mg}\cdot\text{hr}\cdot\text{cm}^{-2}$), where the proportion of viable cells increased from 15% to 50% when treated with NAC. Individually, the percentage of early, late apoptotic cells and dead cells were all reduced as viability was restored when cells were exposed to ZnO NPs in the presence of NAC compared to those exposed to ZnO alone as shown in figure 6c.

4. Discussion

Sunscreens, with NPs as their active ingredient, are widely used on a daily basis to shield from the sun's harmful UV radiation. It was estimated that teenage females could be exposed to an average daily dose of as high as $1.61 \text{ mg}\cdot\text{day}^{-1}$ of TiO_2 NPs, per kg of their body weight (Lorenz et al. 2011). In absolute terms, this would amount to about 23.5 g or $2.85 \text{ mg}\cdot\text{cm}^{-2}$ of NPs every year over the skin of an average sized teenage female (40 kg) [see supplementary notes]. Assuming application of sunscreen is on the skin of the arms, legs and face/neck, the exposure of NPs can be estimated to be about $96.9 \mu\text{g}\cdot\text{cm}^{-2}$ per application [see supplementary notes]. Previous analysis of sunscreen formulations have confirmed that NPs are present as agglomerates, and the effective size of NPs is known to influence their dermal penetration. Though a direct correlation has yet to be made, a holistic sense from previous published reports does suggest that a sweet spot of particle size does exist which is ideal for penetration. TiO_2 NPs of 10 to 60 nm penetrated across hairless mice skin after subchronic dermal exposure for 60 days (Wu et al. 2009). On the other hand, TiO_2 NPs of agglomerated size 100 to 500 nm were found to largely remain at the upper layers of the stratum corneum, however, 0.39% of these NPs were found in viable epidermis and dermis (Miquel-Jeanjean et al. 2012). This would suggest that at

least a fraction of the NPs, possible those of lower agglomerated size can penetrate across the epidermis.

Using our initially computed dermal NPs payload of $96.9 \mu\text{g}\cdot\text{cm}^{-2}$ per application and the reported penetration fraction of 0.39%, the amount of NPs that could potentially penetrate across healthy skin to reach viable cells in the epidermis and dermis would be approximately $1.13 \pm 1.06 \mu\text{g}\cdot\text{cm}^{-2}$ [calculated from skin penetration data reported in the literature (Miquel-Jeanjean et al. 2012); see supplementary notes]. In skin models used to mimic damaged and/or irradiated skin, a scenario that is probable in the context of sunscreen users, the penetration would be higher, at up to $3.20 \pm 2.98 \mu\text{g}\cdot\text{cm}^{-2}$ (Miquel-Jeanjean et al. 2012). Similar findings have been reported for ZnO NPs, albeit it is not entirely clear if ZnO effects are due to ionic or particulate penetration (Gulson et al. 2010; Ilves et al. 2014). It is important to note that even though the fraction is small, in terms of particle number concentration this can still equate to millions (if not more) of NPs penetrating to the viable layers of the skin where they could induce damage on skin cells and tissues, and could also translocate to other parts of the body (Wu et al. 2009; Osmond-McLeod et al. 2014). As such, it is necessary to further our understanding of the effects of skin cell-nanomaterial interactions in the face of potential long term, low-level penetration in real-life scenarios, in order to identify potential hazards. Basic toxicity of TiO_2 and ZnO NPs to different cell types have been extensively investigated. Most of these studies positioned ROS production as the primary regulatory mechanism of NP-mediated toxicity (Zhao and Ng 2014). However, mechanistic details including the cross talk between ROS induction and other physiological events such as autophagy, apoptosis, and inflammation are still obscure. The aim

of our study is therefore to plug this knowledge gap and to show the potential multitude of cytotoxic effects at physiologically relevant levels of exposure to both TiO₂ and ZnO NPs.

In this study, we showed, that TiO₂ and ZnO NPs exposure to human epidermal keratinocytes resulted in a range of cytotoxic responses which are consistent with previous published reports (Sharma et al. 2009; Kocbek et al. 2010). ZnO NPs were found to be more cytotoxic to keratinocytes than TiO₂ NPs. This was despite the fact that in our study, most of the crucial physicochemical properties were comparable for the two NPs used. The various physiochemical properties such as, size and shape, aggregation/agglomeration, solubility, surface area, surface charge, surface chemistry and hydrophilicity (Yang et al. 2018), has been reported to play a role in toxicity (Gatoo et al. 2014). However, both NPs used in our study have similar primary sizes and shapes and both exhibited similar zeta potential and significant agglomeration in media (Table 1). Interestingly, while both NPs are known to endocytose into cells, most reports show that the cellular uptake of ZnO NPs is lower compared to TiO₂ (Miquel-Jeanjean et al. 2012; Demir et al. 2017; Wang et al. 2018). Despite that, however, ZnO NPs are inevitably reported to cause higher toxicity (Kocbek et al. 2010). Therefore, the eventual cellular fate was thought to play a bigger role in ZnO NPs' higher toxic potency. While TiO₂ NPs remain in particulate form after being taken up by the cells, ZnO NPs dissolve readily in the acidic environment of lysosomes and result in a burst release of Zn²⁺. This can disturb the carefully maintained balance of Zn²⁺ in the cell and cause a host of negative effects like damage to mitochondria, lysosomes and impairment of the autophagic flux. Impaired autophagic flux can result in accumulation of damaged mitochondria, which could lead to excessive ROS generation (Zhang et al. 2017). Impaired autophagic flux and excessive ROS generation by ZnO NPs were

also observed in this study. Finally, Zn^{2+} is also able to enter the nucleus and cause DNA damage, which has been shown by our previous studies (Kathawala et al. 2015b). Interestingly, our previous study have also shown that when both TiO_2 and ZnO NPs were exposed together to cells, TiO_2 attenuated the toxicity of ZnO by reducing the concentration of free intracellular Zn^{2+} . TiO_2 also mitigated the Zn^{2+} from entering the nucleoplasm to interact with DNA and protected the keratinocytes from genotoxicity induced by ZnO NPs (Kathawala et al. 2015a; Kathawala et al. 2015b). It is, therefore, more probable that biochemical interactions between cells and NPs are better readouts than physicochemical properties of the NPs, in understanding differences in NP induced toxicity.

Although keratinocytes could survive exposure to lower concentrations of NPs, our results show that subtoxic concentrations of TiO_2 and ZnO NPs can induce significant levels of ROS generation. Mitochondria are often a major target of NP-mediated ROS, causing damage to membrane phospholipids and consequent mitochondrial membrane depolarization (Xia et al. 2006), as observed in the current study with increased generation of MSO. Both TiO_2 and ZnO NPs have been reported to cause oxidative stress-mediated apoptosis in human epidermal keratinocytes (Shukla et al. 2011; Ryu et al. 2014). By using the antioxidant NAC, we confirmed that oxidative stress was an important mediator of TiO_2 and ZnO NP-induced cell death. However, it was interesting to note that NAC regulated recovery was more significant in ZnO exposed than in TiO_2 exposed cells, suggesting mechanistic differences in cell interaction between the two NPs.

Induction of autophagy was observed at administered dosages as low as $100 \text{ fg}\cdot\text{ml}^{-1}$ for TiO_2 and $50 \text{ pg}\cdot\text{ml}^{-1}$ for ZnO. This is evidenced by increased levels and enhanced degradation of LC3 II and p62, which appears to be a cell survival response according to the results of the current study (supplementary figure 7). According to the literature, both particles can be uptaken by the endosomes and accumulate in early endosomes, late endosomes and amphisomes (Kocbek et al. 2010). Interestingly, amphisomes are cytoplasmic compartments that play a functional role in collecting autophagic substrates from endosomes and delivering them to the lysosomes (Gordon and Seglen 1988; Fader and Colombo 2009). This corroborates with the increased LC3 II and p62 degradation profiles that we observed. The current study further indicated the roles of TiO_2 and ZnO NPs induced autophagy on the inflammatory pathway. The NF- κ B pathway has long been implicated as a prototype pro-inflammatory signaling pathway which is activated by cytokines like IL- 1α and TNF- α (Karin and Ben-Neriah 2000). Triggering of NF κ B-p65 (Rel A) - a subunit of NF κ B transcription complex, in turn, leads to activation of pro-inflammatory genes (Baeuerle and Henkel 1994; Barnes and Adcock 1997; Tak and Firestein 2001). Hence, the effects of TiO_2 and ZnO on the activation of p65 were investigated. Our studies identified p65 as an autophagic substrate at the high administered dosage of $500 \text{ }\mu\text{g}\cdot\text{ml}^{-1}$ of TiO_2 NPs. This was corroborated by the outcome that at the low administered dosage of $100 \text{ fg}\cdot\text{ml}^{-1}$, there was a significant increase in p65 levels, which reduced to basal levels when the cells were exposed to higher administered dosage of $500 \text{ }\mu\text{g}\cdot\text{ml}^{-1}$. The activation of p65 entails its phosphorylation and subsequent nuclear translocation. We encountered a significant reduction in phospho-p65 levels at the high administered dosage of $500 \text{ }\mu\text{g}\cdot\text{ml}^{-1}$ of TiO_2 NPs, which would result in reduced inflammation. It is also possible that the activation of autophagy at different administered dosages of the NPs, degrades various components of the inflammatory pathway, and thus also

prevents phosphorylation of p65 (Trocoli and Djavaheri-Mergny 2011). This agrees with the significant reduction of phospho-p65 levels at the high administered TiO₂ dosage of 500 µg·ml⁻¹, when autophagy is maximally activated. Interestingly, herein lies the difference in the effect of ZnO NPs, whereby we do not see significant autophagic degradation of p62 although there is significant degradation of LC3 II. p62 is a multidomain adaptor protein at the crossroads of autophagy and proteasomal pathways. Inhibition of proteasomal activity causes induction of p62 (Ishii et al. 1997; Ishii et al. 2000; Kuusisto et al. 2001; Thompson et al. 2003). Moreover, it is reported that increased oxidative stress activates transcription factor Nrf2 which results in increased mRNA and protein levels of p62 (Ishii et al. 2000). Hence, we postulate that the increase in p62 could be a result of increased oxidative stress due to exposure to the higher administered dosage of ZnO NPs or inhibition of the proteasomal pathway. Additionally, it is known that increased ROS generation activates redox-sensitive transcription factor NF-κB as well as secretion of the pro-inflammatory cytokine TNF-α (Tsou et al. 2010). However, our studies indicated no change in the activation of NF-κB in response to different concentrations of ZnO NPs, in contrast to TiO₂ exposure. In essence, NP-induced autophagy may provide a protective mechanism to overcome oxidative stress, DNA damage and also inflammation.

At low administered dosages of either TiO₂ or ZnO, a secondary inflammatory pathway was also activated, as observed by increased TRAF6 levels. TRAF6 is an important player in the alternative inflammatory pathway, which is triggered by binding of the CD40 ligand to its receptor, recruiting TRAF6 proteins and downstream processing of p100 protein to p52, thus regulating transcriptional activation of inflammatory proteins (Viatour et al. 2005). Although there is no direct evidence in keratinocytes, TNF-α and IL-1β are known to increase TRAF6

expression in cultured astrocytes (Lu et al. 2014). TRAF6, in addition to activating inflammation, also activates autophagy in a beclin-mediated pathway (Shi and Kehrl 2010). Interestingly, our results indicate that over-activation of autophagy due to acute exposure to the NPs also led to the degradation of TRAF6, forming a feedback loop. Hence, at higher dosages of TiO₂ or ZnO, TRAF6 levels are significantly reduced. This study demonstrates, for the first time, the degradation of TRAF6 by autophagy under the influence of TiO₂ or ZnO NPs. The effects of low and high administered dosages of TiO₂ and ZnO NPs on keratinocytes is summarized schematically in figure 7.

Accompanied by the increase in autophagy levels, there was also increased secretion of certain pro-inflammatory cytokines at high administered dosages of the NPs. Interestingly, at a very high administered dosage of 25 µg·ml⁻¹ of ZnO NPs, the secreted levels of several pro-inflammatory cytokines like IL-6 and IL-6sR were observed to be depleted. This can be due to various reasons, namely 1) high toxicity of the ZnO particles leading to cell death, which is the likely reason here as supported by cell viability and apoptosis results; 2) increased degradation of cytokines like TGF-β1 by increased autophagy activity (Ding et al. 2014); 3) reduced TRAF-6 levels due to its autophagic degradation, which may impair the production of certain cytokines like IL-6 and TNF (Yang et al. 2008). Consequently, it can be remarked that TiO₂ and ZnO NPs induce an inflammatory response in epidermal keratinocytes which may lead to apoptosis and cell death at higher dosages.

The current study provides evidence that induction of autophagy by TiO₂ NPs plays inhibitory roles against both classical p65 NF-κB-mediated and TRAF6-mediated pathways

whereas ZnO NPs play an inhibitory role against TRAF6-mediated pathway only, to reduce the inflammatory response. This supports our observation that there were overall lower levels of secreted cytokines in keratinocytes exposed to TiO₂ NPs compared to ZnO NPs, and hence the observed outcome that ZnO NPs exerted greater acute toxicity on keratinocytes compared with TiO₂ NPs.

5. Conclusion

It is generally accepted that dermal exposure to NP does not constitute significant health risks due to low levels of NP penetration across the skin barrier. Despite this, current understanding of the physiological influence of such low levels of penetration is lacking and the jury is still out with regards to whether such influences may influence skin health and beyond. In this study, we, therefore, evaluated a range of physiological effects of TiO₂ and ZnO NPs exposure on human primary keratinocytes. We did this with special consideration given to mimic real-life exposures by using a range of NP concentrations modeled after reported levels of NP exposure and penetration, and correlating effects against the TWE which is a more relevant parameter for *in vitro* formats. We found that TiO₂ and ZnO NP exposure leads to the activation of inflammatory responses in primary keratinocytes, primarily through the TRAF6-mediated pathway. NP exposure concurrently initiates autophagy induction in keratinocytes, as a pro-survival response to NP-induced oxidative stress. Specifically, sustained NP induced autophagy promotes degradation of TRAF6, and in the case of TiO₂, reduces NF-κB activation, thereby contributing to cell survival. Nonetheless, with increasing levels of NP exposure, oxidative stress induced by TiO₂ and ZnO NPs plays a central role in the onset of inflammation and eventual cell

death by apoptosis. The physiological influence associated with dermal exposure and subsequent penetration of TiO₂ and ZnO NPs at the cellular level, therefore, hinges on the balance between autophagy and oxidative stress induction.

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Conflict of interest

The authors declare that they have no conflicts of interest.

Appendix A. Supplementary Data

Supplementary information (SI) contains additional method and result sections. Supplementary data to this article can be found online at:

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892 **Table**

893

894 Table 1. Hydrodynamic size of TiO₂ and ZnO NPs in DI water and cell culture medium

Hydrodynamic properties	TiO₂ (500 µg·ml⁻¹)		ZnO (500 µg·ml⁻¹)	
	DI Water	Medium	DI Water	Medium
Size (nm)	408	3110	440	2128
Zeta potential (mV)	35	-12	23	-11

895

896

897 Table 2. Concentration of Zn²⁺ dissolved from ZnO NPs dispersed in cell culture medium

ZnO NPs (µg·ml ⁻¹)	Zn ²⁺ (µg·ml ⁻¹)
100	9.336
50	7.144
10	3.337
5	1.868
1	0.371

898

Figure Captions

Fig. 1. *Schematic representation of the flow of calculating realistic dosage for the in vitro assessments.* Real-life Exposure was obtained from (Lorenz, Von Goetz et al. 2011). (A-B) depict the sunscreen exposure and tabulate key numbers derived from that study regarding yearly exposure to NPs in the low and high exposure individuals. (C-D) depict the penetration of the applied NPs. Penetration dynamics were obtained from (Miquel-Jeanjean, Crepel et al. 2012). (E) shows the range of doses that we test out in our in vitro toxicity experiments. As shown by the red line in the scale bar E, our tested range covers the realistic range of exposure.

Fig. 2. *Effects of NPs on viability, proliferation and oxidative stress at different dose and exposure time.* **a.** Cell viability and proliferation of keratinocytes exposed to TiO₂ and ZnO NPs for 4 hours measured by Alamar blue and Picogreen assay, **b.** Cell viability and proliferation of keratinocytes exposed to TiO₂ and ZnO NPs for 24 hours measured by Alamar blue and Picogreen assay, **c.** ROS and MSO induced in keratinocytes exposed to TiO₂ and ZnO NPs for 4 hours, **d.** ROS and MSO induced in keratinocytes exposed to TiO₂ and ZnO NPs for 24 hours. *p < 0.05, against control (untreated cells).

Fig. 3. *Effects of TiO₂ and ZnO on LC3 II, p62 and TRAF6 turnover by autophagy.* **a.** Representative immunoblots showing levels of LC3 II, p62 and Actin in keratinocytes exposed to tamoxifen (Tam) and various administered doses of TiO₂ and ZnO NPs. Graphs show the fold change in the levels of **b.** LC3 II and **c.** p62 in keratinocytes exposed to tamoxifen (Tam) and various administered doses of TiO₂ and ZnO NPs. **d.** Representative immunoblots showing levels of LC3 II, p62, TRAF6 and Actin in keratinocytes exposed to tamoxifen (Tam) and

various administered doses of TiO₂ and ZnO NPs with or without NH₄Cl/Leupeptin (NL). Graphs show the fold change in the levels of **e.** LC3 II, **f.** p62 and **g.** TRAF6 in NL treated (+) condition against without NL (-) treatment in keratinocytes exposed to tamoxifen (Tam) and various administered doses of TiO₂ and ZnO NPs. *p < 0.05, against non-NL treated group (-).

Fig. 4. *Differential inflammatory effects of low and high dosage NPs exposure in keratinocytes.* **a.** Representative immunoblots showing levels of TRAF6, phospho-p65, p65 and Actin in keratinocytes exposed to tamoxifen (Tam) and various administered doses of TiO₂ and ZnO NPs. Graphs show the fold change in the levels of **b.** p65, **c.** phospho-p65 and **d.** TRAF6 in keratinocytes exposed to tamoxifen (Tam) and various administered doses of TiO₂ and ZnO NPs. *p < 0.05, against (-), #*p* < 0.05 against TiO₂ treated. **e.** Levels of inflammatory cytokines secreted by keratinocytes at 24 hours exposure to various administered doses of TiO₂ NPs. **f.** Levels of inflammatory cytokines secreted by keratinocytes at 24 hours exposure to various administered doses of ZnO NPs. Values are expressed as mean ± S.D, n=2.

Fig. 5. *Reduction in ROS and apoptosis in keratinocytes under TiO₂ exposure with NAC for 24 hours.* **a.** ROS induced in keratinocytes treated with TiO₂ NPs with and without antioxidant NAC, **b.** Apoptosis profile of keratinocytes treated with TiO₂ NPs at administered dose 100 µg·ml⁻¹ with and without NAC, **c.** Percentage of viable cells, early apoptosis, late apoptosis and dead cells induced by TiO₂ NPs exposure at various administered doses with and without antioxidant NAC, AD- Administered dose (µg·ml⁻¹), TWE- Time Weighted Exposure (mg·hr·cm⁻²), *p < 0.05 against cells treated with TiO₂ without NAC.

Fig. 6. *Reduction in ROS and apoptosis in keratinocytes under ZnO exposure with NAC for 24 hours.* **a.** ROS induced in keratinocytes treated with ZnO NPs with and without antioxidant NAC, **b.** Apoptosis profile of keratinocytes treated with ZnO NPs at administered dose 100 $\mu\text{g}\cdot\text{ml}^{-1}$ with and without NAC, **c.** Percentage of viable cells, early apoptosis, late apoptosis and dead cells induced by ZnO NPs exposure at various administered doses with and without antioxidant NAC, AD- Administered dose ($\mu\text{g}\cdot\text{ml}^{-1}$), TWE- Time Weighted Exposure ($\text{mg}\cdot\text{hr}\cdot\text{cm}^{-2}$)* $p < 0.05$ against cells treated with ZnO without NAC.

Fig. 7. *Protective role of autophagy against TiO_2 and ZnO induced inflammation.* Schematic diagram depicting the effects of low and high administered doses of TiO_2 and ZnO NPs on keratinocytes. At low administered dosages of both NPs, there is an upregulation of autophagy and TRAF6 levels. Both the NPs exert inflammation by the release of various cytokines at higher administered dosages. Autophagy is activated in a dose-dependent manner under the influence of the NPs and plays a protective role against inflammation by (1) degradation of TRAF6 upon maximum activation. In addition to TRAF6 degradation, (2) autophagy also degrades p65, thus preventing its activation in the TiO_2 treated condition.

**Understanding the Implications of Engineered Nanoparticle Induced Autophagy in Human
Epidermal Keratinocytes In Vitro**

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Correlating in vitro dosimetry to real-life exposure

The objective of this section is to link exposure of sunscreen to dosimetry for *in vitro* testing. First we analyzed the data reported by an exposure assessment study to understand the amounts of nanoparticles that a representative sunscreen user can come into direct skin contact with. Next, we utilized data published from a penetration analysis study to assess the realistic quantities of nanoparticles that can reach viable layers of the skin and have a direct influence on cell health. Last we align the penetrative load of nanoparticles to the kinds of dosage which are usually tested by *in vitro* studies including our own, to establish the relevance of such studies and ascertain that the doses tested herein were realistic.

In the context of sunscreen application, two parameters together define dose. The first is the surface area of the skin over which a sunscreen is applied as the more it is spread, the less payload each portion the skin has to bear. Second is the amount of sunscreen which is applied over that area. To address the first parameter, we employed the Du Bois formula (Du and Du 1916) to calculate the total body surface area (TBSA):

$$TBSA = 0.007184 \times Height^{0.725} \times Weight^{0.425}$$

The average height of individuals was based on data reported by The World Health Organization (WHO), Gerontology Research Center (National Institutes of Health, USA), and the U.S. Centers for Disease Control and Prevention (CDC) (2018). The surface area for individual body parts was based on the modified Lund and Browder chart developed by the Department of Surgery, Lautoka Hospital, Lautoka, Fiji (Murari 2017).

Assessing amounts of topical nanoparticle exposure on sunscreen users

A comprehensive assessment on nanoparticle exposure through commercially available topical products was performed by Lorenz et al. (Lorenz et al. 2011). The study categorized teenage females based on two

tiers of exposure considerations. The first was based on the amount per application with three categories: low, medium and high. “Low” referred to those who use small amounts per application and have a larger body weight while “high” are those who use large amounts per application and have a smaller body weight. “Medium” lie in the middle. The second tier was based on the frequency with which the individuals use sunscreens, which ranged from 0, 10, 10-20 and more than 20 days per year.

To cover the entire spectrum, we assessed both the extremes: (A) “High Exposure” - large product amounts with highest frequency of application, and (B) “Low Exposure” - small product amounts with lowest frequency of application (Supplementary Notes Table 1).

Supplementary Notes Table 1- Estimating the exposure of sunscreen users to nanoparticles on a daily and yearly basis.

Parameter	High Exposure	Low Exposure
Body weight for this category, m_{bw}	40 kg	68 kg
Height for this category, H	147 cm [*]	178 cm [*]
Total Body Surface Area, TBSA	$1.28 \times 10^4 \text{ cm}^2$ [†]	$1.85 \times 10^4 \text{ cm}^2$ [†]
Fraction of TBSA where sunscreen is applied	4.5% (Face/Neck) 10% x 2 (Arms) 20% x 2 (Legs) <u>Total: 64.5% of TBSA</u>	4.5% (Face/Neck) 10% x 2 (Arms) 20% x 2 (Legs) <u>Total: 64.5% of TBSA</u>

^{*} Heights for females with body weights of 36.4-44.9 kg & 61.2-74.8 kg corresponds to 147cm & 178 cm respectively: <https://www.disabled-world.com/calculators-charts/height-weight.php>

[†] Du Bois Formula

Body Surface Area of Application, BSA_{app}	$8.26 \times 10^3 \text{ cm}^2$	$1.19 \times 10^4 \text{ cm}^2$
Mass of product per application, q_{Prod} (reported)	16 g	3.2 g
Product per application (per unit surface), $q_{P/SA} = q_{Prod} \div BSA_{app}$	1.94 mgcm^{-2}	$2.68 \times 10^{-1} \text{ mgcm}^{-2}$
Weight fraction of Nanoparticles, w_{Prod}	5%	5%
Nanoparticle per application (absolute), $E_{Appl} = q_{Prod} \times w_{Prod}$	0.8 g	0.16 g
Nanoparticle per application (per unit surface), $E_{Appl/SA} = E_{Appl} \div BSA_{app}$	$96.9 \text{ } \mu\text{gcm}^{-2}$	$13.4 \text{ } \mu\text{gcm}^{-2}$
Frequency of Application (daily), f_{Event}	0.082 (over 20 days per year)	0.025 (less than 10 days per year)
Nanoparticle Exposure (daily per kg body mass), E_{Total}	$1.61 \times 10^3 \text{ } \mu\text{g/day/kg}$	$5.76 \times 10^1 \text{ } \mu\text{g/day/kg}$
Yearly Nanoparticle Exposure (absolute), $E_Y = E_{Total} \times m_{bw} \times 365 \text{ days}$	23.5 g/year	1.43 g/year
Yearly Nanoparticle Exposure (per unit surface), $E_{Y/SA} = E_Y \div BSA_{app}$	$2.85 \text{ mgcm}^{-2}/\text{year}$	$0.12 \text{ mgcm}^{-2}/\text{year}$

The key takeaway from the above calculation is the amount of nanoparticles which a typical sunscreen user applies on his/her body: 1.43 g to 23.5 g every year. In terms of surface specific yearly exposures for the two extreme cases of sunscreen users the numbers are 0.12 and 2.85 mgcm⁻².

Assessing amounts of nanoparticles that penetrate the skin to reach viable cell layers

The abovementioned numbers are the absolute amounts of NPs that sunscreen users potentially apply on their skin. To evaluate the portion that could penetrate through the topical barrier to the viable layers of the skin, the dermis and epidermis, data from a penetration study performed on human skin explants was utilized (Miquel-Jeanjean et al. 2012). The study suggests that by applying realistic amounts of sunscreen (4 mgcm⁻²), only 0.19 ± 0.15 % of the TiO₂ NPs penetrated through to the viable layers of the dermis and epidermis of healthy skin. In the case of damaged and/or UV irradiated skin explants, the penetrated amounts were higher, up to 0.55 ± 0.46 %. The following table (Supplementary Notes Table 2) shows the absolute amounts penetrated per unit surface area of the skin which are reported in the study as well as those calculated from the abovementioned percent penetration for the “High Exposure” and “Low Exposure” groups.

Supplementary Notes Table 2- Calculating exposure of sunscreens and NPs in sunscreens in terms of mass per surface area of skin.

	Data Reported in (Miquel-Jeanjean et al. 2012)		Data Modelled Using Penetration Percentage Reported in (Miquel-Jeanjean et al. 2012)					
			High Exposure Group		Low Exposure Group		Recommended ^Y	
	Intact	Damaged	Intact	Damaged	Intact	Damaged	Intact	Damaged

	Skin	Skin	Skin	Skin	Skin	Skin	Skin	Skin
Sunscreen per Application (μgcm^{-2})	4×10^3	4×10^3	1.45×10^3	1.45×10^3	0.291×10^3	0.291×10^3	2×10^3	2×10^3
NP amount per Application (μgcm^{-2})	603 ± 68	590 ± 53	96.9*	96.9*	13.4*	13.4*	100*	100*
NP% in Dermis & Epidermis, (%)	0.19 ± 0.15	0.55 ± 0.46	$0.19 \pm 0.15^\dagger$	$0.55 \pm 0.46^\dagger$	$0.19 \pm 0.15^\dagger$	$0.55 \pm 0.46^\dagger$	$0.19 \pm 0.15^\dagger$	$0.55 \pm 0.46^\dagger$
NPs in Dermis & Epidermis, ($\times 10^{-3} \mu\text{gcm}^{-2}$)	1130 ± 1060	3200 ± 2980	$184 \pm 145^\ddagger$	$533 \pm 445^\ddagger$	$25.5 \pm 20.1^\ddagger$	$73.7 \pm 61.6^\ddagger$	$190 \pm 150^\ddagger$	$550 \pm 460^\ddagger$

75

76 *Based on 5% weight fraction of nanoparticles; † Based on (Miquel-Jeanjean et al. 2012); ‡ Calculated using NP%
77 in Dermis & Epidermis and NP per Application amounts; $^\gamma$ Based on standard sunscreen application guidelines
78 recommended by manufacturers to achieve the marketed and claimed SPF value.

79 The first two columns of the table show data which had been reported in the penetration study mentioned
80 above (Miquel-Jeanjean et al. 2012). For the other three groups, “High Exposure”, “Low Exposure” and
81 “Recommended”, the sunscreen applications were either based on the calculations performed above for

the two extreme cases of sunscreen users or based on the manufacturer recommended sunscreen application amounts from which sun protection factors (SPF) were calculated. Data was then modelled using the penetration percentage reported by (Miquel-Jeanjean et al. 2012) to arrive at the amounts of nanoparticles which could potentially reach the viable layers of skin.

The key takeaway, therefore, are the numbers in the last row which show the per unit surface area amount of nanoparticles which reach the dermis and the epidermis and can therefore have a direct influence on viable cells. Interestingly, the High Exposure group experience a lower payload compared to that tested in the penetration study as well as the amount which would reach the viable cells if the recommended sunscreen amounts are applied.

Comparing real penetration amounts to in vitro test doses

Typical *in vitro* tests use cellular monolayer models in which test samples are prepared as nanoparticle suspensions and the concentrations are reported as the dose. In order to compare the *in vitro* doses to the real exposure amounts discussed above, we performed two calculations. First we converted the absolute doses, previously in volumetric concentrations, into mass per surface area equivalents. Second, we calculated the Time-Weighted Exposure which is the cumulative *effective exposure* time that every fraction of the administered dose spends directly exposed to the cells after deposition using the “*In Vitro Sedimentation, Deposition and Dosimetry*” (ISDD) model to draw comparisons with the abovementioned exposure levels.

In order to convert volumetric concentrations to mass per surface area, we performed the following operation:

$$\text{mass per surface area } (\mu\text{gcm}^{-2}) = \text{concentration } (\mu\text{gml}^{-1}) \times \frac{\text{volume of suspension (ml)}}{\text{cell culture plate area}(\text{cm}^2)}$$

For example, in a typical experiment using 48-well plates (0.875 cm^2) cells were incubated with a media volume of 0.25 ml. So for a concentration of $10 \text{ } \mu\text{gml}^{-1}$, the equivalent mass per surface area would be $2.86 \text{ } \mu\text{gcm}^{-2}$. The next step in making dose relevant to reality was to consider not just the absolute amounts that cells were treated with but also the fractions of nanoparticles that would sediment over time and come into direct contact with the cell monolayers at the bottom of the cell culture vessel. The ISDD model used simulates the particle deposition profile considering two driving forces to particle motion: isotropic Brownian motion and anisotropic gravitational force. Supplementary Notes Table 3 lists the fraction of particles deposited at hourly time-points modelled for an absolute dose of $10 \text{ } \mu\text{gml}^{-1}$ for all the various cell culture templates used in this study. The deposition rate is comparable across the culture plate formats but is highly sensitive to the particle type because of variation in the agglomerate effective density – ZnO NPs having a higher effective density than TiO_2 NPs deposit much faster than the latter.

Supplementary Notes Table 3- ISDD simulations for various cell culture templates modelled at $10 \text{ } \mu\text{gml}^{-1}$. For other concentrations, the percentage values in the brackets can be used instead.

	Mass per unit surface, $\times 10^{-3} \text{ } \mu\text{gcm}^{-2}$ (percent deposited, %)					
Cell culture template	48 well plate		96 well plate		T 150 cm^2 flask	
Volume (μL)	250	250	100	100	30000	30000
Culture Surface Area (cm^2)	0.875	0.875	0.322	0.322	150	150
Nanoparticle Time (hours)	TiO_2	ZnO	TiO_2	ZnO	TiO_2	ZnO
0	1 (0.05%)	1 (0.05%)	2 (0.05%)	2 (0.05%)	1 (0.05%)	1 (0.05%)
1	364 (12.73%)	713 (24.95%)	361 (11.64%)	708 (22.81%)	364 (18.19%)	713 (35.64%)

	724	1424	719	1415	724	1424
2	(25.33%)	(49.83%)	(23.16%)	(45.55%)	(36.19%)	(71.18%)
	1084	2134	1077	2121	1084	1996
3	(37.94%)	(74.7%)	(34.68%)	(68.29%)	(54.19%)	(99.82%)
	1444	2806	1435	2827	1444	2000
4	(50.54%)	(98.22%)	(46.2%)	(91.03%)	(72.19%)	(100%)
	1804	2857	1793	3106	1802	2000
5	(63.14%)	(100%)	(57.72%)	(100%)	(90.09%)	(100%)
	2164	2857	2150	3106	1994	2000
6	(75.74%)	(100%)	(69.25%)	(100%)	(99.72%)	(100%)
	2524	2857	2508	3106	2000	2000
7	(88.33%)	(100%)	(80.77%)	(100%)	(100%)	(100%)
	2812	2857	2863	3106	2000	2000
8	(98.41%)	(100%)	(92.19%)	(100%)	(100%)	(100%)
	2857	2857	3086	3106	2000	2000
9	(99.99%)	(100%)	(99.38%)	(100%)	(100%)	(100%)
	2857	2857	3106	3106	2000	2000
10	(100%)	(100%)	(100%)	(100%)	(100%)	(100%)
	2857	2857	3106	3106	2000	2000
11	(100%)	(100%)	(100%)	(100%)	(100%)	(100%)
	2857	2857	3106	3106	2000	2000
12	(100%)	(100%)	(100%)	(100%)	(100%)	(100%)
	2857	2857	3106	3106	2000	2000
13	(100%)	(100%)	(100%)	(100%)	(100%)	(100%)
14	2857	2857	3106	3106	2000	2000

	(100%)	(100%)	(100%)	(100%)	(100%)	(100%)
15	2857 (100%)	2857 (100%)	3106 (100%)	3106 (100%)	2000 (100%)	2000 (100%)
16	2857 (100%)	2857 (100%)	3106 (100%)	3106 (100%)	2000 (100%)	2000 (100%)
17	2857 (100%)	2857 (100%)	3106 (100%)	3106 (100%)	2000 (100%)	2000 (100%)
18	2857 (100%)	2857 (100%)	3106 (100%)	3106 (100%)	2000 (100%)	2000 (100%)
19	2857 (100%)	2857 (100%)	3106 (100%)	3106 (100%)	2000 (100%)	2000 (100%)
20	2857 (100%)	2857 (100%)	3106 (100%)	3106 (100%)	2000 (100%)	2000 (100%)
21	2857 (100%)	2857 (100%)	3106 (100%)	3106 (100%)	2000 (100%)	2000 (100%)
22	2857 (100%)	2857 (100%)	3106 (100%)	3106 (100%)	2000 (100%)	2000 (100%)
23	2857 (100%)	2857 (100%)	3106 (100%)	3106 (100%)	2000 (100%)	2000 (100%)
24	2857 (100%)	2857 (100%)	3106 (100%)	3106 (100%)	2000 (100%)	2000 (100%)

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118 In order to derive the Time Weighted Exposure levels, the fraction of NPs deposited was plotted against
119 incubation time and the area under the curve was calculated. Supplementary figure depicts the rationale
120 behind this integration. As can be seen, each particle is only effectively exposed to the cells after it is

deposited. It is therefore reasonable to only consider the time the particle has spent incubated with the cells *after* being sedimented. The area-under-curve essentially measures just that for each and every particle or fraction of the administered dose. This parameter can effectively differentiate between particles of varying rate of deposition, something which traditional administered dose reporting lacks. This parameter has units representing both administered dose (in terms of particle mass, particle surface area, particle number, etc.) as well as time (in hours). For example, 1 $\mu\text{g}\cdot\text{hour}$ is equivalent to 1 μg of particles being *effective* exposed to the cells for 1 hour. If the fraction deposited against time curve has no unit for the fraction deposited (i.e. it is a percentage of administered dose), then the area-under-curve also has no units for the dose component. In this case the area-under-curve is called the TWE Factor. This can be converted to TWE by simply multiplying to the administered dose but more importantly the TWE factors can be used to compare the potency of different particles due to their deposition rate. Supplementary Notes Table 4 shows the list of TWE factors for ZnO and TiO₂ across different cell culture formats

Supplementary Notes Table 4- Time Weighted Exposure (TWE) factor values obtained from ISDD simulation for Dosimetry. The TWE factors were derived by calculating the areas under the “fraction of NPs deposited against incubation time” curve.

Nanoparticle	TiO ₂		ZnO	
Time point Culture template	4 hr	24 hr	4 hr	24 hr
96 well plate	0.926	19.650	1.822	21.777
48 well plate	1.013	20.021	1.991	21.977
150 cm ² flask	1.447	21.205	2.592	22.566

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Supplementary figure 1. Characterization of NPs

TEM observation of commercial grade **a.** TiO_2 NPs, **b.** ZnO NPs, Scale bar = 100nm, **c.** XRD diffraction peaks showing Anatase and Rutile phases of TiO_2 NPs, **d.** XRD diffraction peaks showing polycrystalline Zincite phase of ZnO NPs.

Supplementary figure 2. Simulated transport rate of NPs calculated using ISDD model

a. Fraction of administered dose of TiO₂ NPs sedimented over a given time period, b. Fraction of administered dose of ZnO NPs sedimented over a given time period.

Supplementary figure 3. Schematic depicting the derivation of Time Weighted Exposure (TWE).

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194 *Supplementary figure 4. Light microscopy images of HEK treated with NPs at different concentrations of*

195 *a. TiO_2 NPs, b. ZnO NPs*

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Supplementary figure 5. Comparison of cytotoxic effects in HEK treated with ZnO and Zn²⁺ ions at 24 hours

a. Cell proliferation of HEK exposed to various concentration of ZnO and equivalent amount of aqueous Zn²⁺ ions dissolved from ZnO NPs at that concentration, **b.** Cell metabolism of HEK exposed to various concentration of ZnO and equivalent amount of aqueous Zn²⁺ ions dissolved from ZnO NPs at that concentration. *p < 0.05 compared to cells treated to ZnO NPs using one way ANOVA.

Supplementary figure 6. Visualization of autophagosomes in keratinocytes exposed to TiO₂ and ZnO NPs by monodansylcadaverine staining.

Supplementary figure 7. Inflammatory cytokines induced in HEK under NPs exposure

Cytokine profile of HEK exposed to various administered doses of **a.** TiO_2 NPs **b.** ZnO NPs, few representative cytokines are labeled in the array.

Supplementary figure 8. Representative Immunoblots showing the effects of TiO₂ and ZnO NPs in p62 levels

a. showing levels of p62 and Actin in keratinocytes exposed to tamoxifen (Tam) and various administered doses of TiO₂ and ZnO NPs with or without NH₄Cl/Leupeptin (NL), **b.** Graph shows the fold change in the levels of p62 in NL treated (+) condition against without NL (-) treatment in keratinocytes exposed to tamoxifen (Tam) and various administered doses of TiO₂ and ZnO NPs. Values are expressed as mean ± S.E.M, N=3. Differences are significant for *p<0.05 against (-).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7