Support for the Safe Use of Zinc Oxide Nanoparticle Sunscreens: Lack of Skin Penetration or Cellular Toxicity after Repeated Application in Volunteers

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Zinc oxide is a widely used broad-spectrum sunscreen, but concerns have been raised about the safety of its nanoparticle (NP) form. We studied the safety of repeated application of agglomerated zinc oxide (ZnO) NPs applied to human volunteers over 5 days by assessing the skin penetration of intact ZnO-NPs and zinc ions and measuring local skin toxicity. Multiphoton tomography with fluorescence lifetime imaging microscopy was used to directly visualize ZnO-NP skin penetration and viable epidermal metabolic changes in human volunteers. The fate of ZnO-NPs was also characterized in excised human skin in vitro. ZnO-NPs accumulated on the skin surface and within the skin furrows but did not enter or cause cellular toxicity in the viable epidermis. Zinc ion concentrations in the viable epidermis of human skin were slightly elevated. In conclusion, repeated application of ZnO-NPs to the skin, as used in global sunscreen products, appears to be safe, with no evidence of ZnO-NP penetration into the viable epidermis or toxicity in the underlying viable epidermis. It was associated with the release and penetration of zinc ions into the skin, but this did not appear to cause local toxicity.


INTRODUCTION

Repeated long-term exposure to UVR causes skin aging and increased risk of skin cancer (Seite et al., 2010a, 2010b). Strategies to protect against the harmful effects of UVR, including regular use of efficient sunscreens, are strongly advocated as likely to lead to better health outcomes for individuals and cost savings for health care systems (Gordon et al., 2009; Mancuso et al., 2017). Although there is no direct evidence to show that zinc oxide (ZnO) nanoparticles (NPs) are effective in preventing human cancers, they are commonly used in sunscreen products, often in combination with chemical sunscreens. However, some public advocacy groups have questioned the safety of nanoparticle-based sunscreens, and a 2017 National Sun Protection Survey by Cancer Council Australia found that only 55% of Australians believed it was safe to use sunscreen every day, down from 61% in 2014 (Cancer Council Australia, 2017). A key concern is whether avoiding the use of such sunscreens could increase the incidence of skin cancer.

This article addresses the human safety of ZnO-NP, a broad-spectrum sunscreen that blocks exposure to UVA (320–400 nm) and UVB (290–320 nm) radiation and can contribute to a high sun protection factor rating when formulated optimally. Engineered ZnO-NPs are transparent sunscreen products that provide high UVR protection and cosmetic elegance (Nohynek et al., 2007) and reduce the reliance on chemical sunscreen agents that can be absorbed systemically (Gonzalez et al., 2006; Hayden et al., 1997).

A key unresolved issue with ZnO-NPs is their local safety, with suggestions that they may penetrate the stratum corneum barrier, gain access to viable epidermis cells, and cause potentially toxic responses (European Commission Scientific Committee on Consumer Safety, 2012; Senjen, 2012; Toxicology Section, Office of Scientific Evaluation, Therapeutics Goods Administration of Australia, 2017). Toxicity concerns are largely based on cell culture studies showing that ZnO-NPs can induce cytotoxicity, oxidative stress, and DNA and metabolic damage when applied for less than 6 hours to human liver cells (HepG2), epithelial cells (A431), keratinocytes (NCTC2544), and fibroblasts (Akhtar et al., 2012; Alarifi et al., 2014; Kochek et al., 2010; Sharma et al., 2009). The only study of repeated topical application of 68Zn-O-NP sunscreen to human volunteers found 68Zn ions in the blood and urine (Gulson et al., 2010, 2012), although the penetration of intact ZnO-NPs was not proven.

At this time, no studies have directly assessed the skin penetration and local safety of ZnO-NP after repeated topical
application to humans in vivo. The published studies have been limited to healthy volunteers (Filipe et al., 2009; Leite-Silva et al., 2013, 2016a, 2016b; Lin et al., 2011; Zvyagin et al., 2008) or psoriatic patients (Prow et al., 2011) after a single application and in vitro studies with excised human skin (Baroli et al., 2007; Cross et al., 2007; Holmes et al., 2016; Zvyagin et al., 2008). These have indicated that skin penetration after a single topical application of ZnO-NP is limited to the superficial layers of the stratum corneum, with minimal or no penetration into the viable epidermis. However, these single-application reports have been criticized as being nonrepresentative of actual consumer use (Senjen, 2012). This concern is exacerbated by the potential of ZnO-NP lipophilic coatings to promote skin penetration (Osmond-McLeod et al., 2013; Smijs and Pavel, 2011) and the observations of penetration of other nanomaterials into the viable epidermis (Tinkle et al., 2003) and animal (Monteiro-Riviere et al., 2011) skin.

We assessed the presence of intact ZnO-NP in skin layers by multiphoton tomography (MPT), with detection of a second harmonic generation (SHG) signal. SHG occurs in specific nonlinear materials such as ZnO-NP when two identical excitation photons interact with the matrix and combine to form a single emission photon with exactly twice the frequency and half the wavelength (Darvin et al., 2012). Changes in skin properties due to the presence of exogenous ZnO-NPs were evaluated by the addition of fluorescence lifetime imaging microscopy (FLIM) to multiphoton tomography (MPT). In this technique, fluorophores, including endogenous fluorophores in the skin, are excited by laser irradiation, and the time taken for them to return to the ground state is measured as the fluorescence lifetime, typically measured in picoseconds to nanoseconds. Changes in fluorescence lifetimes can be indicative of interactions with exogenous materials, cellular degeneration, or cellular toxicity resulting from exposure (Sanchez et al., 2015). To further explore the fate of ZnO-NP on the skin surface, parallel studies were carried out in excised human skin using a labile zinc-specific probe, ZinPyr-1, to detect zinc ions in the skin.

RESULTS
The uncoated and coated ZnO-NPs had a mean size ± standard error of the mean of 74.0 ± 3.1 nm and 65.0 nm ± 3.4 nm, respectively. Scanning electron cryomicroscopy showed that the ZnO-NPs were agglomerated in the topical product.

Localization and distribution of uncoated and coated ZnO-NPs in human skin in vivo after repeated topical application
Representative multiphoton tomography multispectral images of in vivo human skin after repeated hourly (over 6 hours) and daily (over 5 days) topical application of uncoated and coated ZnO-NPs are shown in Figures 1a and 2a, respectively. The lack of a ZnO-NP SHG signal (red) at any depth in the viable epidermis (pseudocolored green-blue) shows that ZnO-NPs were not present in the viable epidermis after either of these treatment regimes. Rather, the ZnO-NP were localized on the stratum corneum surface and in the skin furrows.

There were no statistically significant differences in the free-to-bound ratios ($\alpha_1$/$\alpha_2$) (Figures 1b and 2b) and average amplitude-weighted lifetimes ($\tau_m$) (Figures 1c and 2c) of reduced nicotinamide adenine dinucleotide phosphate (NAD(P)H) and flavin adenine dinucleotide (FAD) from the viable epidermis at various strata between untreated/vehicle controls and repeated ZnO-NP treatments. In addition, no morphological changes in the viable epidermis keratinocytes associated with cellular toxicity or apoptosis (i.e., cell shrinkage, blebbing) were observed.

Concentration profile of ZnO-NP within the skin after repeated topical application
Representative pseudocolored MPT-FLIM images of the stratum granulosum after repeated hourly and daily treatments of ZnO-NP are shown in Figures 3a and 4a, respectively; the top row shows average amplitude-weighted lifetime, the middle row displays only the ZnO-NP SHG signal (pseudocolored to indicate concentration), and the bottom row is an overlay of the two (with autofluorescence in gray).

The ZnO-NPs were detected with SHG/hyper Rayleigh scattering optical phenomena, which can be detected as an ultrafast fluorescence lifetime (almost instantaneous) emission at half the excitation wavelength. This SHG signal appeared to be localized in the skin furrows at concentrations comparable to the administered dose, with no evidence of a ZnO-NP SHG signal within the viable epidermis. Figures 3 and 4b display line intensity plots of the ZnO-NP SHG signal corresponding to each treatment group. The data show signal intensity peaks corresponding to the furrows, due to accumulation of both uncoated and coated ZnO-NPs. Within the viable epidermis, ZnO-NP signal intensities in treated groups are comparable to the nonspecific background levels in the untreated and vehicle control groups. Scatterplots of ZnO-NP concentrations after hourly and daily repeated topical application are displayed in Figures 3d and 4d, respectively.

For repeated hourly topical application (Figure 3d), the mean concentrations measured in the viable epidermis from representative regions of interest of the five volunteers were 0.48% (95% confidence interval [CI] = −0.6 to 1.57) and 0.71% (95% CI = −1.0 to 2.4) of the topical dose for uncoated and coated ZnO-NPs, respectively. These levels were not significantly different from nonspecific background untreated (0.06%, 95% CI = 0.03–0.1) and vehicle (0.08%, 95% CI = 0.15) controls.

After repeated daily topical application (Figure 4d), the mean levels of uncoated and coated ZnO-NPs in the viable epidermis measured from the representative regions of interest of five volunteers were 0.1% (95% CI = 0.07–0.14) and 0.08% (95% CI = 0.06–0.09), respectively. Again, the levels of ZnO-NPs in the viable epidermis after daily repeated treatment were not significantly different from the nonspecific background signals measured in the untreated (0.09%, 95% CI = 0.06–0.1) or vehicle (0.08%, 95% CI = 0.04–0.12) controls.

In summary, hourly or daily repeated topical treatments of ZnO-NPs did not lead to significantly elevated ZnO-NP penetration into the viable epidermis.

Labile zinc distribution in ex vivo human skin samples
ZinPyr-1 is a bright fluorescent probe based on the fluorescein structure that is cell permeable and fluoresces in the
presence of labile zinc in biological systems (Walkup et al., 2000). MPT images of ZinPyr-1–stained full-thickness skin overlaid with the SHG channel corresponding to both ZnO-NP and collagen show increased labile zinc in the viable epidermis (Figure 5). White arrows illustrate the presence of ZnO-NP on the stratum corneum surface in the ZnO-NP–dosed skin and not in control skin, although SHG is also present because of the collagen fibers of the dermis (Figure 5a–c, e–g). After repeated hourly application of either coated or uncoated ZnO-NPs, there was an increase in solubilized zinc species within the stratum corneum and the viable epidermis compared with the control (Figure 5a–c). The signal intensity for ZinPyr-1 for two selected regions of interest shows that the solubilized zinc species increased in the skin after repeated dosing of coated ZnO-NP (P < 0.05, n = 6) compared with control skin (Figure 5d).

After repeated daily ZnO-NP dosing (Figure 5e–g), an increase of solubilized zinc species was observed compared with the control. Figure 5h shows the increased ZinPyr-1 signal intensity after application of ZnO-NP in caprylic/capric triglycerides compared with control. It is evident that repeated daily application of ZnO-NP leads to an increase in the concentration of zinc species in the skin. The greatest increase was observed when coated ZnO-NPs were applied daily, compared with uncoated ZnO-NPs, although both coated and uncoated ZnO-NPs resulted in a higher solubilized zinc signal in the viable epidermis; in all but one case the differences were not statistically significant.

DISCUSSION
In summary, repeated application of ZnO-NPs to human skin in vivo over several days did not result in NP penetration through the stratum corneum or cause any visible morphological or redox changes. This finding extends the earlier observations that single ZnO-NP applications to human skin in vivo are associated with minimal skin penetration and local toxicity (Leite-Silva et al., 2013, 2016a, 2016b; Lin et al., 2011). These findings contrast with those of Bakand et al. (2017), who suggested that cellular toxicity arises after ZnO-NPs are applied to human skin fibroblasts in cell culture. Deng et al. (2015) also suggested that NP-based sunscreens can penetrate skin and cause local toxicity. However, these researchers used murine models that poorly represent the effective barrier property of human skin (Scott et al., 1986). Deng et al. (2015) argued that NP skin penetration could be minimized by using sunscreen NPs that adhere to the skin surface, yet the available evidence from single and repeated dosing studies on human skin shows that ZnO-NPs do remain on the skin surface.

This study adds to the body of knowledge used by various health-related regulatory bodies to assess the safety of topically applied sunscreen NPs (European Commission Scientific Committee on Consumer Safety, 2012; Senjen, 2012; Toxicology Section, Office of Scientific Evaluation, Therapeutics Goods Administration of Australia, 2017). In particular, we addressed the knowledge gap of what happens when coated and uncoated ZnO-NPs are applied by repeated topical administration under in-use conditions, using a highly...
sensitive, in vivo, noninvasive, multiphoton approach to evaluate NP skin penetration and distribution after realistic in-use administration of sunscreen products. Repeated hourly applications simulate in-use conditions of outdoor activities such as sports or swimming, and repeated daily application rates simulate standard everyday activities. The approach also enabled potential local skin toxicity in the various skin layers of human volunteers to be assessed for changes in skin morphology and redox state, an early marker of apoptosis change. Finally, we corroborated our in vivo findings with those of in vitro studies, in which the same ZnO-NP formulations were repeatedly applied to excised human skin and the penetrations of solid ZnO-NP and labile zinc species were quantified.

Our repeated application results extend the earlier proposition that single-dose administration of ZnO-NP (Leite-Silva et al., 2013, 2016a, 2016b; Lin et al., 2011) is not associated with any skin penetration and local toxicity. Our work also brings into context the work of Gulson et al. (2010, 2012), who reported small but significantly elevated zinc levels in the blood and urine of volunteers after repeated applications of ZnO-NP that were more than 99% enriched with the stable isotope \(^{68}\)Zn. Localized skin toxicity was not examined in the blood and urine of volunteers after repeated applications of ZnO-NP to humans under realistic in-use conditions. This is supported by the findings of O’Keefe et al. (2016) that despite causing similar levels of in vitro cytotoxicity in human monocytes and macrophages compared with chemical UV filters, ZnO-NPs were likely to be at least one order of magnitude less cytotoxic in vivo, because of their very low rates of skin penetration.

Figure 2. Effect of daily application of uncoated and coated ZnO-NPs on human skin in vivo for 5 days. Negative and vehicle controls are included. (a) Representative pseudocolored multispectral images (\(\lambda_{em} = 370–390\) nm [red], \(\lambda_{em} = 450–515\) nm [green], \(\lambda_{em} = 515–620\) nm [blue]) of different skin strata after treatment. The images were pseudocolored to show cellular autofluorescence (green/blue) and ZnO-NP (red). (b, c) Metabolic- and redox-associated changes in FLIM channel 2 (\(\lambda_{em} = 450–515\) nm) in the stratum granulosum, stratum spinosum, and stratum basale after treatment. (b) Average redox (\(a_1/a_2\)) ratios obtained after treatment. (c) Average fluorescence NAD(P)H/FAD lifetime (\(\tau_{avg}\)) obtained after treatment. Data represent mean lifetime (ps) or ratio ± 95% confidence interval (n = 5). Scale bar = 40 µm. Em, emission; FAD, flavin adenine dinucleotide; FLIM, fluorescence lifetime imaging microscopy; NAD(P)H, reduced nicotinamide adenine dinucleotide phosphate; NP, nanoparticle; ZnO, zinc oxide.
It is important to note the health care importance of our findings. The benefits of providing regular and ongoing skin protection from UVR exposure are well documented (Green et al., 2011; Hughes et al., 2013; Thompson et al., 1993; van der Pols et al., 2006), yet chemical sunscreens have been shown to be systemically absorbed (Gonzalez et al., 2006). A real concern is that the conflicting views provided to the public regarding sunscreen and NP safety may be inhibiting sunscreen use. Indeed, the recent National Sun Protection Survey by the peak Australian nongovernment cancer control organization, Cancer Council Australia, recorded a decline in the past 3 years from 61% to 55% of Australians who believe it is safe to use sunscreen every day (Cancer Council Australia, 2017). This study supports the stated position of Cancer Council Australia that on the current weight of evidence, ZnO-NPs remain on the surface and outer layers of the stratum corneum and do not reach viable cells. The potential risk of increased skin cancer due to a reduction in sunscreen use is far greater than could conceivably result from NP toxicity (Slevin, 2012). The significance of our study, in the context of a need for confidence in sun protection measures such as the use of aesthetically acceptable and effective ZnO-NP products, is evident in the estimated 13,941 new cases of and 1,839 deaths from melanoma in Australia in 2017 (Cancer Australia, 2018).

MATERIALS AND METHODS

Uncoated (Z-COTE; BASF, Ludwigshafen, Germany) and coated (Z-COTE HP1 ZnO-NP, manufacturer estimated particle size < 200 nm) were supplied as a dry white powder. Z-COTE HP1 is composed of 98% ZnO and 2% triethoxycaprylylsilane, a silicone derivative-based hydrophobic coating, whereas Z-COTE is composed of 100% ZnO. These NPs are commonly used in commercial sunscreen products. ZnO-NPs were dispersed in caprylic/capric triglyceride (10% weight/weight) for application to the skin. ZnO-NPs were characterized using a JEOL (Munich, Germany) scanning electron microscope (JSM7100F) and JEOL 1011 transmission electron microscope, as described previously (Leite-Silva et al., 2016a).

Repeated topical application of ZnO-NP

Five healthy volunteers (ages 20–30 years) with undamaged skin and no active skin disease gave written informed patient consent (University of Queensland Human Research Ethics Committee approval number 2007/197-2008001342) and abstained from using sunscreen or cosmetic skin product use for 24 hours before the experiment.

Treatments of the vehicle (caprylic/capric triglyceride) control and uncoated and coated ZnO-NPs were topically applied at a rate of 2 mg/cm² (based on the standard protocol for determination of the sun protection factor of sunscreen products) over 4-cm² areas on each volunteer’s volar forearm repeatedly over two time periods: one application every hour to the same site for 6 hours and one application per day to the same site for 5 days. The hourly and daily applications were performed at comparable sites on opposite arms.

MPT-FLIM images were acquired at the end of the hourly treatment on the first day and approximately 6 hours after the final treatment on day 5.

MPT with fluorescence lifetime imaging

A multiphoton tomograph DermalInspect (JenLab, Jena, Germany) equipped with a tunable titanium sapphire femtosecond laser Mai
Tai (Spectra Physics, Mountain View, CA) and a time-correlated single photon-counting module SPC-830 (Becker & Hickl, Berlin, Germany) was used for imaging. Time-correlated single photon-counting was performed by building up a photon distribution based on the arrival times of the detected photons for each pixel in the scanned area, constructing a decay curve over 256 time channels within a 10-ns interval (less than the 13-ns pulse width) between two laser pulses. Three Becker & Hickl photo multiplier detectors PMC-100 were integrated into the MPT system, enabling splitting of the fluorescence emission into three wavelength ranges. The bandpass filters were 400/100 nm, 482/65 nm, and 567/105 nm. To spectrally select the ZnO-NP SHG signal (and, to a degree, hyper

Figure 4. Visualization and quantification of ZnO-NPs in the viable epidermis and furrows after daily application of uncoated and coated ZnO-NP for 5 days. Negative (untreated) and vehicle controls are included. (a) First row: representative multiphoton tomography with fluorescence lifetime imaging microscopy images of the viable epidermis, pseudocolored to indicate fluorescence lifetimes (0–1,500 ps). Second row: Pseudocolored spectral images ($\lambda_{Em} = 370–390$ nm) to indicate the concentration (mg/ml) of uncoated and coated ZnO-NP. Third row: Merging of multiphoton tomography with fluorescence lifetime imaging microscopy and spectral images. (b) Line intensity plot of the ZnO-NP signal profile along a dotted line the viable epidermis after treatment. (c) Average (percentage of applied dose) ZnO-NP quantified in the regions of interest in the viable epidermis (purple box) and (d) in the furrows (green box). Scale bar = 40 $\mu$m. CCT, caprylic/capric triglyceride; Em, emission; NP, nanoparticle; ROI, region of interest; SHG, second harmonic generation; ZnO, zinc oxide.

Figure 5. Hourly (a–d) and daily (e–h) topical application of uncoated and coated ZnO-NPs on ex vivo human skin. Representative overlay images of ZinPyr-1 fluorescence (gray; $\lambda_{Ex} = 488$ nm, $\lambda_{Em} = 520–560$ nm) and second harmonic generation (pink; $\lambda_{Ex} = 800$ nm; $\lambda_{Em} = 405/10$ nm) for skin treated with (a, e) vehicle control, (b, f) coated ZnO-NP (10% weight/weight in caprylic/capric triglycerides), and (c, g) uncoated ZnO-NPs (10% weight/weight in caprylic/capric triglyceride). White arrows indicate ZnO-NP on the stratum corneum surface. Scale bar = 20 $\mu$m. (d, h) ZinPyr-1 signal intensities for the stratum corneum and viable epidermis after hourly and daily applications, respectively. Data represent the mean fluorescence $\pm$ standard error of the mean (n = 6). **P < 0.05. AU, arbitrary unit; DE, dermis; Em, emission; Ex, excitation; NP, nanoparticle; SC, stratum corneum; VE, viable epidermis; ZnO, zinc oxide.
In vitro Franz diffusion cell study using ex vivo human skin

Written informed patient consent was obtained from a female patient (age = 36 years) to donate skin after elective abdominoplasty (Queen Elizabeth Hospital Protocol-2009208). Subcutaneous fat was removed by blunt dissection, and the skin was mounted in a Franz-type diffusion cell. After barrier integrity check by transepithelial electrical resistance (Cross and Roberts, 2008), 0.1% sodium azide solution was placed in the receptor, and dose formulations were applied at 2 mg/cm² to the skin (replicating in vivo dosing). Franz cells were unoccluded, placed in a water bath at 35°C, and constantly agitated by a magnetic flea. At completion, excess formulation was removed with a moist swab, and the skin surface was blotted dry. Skin samples were then snap frozen (−80°C), embedded in optimal cutting temperature media, cryosectioned to 20 μm (CM1950 cryostat; Leica, Wetzlar, Germany), placed on a poly-l-lysine coated microscope slide, and kept frozen on dry ice until staining. A 10-μmol/L aqueous solution of ZinPyr-1, a selective fluorescent probe for labile zinc, was applied to skin sections (10 μl) and left at ambient temperature in the dark for 15 minutes.

Labile zinc distribution within ex vivo human skin samples

Imaging of ex vivo skin cryosections stained with ZinPyr-1 (n = 6) was conducted on a Zeiss LSM 710 multiphoton microscope equipped with an argon gas laser (488 nm) and a tunable titanium sapphire laser (Mai Tai, Spectra Physics). ZinPyr-1 was detected at excitation wavelength 488 nm and emission wavelengths 520–560 nm (laser power of 1.2 μW and 500 gain). SHG was detected at 800 nm using a bandpass filter of 405/10 nm at a constant laser power of 9 mW and 700 gain.

Statistics

The data presented in Figures 1–4 (n = 5) and Figure 5 (n = 6) are expressed as mean with 95% CI. Statistical differences between the means were evaluated using a Kruskal-Wallis test with post hoc Dunn multiple comparisons test. Statistical differences between the ZinPyr-1 intensities were assessed using one-way analysis of variance with post hoc Bonferroni multiple comparison test. All analyses were conducted using GraphPad Prism (La Jolla, CA), with a P value of less than 0.05 defined as statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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