Literature Review on the safety of titanium dioxide and zinc oxide nanoparticles in sunscreens
Scientific review report
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Summary

This scientific review report is limited to the review of safety concerns surrounding zinc oxide (ZnO) and titanium dioxide (TiO₂) nanoparticles (NPs) present in sunscreens. The two main issues considered in this review are the evidence for the ability of these NPs to penetrate the skin to reach viable cells and the potential toxicity exerted by them.

The TGA has been continuously monitoring the emerging scientific literature in this area and working cooperatively with international regulatory agencies to ensure that appropriate regulatory action is undertaken if any unacceptable risk of harm/toxicity is identified.

A review on these issues was first published by the Therapeutic Goods Administration (TGA) in 2006 which was updated in May 2013. This review is a further update to include relevant literature that has been published between May 2013 and August 2016.

The majority of in vitro studies (using both animal and human skin) and in vivo studies have shown that both ZnO and TiO₂ NPs either do not penetrate or minimally penetrate the stratum corneum and underlying layers of skin. This suggests that systemic absorption, hence toxicity, is highly unlikely.

In conclusion, on current evidence, neither TiO₂ nor ZnO NPs are likely to cause harm when used as ingredients in sunscreens and when sunscreens are used as directed.

1. Introduction

The inorganic UV filters ZnO and TiO₂ have been used as ingredients in sunscreens for over three decades. However, one apparent disadvantage of ZnO and TiO₂ is that in their macroparticulate (bulk) form in sunscreens, they are visible on the skin as an opaque layer resulting in reluctance of consumers to use the products. This undesirable visual effect has been addressed by decreasing the particle size of these metal oxides to nanoparticle (NP) form (see Section 2). When used in this NP form, these oxides cannot be seen on the skin but retain or even augment their UV-sunscreening properties.

In the USA, patents on TiO₂ and ZnO NPs were filed in the 1980s (Wang & Tooley 2011), although in Australia the use of TiO₂ and ZnO NPs in sunscreens began later. These NPs are particularly useful in sunscreens because of their intrinsic ability to filter ultraviolet (UV)A as well as UVB wave length spectra, thus providing broader protection than any other sunscreening agent.

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This scientific review report is limited to the review of safety concerns surrounding ZnO and TiO₂ NPs present in sunscreens. The two main issues considered in this review are the evidence for ability of these NPs to penetrate the skin to reach viable cells and the potential toxicity induced by them.

(Nanoparticle OR nanoparticles OR nanoparticulate OR nanoscale OR nanosize OR nanomaterials) AND (zinc oxide OR titanium dioxide) AND (sunscreen OR sunblock OR sun block OR sun screen OR UV blockers OR physical sunscreen) AND (safety OR toxicology OR toxic OR safe)
2. Nanoparticle characteristics

For the purpose of this report, the definition of TiO$_2$ and ZnO NPs includes materials within the nanosize range of 1 to 100 nm. Nanosized TiO$_2$ and ZnO exist in three separate states: primary particles (5-20 nm), aggregates (30-150 nm) and agglomerates (1-100 microns). Primary particles cluster together to form aggregates and are the smallest units present in a final sunscreen formulation (Butler et al., 2012; SCCP, 2007; Schilling et al., 2010; Wang & Tooley, 2011). The larger agglomerates form when aggregates bind loosely during the manufacturing process (Schilling et al., 2010). These are not efficient UV absorbers so they need to be broken down into the more efficient aggregates, which are chemically bound. This review includes assessments of NP preparations that contain aggregates and agglomerates. The latter have been included because of their yet unclear potential to disaggregate and disagglomerate when applied on the skin in a sunscreen formulation.

Although agglomerates are not normally found in sunscreen formulations, they may form on the skin surface after application of sunscreens, suggesting fewer primary NPs would be available for skin penetration (Tran & Salmon, 2010). A preliminary study by Bennett et al. (2012) suggests that exposure to sunlight can lead to disaggregation of TiO$_2$ NPs which facilitated penetration when tested on isolated porcine skin sections. Thus, any potential hazard linked with nanosized particles is likely to be reduced if these particles aggregate and form structures above the nanoscale, which then do not dissociate into smaller NPs, thus reducing their ability to penetrate the skin.

In addition to changes in physico-chemical properties due to aggregation and agglomeration, TiO$_2$ NPs (but not ZnO NPs) adopt different crystal forms: rutile, anatase and amorphous. The rutile form, or a mixture of rutile and anatase, is generally used in sunscreens (Dussert & Gooris, 1997). The anatase form is substantially more photocatalytic and adheres more strongly to skin than the rutile form (Turci et al., 2013; Osmond-McLeod et al., 2016). The form and size of the NPs used in the studies summarised in this review are specified if these parameters were described in the study.

3. Dermal exposure

3.1 Skin irritation/sensitisation

The potential effects of photoirritation and photosensitisation of ZnO were discussed in the European Commission’s Scientific Committee on Consumer Safety (SCCS) report (SCCS, 2012); there was no evidence of any positive findings in two photoirritation studies and two photosensitisation studies after topical application to intact skin of human volunteers. Furthermore, in a review of photoprotection, Lautenschlager et al. (2007) reported that neither TiO$_2$ nor ZnO NPs possess notable skin irritation or sensitisation properties when used in sunscreens on humans.

A number of studies published since these safety reviews have assessed the potential of ZnO NPs or TiO$_2$ NPs to cause skin irritation (reversible skin damage), corrosion (irreversible necrotic damage extending into the dermis) or sensitisation in the absence of UV or non-UV light. In a mouse model of atopic dermatitis employed to determine whether ZnO NPs could exacerbate the inflammatory pathology associated with allergic skin conditions, ZnO NPs were applied to the skin of mice sensitised to ovalbumin (Ilves et al., 2014). ZnO NPs were detected in the epidermal and dermal layers of the skin of both sensitised and non-sensitised mice in regions where skin had been tape-stripped (a procedure employed to mimic skin damage). The number of immune cells (T lymphocytes, neutrophils, eosinophils and mast cells) infiltrating the skin in antigen-sensitised mice was attenuated in the presence of ZnO NPs. Serum levels of total IgE and ovalbumin-specific IgE, however, were enhanced by ZnO NPs in antigen-sensitised mice. These
data demonstrate that ZnO NPs can potentially penetrate the dermis, exert an anti-inflammatory effect on allergic skin but enhance IgE generation. Despite the potential for an allergic response, the authors conclude that the benefits of using sunscreens to combat the development of skin malignancies outweigh the possible risks of exacerbating allergic symptoms (Ilves et al., 2014).

In other studies, ZnO NPs failed to cause irritation or sensitisation. ZnO NPs (20-50 nm in diameter) did not cause acute dermal toxicity (when applied topically at a dose of 2g/kg for 24 h to rats) or dermal irritation (when applied topically at a dose of 0.5g for up to 4 h) in rabbits. ZnO NPs also failed to induce skin sensitization in guinea pigs during or up to 48 h after the elicitation phase after an induction phase that involved weekly application of NPs for 3 weeks (Kim et al., 2016).

A dose-dependent increase in ear swelling (used as an indicator of skin irritancy) was observed in female balb/c mice after dermal administration of TiO2 NPs (50 µl, up to 10 % (w/v)) but skin sensitisation was not observed (Auttachoat et al., 2014). In balb/c mice, pre-treatment with TiO2 NPs (dermal application; 4 mg/mL) exacerbated subsequent sensitisation induced by dinitrochlorobenzene (Smulders et al., 2014). Exposure of EpiDerm™, a commercially available human 3D skin model composed of human-derived epidermal keratinocytes, to TiO2 NPs (1 mg/mL every minute for 1 h) did not induce signs of dermal irritation (Miyani et al., 2016). TiO2 NPs, ZnO NPs or a mixture of the two (at a final concentration of 25% (w/v)) did not cause irritation or corrosion when applied to a 3D human skin model, KeraSkin, or to intact rabbit skin (Choi, et al., 2014).

3.2 Skin penetration

Skin penetration of TiO2 and/or ZnO NPs through the outer layer to the viable cells within the deeper skin layers was investigated in a large set of in vitro and in vivo studies assessed for this report. As outlined below, the findings of the great majority of these studies indicated an apparent inability of TiO2 and ZnO NPs to reach viable cells in the dermis. In addition, the SCCS reported in 2012 that “in sunscreens, ZnO NPs can be considered to not pose any risk of adverse effects in humans after application on healthy, intact or sunburnt skin”. Similarly, the Committee concluded that “TiO2 nanomaterials in a sunscreen formulation are unlikely to lead to systemic exposure to nanoparticles through human skin to reach viable cells of the epidermis, dermis, or other organs” (Chaudhry et al., 2015).

3.2.1 In vitro studies

Numerous in vitro studies have investigated the properties of nanoparticulate TiO2 and ZnO, using various cultured cell lines and different methodological approaches. This multiplicity and variability in methodology has generated uncertainty regarding the relevance of individual study findings. For example, Lanone et al., (2009) and Park et al., (2009) identified a clear difference in the sensitivity between cytotoxicity assays with cell type being investigated. Further, numerous issues were identified by Park et al., (2009) concerning the conduct of in vitro studies leading to a conclusion that “much work has yet to be done before in vitro toxicity assays can play any role of importance within the risk/hazard assessment of nanomaterials”.

The complex nature of interactions of nanomaterials (nanoparticles/quantum dots) with cell cultures was highlighted in an in vitro study (Ryman-Rasmussen et al., 2007), where difficulties with interpretation of results from this type of study were identified. A review (Stone et al., 2009) on the development of in vitro systems for nanotechnology highlighted the complexities associated with in vitro studies investigating safety of nanoparticles and the need to establish validated experimental approaches to determine any biologically relevant nanoparticle-induced hazard.

For example, Nohynek et al. (2008) provided a comparative analysis of dermal penetration between different animal species, rating them in the order: rabbit > rat > pig > monkey > man.
They noted that pig and rat skin is up to 4 and 9-11 times, respectively, more permeable than human skin.

This inter-species variability in skin penetration demands caution when extrapolating positive findings from animal studies to potential for hazard in humans. The results of Wu et al., (2009) can at present only be accepted as preliminary, and speculative in terms of the hazard in humans, particularly since their findings in porcine skin in vitro and in vivo clearly indicate that TiO$_2$ NPs penetrated into the stratum corneum, stratum granulosum, prickle cell layer and basal cell layer, but not into the dermis. Importantly, only 4 nm TiO$_2$ NPs reached the basal cell layer; hence the ability of TiO$_2$ NPs to penetrate porcine skin was shown to be size-dependent. This is an important finding considering that a great majority of TiO$_2$ NPs exist in sunscreen formulation as relatively large aggregates or even larger agglomerates after the skin application (Schilling et al., 2010).

A few studies that reported findings suggesting that TiO$_2$ NPs could penetrate beyond the stratum corneum suffered from important methodological limitations that put the validity and extrapolation of these findings to humans in doubt (Tan et al., 1996, effect not statistically significant; Wu et al., 2009, effect observed in hairless mouse only; Sadrieh et al., 2010, tissue contamination likely).

Evidence that other forms of nanomaterials can penetrate into the skin has been presented. Vogt et al. (2006) examined dermal penetration of NP fluospheres and showed that 40 nm particles can penetrate hair follicles and reach the Langerhans cells surrounding the follicles in the dermis. However, this penetration was only achieved after tape-stripping debris from the follicle opening, as well as removing a significant proportion of the protective stratum corneum and subjecting the human skin samples in vitro to a prolonged (16 h) exposure to the test material in a special humidified chamber. Such conditions are artificial and unlikely to mimic typical use of sunscreens. Zhang & Monteiro-Riviere (2008) showed that Fluosphere NPs (quantum dots of 14 and 18 nm) did not penetrate normal, flexed or even tape-stripped skin in rats, while minimal penetration was observed in abraded skin. This was confirmed by Campbell et al. (2012) who demonstrated that Fluosphere polymeric NPs (20-200 nm) penetrated only into the superficial layers of the stratum corneum of partially damaged pig skin strips, again suggesting that the NPs are unlikely to reach viable cells below the stratum corneum.

The effect of absorption enhancers (oleic acid and/or ethanol) on the skin penetration of ZnO NPs was studied (Kuo et al., 2009) in vitro using skin samples from hairless mice. Results indicated that ZnO NPs penetrated into the stratum corneum, but not into viable cells in the presence of these penetration enhancers. This finding suggested that ZnO NPs are unlikely to penetrate into viable skin cells in humans, since this could not be achieved in an animal model with higher dermal permeability compared with human skin, even in the presence of penetration enhancers.

The possibility that hair follicles may play a part in percutaneous absorption of nanomaterials has also been examined. Otberg et al. (2008) found that hair follicles represented very limited sites for potential absorption (up to 0.1% of the skin surface), assuming they were permeable to NPs, although ZnO or TiO$_2$ NPs were not investigated. In another study, Lademann et al. (1999) failed to detect TiO$_2$ in the epidermal or dermal tissue outside of the follicles. Similarly, Pflücker et al. (1999) found TiO$_2$ NPs only in trace amounts in the upper part of the hair follicle without any evidence of uptake into follicular epithelium. Although Bennat & Muller-Goymann (2000) concluded that their findings in vitro suggested that TiO$_2$ NPs seems to penetrate human skin probably via the sebum lipids of the hair follicle, they could not detect any TiO$_2$ NPs within the sebum matrix of hair follicles.

More recent studies have essentially supported these findings that suggest neither ZnO NPs nor TiO$_2$ NPs penetrate the dermis to any significant degree. TiO$_2$ NPs (minimum 90% anatase solution) applied to either healthy or abraded human skin samples in Franz cells (an apparatus that can assess transdermal permeability) at a concentration of 1 g/L was detected only in the
epidermal, and not the dermal, layer (Crosera et al., 2015). Three preparations of ZnO nanoparticles were tested for their ability to penetrate human skin: ZnO NPs suspended in either capric caprylic triglycerides (commonly used in commercial sunscreen preparations), a pH 6 aqueous solution (to mimic natural skin pH) or a pH 9 aqueous solution (which minimises damage to the epidermis) (Holmes et al., 2016). When applied to human skin samples in vitro for 48 h, none of these ZnO NP formulations penetrated beyond the superficial layers of the stratum corneum into the intact viable epidermis. It was shown, however, that ZnO hydrolysis caused an increase in Zn\(^{2+}\) ions within viable epidermal layers. The authors suggest that any systemic increases in zinc detected in humans after sunscreen application in previous studies (such as in Gulson et al., 2010) represent Zn\(^{2+}\) ions and not ZnO NPs (Holmes et al., 2016). Similarly, in another study, zinc was detected only in the stratum corneum and not in the viable underlying skin layers when Nanosun™ 65/30 was applied to porcine skin (Detoni et al., 2014).

Therefore, the currently available evidence suggests that the likelihood of penetration of TiO\(_2\) or ZnO NPs beyond the surface layers into viable cells of the dermis is extremely low.

### 3.2.2 In vivo studies

Potential systemic absorption of ZnO NPs when applied in a sunscreen formulation was assessed under real-life conditions of sunscreen use by human volunteers over a period of five days (Gulson et al., 2010). Blood and urine levels of \(^{68}\)Zn (based on changes in \(^{68}\)Zn/\(^{64}\)Zn ratio) from \(^{68}\)ZnO particles in sunscreens increased in all subjects over the period of exposure, with significantly higher levels of \(^{68}\)Zn in females exposed to a sunscreen containing NPs of \(^{68}\)ZnO, compared to females exposed to larger \(^{68}\)ZnO particles and males exposed to particles of both sizes. However, concerns relating to the methodology and conduct of the study impact on the validity of these findings. Most critically, the determination of \(^{68}\)Zn ion concentration in the blood as evidence of systemic or dermal exposure to ZnO NPs does not necessarily prove the dermal penetration of, or systemic exposure to, ZnO NPs. The method used to detect \(^{68}\)Zn from the sunscreens could not distinguish between \(^{68}\)ZnO particle or \(^{68}\)ZnO\(^{2+}\) ions. However, these observations may still be relevant to human risk assessment because they show that the level of exposure to zinc following short term and real-life use of ZnO NP-formulated sunscreen was negligible compared with the zinc levels normally found in the body and in a typical daily diet. Thus, such exposure is unlikely to be of any concern to human health.

Subsequently, Filipe et al., (2010) investigated dermal penetration of TiO\(_2\) and ZnO NPs in human volunteers after in vivo application, using punch biopsy analysis. Localisation of TiO\(_2\) and ZnO NPs in damaged skin was evaluated using skin tape stripping before sunscreen application. Interestingly, the removal of the stratum corneum resulted in negligible adhesion of the sunscreen NPs were did not penetrate beyond the stratum corneum; neither titanium (Ti) nor Zn\(^{2+}\) was detectable in the dermis after 48 h exposure to the sunscreen under occlusion.

The issue of altered penetration of nanosized ingredients through damaged skin (i.e., diseased or sunburnt) was reviewed (Newman et al., 2009). It was concluded that penetration through compromised skin was likely to be similar to normal skin (findings of other studies were cited). For psoriatic skin, application resulted in the sunscreen only being found on the top layers of the stratum corneum. Additional in vitro data (Senzui et al., 2010) for TiO\(_2\) NPs applied on skin (from micropig) damaged using tape stripping showed no penetration of Ti into viable skin cells.

Sadrieh et al. (2010) conducted a dermal penetration study in minipigs using three different forms of TiO\(_2\) NPs, including one TiO\(_2\) NP currently used in a commercial sunscreen formulation. Using detailed transmission electron microscopy analysis, they detected minimal penetration of TiO\(_2\) NPs into sub-epidermal layers of the skin, but found no evidence of TiO\(_2\) NP penetration via expected routes such as follicular lumens. In concert, these results suggest that the risk of TiO\(_2\) NP penetration into the dermis via hair follicles is very low.

Two-photon microscopy (a fluorescence imaging technique that permits the imaging of living tissue) was used to visualize the distribution of ZnO NPs in vivo after topical application of a
commercially available sunscreen on human skin. There was no penetration of ZnO nanoparticles beyond the stratum corneum, including microscopic skin wrinkles, where this layer is significantly thinner (Breunig et al., 2015). ZnO NPs (< 100 nm in diameter), both non-coated or coated with triethoxycaprylylsilane (to enhance hydrophobicity), did not alter the hydration or barrier function of skin when applied topically to the forearms of human volunteers for 4 h. Coated and uncoated ZnO NPs localized predominantly within the stratum corneum and furrows of the epidermis. There was evidence of minimal penetration into the stratum granulosum of the viable epidermis only for coated ZnO NPs. Neither formulation altered the redox state of the cells in the viable epidermis (Leite-Silva et al., 2013). Furthermore, neither coated nor uncoated ZnO NPs penetrated into the viable epidermis of occluded skin of volunteers (occlusion was achieved by placing impermeable adhesive dressings over the sites of application). Penetration of ZnO NPs into barrier-impaired (tape-stripped) skin, which models damaged/sunburnt skin, was minimal and limited to the outermost layer of viable cells in the epidermis. There were no changes in cellular morphology or evidence of apoptosis in viable epidermal cells after topical application of either NP preparation for 6 h (Leite-Silva et al., 2016).

A study was conducted in mice to determine whether ZnO NPs could be detected in organs after topical application of sunscreen (Osmond-Mcleod et al., 2014). Female, immune-competent, hairless SKH:QS mice were topically administered a total of 0.6 g of sunscreen containing ZnO NPs over a period of four days. Zn$^{2+}$ ions derived from ZnO NPs was detected in the liver, kidneys, brain, heart, lung, blood and in the spleen and in the fetal liver when applied topically to dams. The method of zinc detection could not distinguish between free Zn$^{2+}$ originating from ZnO NPs and zinc present in ZnO NPs, therefore it could not be determined whether ZnO NPs penetrated the skin. Irrespective of the source of the Zn$^{2+}$ ions, there was no increase in the total zinc levels in organs suggesting that endogenous zinc homeostatic mechanisms were not altered by sunscreen application. Serum amyloid A1 and A2 levels (markers for acute phase inflammation) were reduced in ZnO NP-treated mice compared to controls and were equivalent to controls in pregnant mice, indicating that the sunscreens did not induce or exacerbate inflammation. The authors subsequently extended these investigations with longer-term studies (36 weeks) that included groups of mice treated with sunscreens that contained TiO$_2$ NPs and included groups that were subject to UV irradiation in combination with the sunscreens (Osmond-Mcleod et al., 2016). These studies revealed that sunscreen use was associated with significant protection from skin malignancies (which were observed in the UV irradiated mice) and that tissue zinc levels and hepatic titanium levels were not elevated in sunscreen-treated mice. The authors concluded that repeated, long-term application of sunscreen to mice did not result in significant dermal penetration, accumulation in organs and adverse biological outcomes. It is important to consider that these studies were conducted in hairless mice, which have skin that is significantly more permeable to NPs than intact human skin.

The potential for TiO$_2$ NPs to penetrate intact skin was assessed in fair-skinned individuals (Coelho et al., 2016). Sunscreen containing TiO$_2$ NPs was applied once daily for 3 days to 2 participants and once daily for 8 days to 4 participants (2 mg/cm$^2$ over a 5cm$^2$ area). One day after the final sunscreen application, biopsy specimens (approximately 4 mm in diameter) were acquired from each participant. In total, fewer than 30 confirmed TiO$_2$ nanoparticles or their aggregates were detected in all of the skin specimen samples and their abundance did not correlate with skin depth. The majority of NPs were detected mainly in the dermis surrounding hair follicles. Follicular accumulation of NPs has not been associated with penetration into viable skin cells (Filipe et al., 2009; Lademann et al., 1999). In another study conducted in two human volunteers, a commercial sunscreen containing TiO$_2$ NPs was applied (2 mg/cm$^2$ over a total skin area of approximately 600 cm$^2$) 6 times a day for seven consecutive days to both intact and UVB-sunburned skin (Næss et al., 2015). TiO$_2$ NPs were detected in viable cells of the epidermal layer in both intact and damaged skin; 1 to 10 TiO$_2$ NPs were detected in a total of 3–4 skin sections each measuring approximately 200 µm × 60 µm. This study did not ascertain whether the trace levels of NPs detected in viable epidermal cells were translocated into the systemic circulation.
Therefore, based on these *in vitro* studies using both animal and human skin, and *in vivo* studies that included studies with human subjects, it can be concluded that ZnO or TiO$_2$ NPs minimally penetrate the underlying layers of skin, with penetration largely limited to the *stratum corneum*. This suggests that the likelihood of NPs causing cytotoxicity or pathology in internal organs or tissues is very low since systemic absorption is highly unlikely.

## 4. Cytotoxicity and genotoxicity

### 4.1 Photocatalysis and *in vitro* cytotoxicity

Both ZnO and TiO$_2$ NPs can generate, via UV-induced photocatalysis, reactive oxygen species (ROS) such as superoxide anions or hydroxyl radicals (Li *et al*., 2012). ROS can damage cellular components and macromolecules (such as lipids, proteins and nucleic acids) and ultimately cause cell death if produced in excess or if they are not neutralised by innate antioxidant defences (Manke, *et al*., 2013; Redza-Dutordoir *et al*., 2016). ROS derived from the photocatalysis of NPs are cytotoxic to a variety of cell types (Cai *et al*., 1991; Dunford *et al*., 1997; Wamer *et al*., 1997; Afaq *et al*., 1998; Serpone *et al*., 2001; Uchino *et al*., 2002; Long *et al*., 2006; Kang *et al*., 2008; Braydich-Stolle *et al*., 2009; Liu *et al*., 2010; Yin *et al*., 2010 and Xue *et al*., 2010).

TiO$_2$ or ZnO NP-generated ROS have been documented to induce DNA damage and cytotoxicity in variety of cell types. These include Chinese hamster ovary (CHO) cells (Uchino *et al*., 2002), HepG2 cells (hepatocellular carcinoma cells; Shi *et al*., 2015), Caco-2 cells (human colon carcinoma cells; De Angelis *et al*., 2013), mouse bone marrow mesenchymal stem cells (Syama *et al*., 2014), normal mouse or human fibroblasts (Seker *et al*., 2014), HUVECs (human umbilical vein endothelial cells; Chen *et al*., 2014), SW480, DLD-1 and NCM460 cells (human epithelial cells; Setyawati *et al*., 2015), mouse podocytes (Xiao *et al*., 2016), A549 cells (human non-small cell lung cancers; Wang *et al*., 2014; Ivask *et al*., 2015), 16HBE14o- cells (human bronchial epithelial cells; Yu *et al*., 2015), RAW264.7 cells (mouse macrophages) and human glial (D384) and neuronal (SH-SY5Y) cell lines (Cocchi *et al*., 2015). In addition, ZnO NPs affected metabolic parameters, such as glycogenolysis and gluconeogenesis, in hepatocyte cell lines (Filippi *et al*., 2014) and interfered with the cell cycle in human intestinal epithelial cells (Setyawati *et al*., 2015). TiO$_2$ NPs could also induce a pre-malignant phenotype in AGS cells (human gastric epithelial cells) characterised by enhanced DNA damage and proliferation, apoptosis resistance and increased invasiveness (Botelho *et al*., 2014). Treatment with ROS scavengers or antioxidants such as N-mercaptopropionyl-glycine (Xiao *et al*., 2016) or N-acetylcysteine (Setyawati *et al*., 2015) could prevent NP-induced cytotoxicity, highlighting the central role oxidative stress plays in NP-induced cell death induction pathways.

Manufacturers of NP-containing sunscreens attempt to block the potential production of ROS by coating NPs or adding anti-oxidant compounds to the sunscreen formulation (Tran & Salmon, 2010). Coating materials include aluminium hydroxide (Al(OH)$_3$), polymers and inert oxides of silica while anti-oxidant compounds include vitamins (A, E, C). For example, Fisichella *et al.* (2012) demonstrated that Al(OH)$_3$-coated TiO$_2$ NPs that were surface-treated by polydimethylsiloxane polymer (which have been included as an ingredient in some sunscreens) significantly attenuated ROS production compared to unmodified TiO$_2$ NPs and were not toxic to Caco-2 cells. It has been shown, however, that the integrity of the Al(OH)$_3$ surface layer can be disrupted, principally by Ca$^{2+}$ and OCl$^-$ ions (which are present in swimming pool water, for example.). This exposes the TiO$_2$ NPs to photocatalysis and the potential for the generation of free radicals. Thus, NPs may be stripped of their surface modifications under certain circumstances. A study into the cytotoxic properties of ZnO NPs coated with a TiO$_2$ NP shell revealed that the inherent cytotoxic properties of the former were markedly reduced by curtailing the release of Zn$^{2+}$ ions and decreasing the contact area of the ZnO NP by the TiO$_2$ NP
shell (Hsiao & Huang, 2011b). Thus, if cytotoxicity is largely related to the release of Zn\(^{2+}\) ions from the particle surface, it is likely that these potentially harmful effects of ZnO NPs can be effectively controlled by particle coating.

Other approaches to curtailing ROS formation have been investigated. It was demonstrated that coatings based on silicon dioxide were highly effective in ameliorating UV-induced TiO\(_2\) NP-derived ROS (Carlotti et al., 2009; Tsuji et al., 2007). ROS formation can also be attenuated by the addition of additives to the matrix the NPs are suspended in; for example, Nanosun™ 65/30, a commercial product consisting of 30 nm ZnO particles dispersed in medium-chain triglycerides at a concentration of 65% (w/v), significantly attenuated UV-induced ROS generation when applied to porcine skin in vitro (Detoni et al., 2014).

The biochemical and cellular signalling events that have been associated with the induction of ROS, DNA damage and cell death mediated by ZnO or TiO\(_2\) NPs include the activation of NF-κB, a master transcriptional regulator of proinflammatory responses (Setyawati et al., 2015), proinflammatory cytokine secretion (De Angelis et al., 2013), disruption of calcium homeostasis (Yu et al., 2015), activation of executioner caspases (Syama et al., 2014; Wang et al., 2014), induction of endoplasmic reticulum stress and caspase 12 activation (Chen et al., 2014; Yu et al., 2015), and the disruption of mitochondrial membrane potential (Filippi et al., 2014).

Of greater relevance to the potential adverse effects of NPs in sunscreens, the cytotoxicity of TiO\(_2\) and ZnO NPs has been investigated in various human skin models, principally HaCaT cells (transformed keratinocytes derived from histologically normal skin), human or animal-derived skin samples exposed to NPs ex vivo, and in human volunteers. When applied to HaCaT cells, TiO\(_2\) NPs reduced cell viability and induced membrane damage at concentrations at or greater than 0.7µg/cm\(^2\) regardless of the exposure time (24 h, 48 h or 7 days) (Crosera et al., 2015). A significant reduction in cell viability and induction of ROS, which were both enhanced by UVB irradiation, were also observed when HaCaT cells were incubated with 10-500 µg/mL TiO\(_2\) NPs for 2 h (Rancan et al., 2014). It was also demonstrated that ZnO NPs (100 nm in diameter) were internalised and induced dose- and time-dependent cytotoxicity in HaCaT cells; a 50% reduction in cell viability was observed when cells were incubated with 200 µg/mL ZnO NPs for 24 h or 50 µg/mL for 48 h or 72 h. Annexin V and propidium iodide staining confirmed that ZnO NPs induced cell death by apoptosis. Preceding apoptosis, ZnO NPs induced the expression of apoptosis-related genes (such as BAX, PUMA and NOXA), ROS, DNA damage and cell cycle arrest at the G2/M checkpoint (Gao et al., 2016).

Addition of three preparations of TiO\(_2\) NPs (1nM, 100% rutile; 21nm, anatase: rutile 4:1; and 12nm, 100% anatase) to HaCaT cells for 24 h induced oxidative stress (superoxide and hydrogen peroxide induction), activated pro-apoptotic proteins (caspase 8/9), down-regulated anti-apoptotic proteins (bcl-2) and induced apoptosis (which was not enhanced by UV-C irradiation). These effects were comparable in magnitude between the different NP preparations. In addition, all NP formulations did not alter the expression of β-catenin and E-cadherin (which regulate the epithelial to mesenchymal transition where cells undergo complex biochemical and morphological changes, increasing their potential for malignant transformation) and inhibited proliferation of human lung cell lines (Wright et al., 2016). These data suggest that TiO\(_2\) NPs could induce apoptosis but could not induce tumourigenic changes in HaCaT cells.

In contrast, some studies have not demonstrated NP-mediated cytotoxicity in skin or skin-derived cells. Although HaCaT cells simultaneously exposed to anatase TiO\(_2\) NPs and to UVA irradiation exhibited impaired mitochondrial activity, oxidative stress and membrane damage (Miyani et al., 2016), this formulation did not generate phototoxic effects in the EpiDerm™ commercial human 3D skin model, which is composed of human-derived epidermal keratinocytes (Miyani et al., 2016). In another study, NPs were internalised in HaCaT cells treated with TiO\(_2\) NPs (5-100 µg/mL for 24 h) and were distributed throughout the cytoplasm and phagosomes without causing cell death or perturbing cell cycle progression. However, mitochondrial dysfunction was observed that caused ROS formation (Tucci et al., 2013). Finally,
there was no significant increase in oxidative stress in skin samples obtained from human volunteers after a 2 h exposure to sunscreen containing ZnO NPs regardless of whether the treated skin was intact or tape-stripped (Hang, et al., 2015).

### 4.2 In vivo cytotoxicity

The toxicity of ZnO and TiO$_2$ NPs has been assessed in a number of animal models through a variety of administration routes.

In inhalation studies conducted in rodents, ZnO NPs caused mild acute pulmonary and systemic inflammation, pulmonary cell damage and a reduction in the antioxidant capacity of the lungs (Gosens et al., 2015; Liu et al., 2015). A robust pulmonary inflammatory response was also observed in rats administered TiO$_2$ intratracheally that was characterised by extensive leukocyte infiltration and ROS formation (Hurbánková et al., 2013). It has also been shown that inhaled ZnO NPs could potentially cause eosinophilic airway inflammation (Huang et al., 2015). TiO$_2$ NPs caused pulmonary hyperplasia and inflammation in the lungs in a 28 day study conducted in mice (Yu et al., 2015) and have also been implicated in causing kidney pathology in mice, where intratracheal instillation elevated serum blood urea nitrogen levels and caused a significant increase in renal oxidative stress and renal fibrosis markers (Huang et al., 2014). Other studies have shown that ZnO NPs cause only minor changes in the lungs; in a chronic inhalational study conducted in mice, administration of ZnO NPs (daily exposure for 13 weeks) resulted in only minimal pulmonary inflammation and toxicity (Adamcakova-Dodd, et al., 2014).

TiO$_2$ and ZnO NPs can also cause pathological changes *in vivo* when administered via oral, intravenous or intraperitoneal routes. Evidence of cardiovascular pathology was observed in rats after daily oral administration of TiO$_2$ NPs at doses of up to 30 mg/kg for 90 days. Mice that were orally administered TiO$_2$ daily for 6 months exhibited lower body weight gain and significant kidney pathology attributed to NP-induced oxidative stress (Gui et al., 2013). TiO$_2$ NPs caused liver damage and induced oxidative stress, DNA damage and clastogenic changes in hepatocytes when administered intraperitoneally to mice for 14 days at doses of up to 100 mg/kg (Shukla et al., 2014). Rats orally administered ZnO NPs (at up to 500 mg/kg per day; 90 days) had mild perturbations in some haematological and biochemical parameters and mild pathological features in the stomach and pancreas at the highest dose (Kim et al., 2014). Similar findings were reported in a study with a similar protocol, where in addition to these pathological features, retinal atrophy was also observed in the high dose group (Park et al., 2014). Orally or intravenously administered ZnO NPs failed to cross the blood brain barrier in a 28 day repeat dose study conducted in rats (Shim et al., 2014) but both ZnO and TiO$_2$ NPs were detected within neurons in a mouse study where NPs were administered orally for 21 days at a dose of 500 mg/kg (Shrivastava et al., 2014). In the latter study, evidence of oxidative stress was evident in the liver and brain and the levels of some neurotransmitters (norepinephrine, dopamine and 5-hydroxytryptamine) within the cerebral cortex were significantly increased in both ZnO NP- and TiO$_2$ NP-treated mice. Finally, in a study conducted in human volunteers TiO$_2$ NPs (as a single oral 5 mg/kg dose; 15 nm or 100 nm in size) were not absorbed systemically because of particle agglomeration in the gastrointestinal tract (Jones et al., 2015).

### 4.3 Genotoxicity

The genotoxicity and/or mutagenicity of ZnO and TiO$_2$ NPs have been addressed by several studies and reviews. Three unpublished studies investigating photomutagenicity effects (possibly linked to ROS formation) of micronized (≤200 nm) ZnO had been reviewed by the Scientific Committee Cosmetic Non-Food Products (SCCNFP 2003) to the European Commission, which concluded that “[M]icronised material (ZnO) has been found to be clastogenic, possibly aneugenic and inducing DNA damage in cultured mammalian cells in vitro, under the influence of UV light”. In the same review, the SCCNFP (2003) noted that micronised ZnO was non-
photomutagenic in an Ames test. However, the general validity of Ames assay findings in nanoparticulate genotoxicity testing has been questioned by Landsiedel et al., (2009) and Warheit & Donner (2010) because NPs may not penetrate the bacterial cell wall. Nevertheless, the OECD guidance manual on nanoparticle genotoxicity testing includes the Ames assay in the list of in vitro methods that should be included in application dossiers submitted in the EU (Warheit & Donner, 2010).

A review by Nohynek et al., (2008) suggests that possible photogenotoxicity associated with in vitro exposure to ZnO NPs may be influenced by UV radiation-induced increases in background sensitivity of experimental cell cultures. This could partly explain why levels of photogenotoxicity determined by Dufour et al. (2006) using the micronucleus assay (following exposure to ZnO) were relatively small (2 to 4-fold increase) compared with a potent effect of a known photo-clastogenic agent. Consequently, Nohynek et al. (2008) questioned the validity of evidence from in vitro studies presented by Dufour et al. (2006) implicating ZnO NPs as a photogenotoxic agent, and the same review documented data suggesting that the TiO2 NPs were not genotoxic. Furthermore, TiO2 NPs were found to be of low hazard based on a series of toxicity studies conducted by Warheit et al. (2007), and Nohynek et al. (2008) concluded that the hazard associated with the use of TiO2 and ZnO NPs appears to be low. This view was recently confirmed by the review conducted by industry representatives (Schilling et al., 2010), which stated that "the in vitro genotoxic and photogenotoxic profiles of these nano-structured metal oxides are of no consequence to human health".

A variety of transformed cell lines, as well as primary cell cultures, have been used to investigate the potential genotoxic effects of ZnO and TiO2 NPs. The findings of Gopalan et al., (2009), who investigated the photogenotoxic effects of TiO2 and ZnO NPs in human lymphocytes and in sperm cells, were inconsistent. Whereas lymphocytes showed significant levels of photogenotoxicity in a concentration-dependent manner for both oxides, the sperm showed greater photogenotoxicity at the lowest concentrations of both TiO2 and ZnO. The relevance of these findings to dermal exposure remains unclear. As discussed by McCall et al., (2013), a better interpretation of the results could be made if it had been accompanied by physico-chemical characterisation of the NPs used in this biological study.

TiO2 NPs (uncoated anatase and coated rutile forms, average crystal size 20 nm for both) were not cytotoxic or genotoxic in vitro (using rat liver epithelial cells in micronucleus assay) in the presence or absence of UV light (366 nm) (Linnainmaa et al., 1997). The authors concluded that TiO2 NPs do not cause direct chromosomal damage and suggested that tumour responses to TiO2 NP dust observed in experimental animals were most likely a consequence of inflammatory effects rather than genotoxicity. In a more recent study, TiO2 NPs induced clastogenic changes in human lymphocytes at concentrations of up to 300 µg/ml, however, a dose-dependent effect was not observed (Tavares et al., 2014). Both ZnO and TiO2 caused dose-dependent increases in DNA damage (assessed by comet assays) in human lymphocytes at concentrations up to 500 ppm (Khan et al., 2015).

ZnO NPs induced cytotoxicity and genotoxicity (at concentrations of up to 100 µg/mL) in a rat kidney epithelial cell line (NRK-52E) (Uzar et al., 2015). In human peripheral blood lymphocytes, ZnO caused cytotoxicity only at very high concentrations (>0.5 mM for 24 h) but caused DNA damage (both single- and double-strand breaks; assessed by alkaline and neural comet assays, respectively) at 10µM (Swilinska et al., 2015). In this study it was shown that a significant proportion of the DNA damage was attributed to the action of NP-induced ROS. ZnO NPs were not cytotoxic to human embryonic kidney cells (HEK293) or mouse embryonic fibroblast cells (NIH/3T3) but caused dose-dependent genotoxicity and clastogenicity (as assessed by comet and micronucleus assays, respectively) and induced cell-anchorage independent growth (an attribute indicative of metastatic potential) in both of these cell lines (Demir et al., 2014). ZnO NPs induced clastogenic changes in Chinese hamster lung fibroblasts (assessed by cytokinesis block micronucleus assay) but was found not to be mutagenic in the Drosophila somatic mutation and recombination test (which assesses the potential of a substance to induce loss of...
heterozygosity resulting from DNA damage or clastogenic changes) (Reis et al., 2015). Topical application of ZnO NP to female SKH mice caused mild skin damage, ROS generation, apoptosis, leukocyte infiltration, DNA damage and induction of COX-2 (an inflammatory marker) in the skin. All of these parameters were augmented by UV irradiation (Pal et al., 2016a; Pal et al., 2016b).

Other studies have not demonstrated a genotoxic effect by TiO$_2$ or ZnO NPs. TiO$_2$ NPs were internalised by immortalised human skin fibroblasts, but did not induce cytotoxicity (assessed by clonogenic survival assay) or clastogenicity (assessed by scoring chromosomal aberrations during metaphase) after a 24 h exposure at concentrations of up to 100 µg/cm$^2$ (Browning et al., 2014). ZnO NPs (20 nm or 70 nm diameter, positively or negatively charged) did not significantly increase the number of revertant Salmonella typhymurium and Escherichia coli colonies (with or without metabolic activation) in bacterial reverse mutation assays (Kwon et al., 2014). In the same study, all ZnO NP types failed to induce clastogenic changes in vitro in Chinese hamster lung fibroblasts (at a concentrations up to 15µg/mL) and in vivo in rats (as assessed by bone marrow micronucleus assay) administered ZnO NPs at up to 2000 mg/kg. Similarly, in another study, injection of rats with TiO$_2$ NPs (approximately 20 nm in diameter, 5 mg/kg administered once intravenously) did not induce genotoxicity or clastogenicity in the bone marrow, as assayed by comet and micronucleus assays (Dobrzyńska et al., 2014). In Caco-2 cells, only ZnO, and not TiO$_2$, induced micronuclei and DNA damage (Zijno et al., 2015). Finally, there was no evidence of DNA damage in HaCaT cells after exposure to ZnO (presumably micronised form; ≤200 nm) in the presence or absence of UV irradiation (SCCNFP 2003).

The SCCS (2012) comprehensive review of ZnO NPs assessed both in vitro and in vivo studies on photo-mutagenicity/genotoxicity and concluded that there is no conclusive evidence to ascertain whether or not ZnO NPs pose a mutagenic/genotoxic, photo-toxic or photo-mutagenic/genotoxic risk to humans. A similar position was upheld in their review of TiO$_2$ NPs (Chaudhry et al., 2015). Since skin penetration is limited to the upper layers, it is very unlikely that harmful effects would occur at the systemic level in humans.

5. Conclusion

There is conclusive in vitro evidence that in the presence of UV light, ZnO and TiO$_2$ NPs can induce ROS, which have the capacity to damage cellular components. Furthermore, ZnO NP- and TiO$_2$ NP-mediated cytotoxicity and genotoxicity have been demonstrated in a wide range of cell types. In addition, there are numerous studies that demonstrate a diversity of potential pathological sequelae upon administration of ZnO and TiO$_2$ NPs to experimental animals via a range of administration routes.

However, a number of factors need to be considered in order to draw sound conclusions from this evidence with regard to potential NP toxicity from topically applied sunscreens in humans. Of paramount importance is the finding that the vast majority of studies do not demonstrate NP skin penetration; the current weight of evidence suggests that TiO$_2$ and ZnO NPs do not reach viable skin cells (even in compromised skin) or the general circulation, but rather remain on the skin surface and in the outer layer of the stratum corneum, a surface layer of non-viable, keratinized cells. It is therefore highly likely that if sunscreens are used as is intended, NPs from sunscreens applied dermally will not achieve significant concentrations in the systemic circulation.

Consequently, it is highly unlikely that NPs will induce the cytotoxic responses or pathological outcomes outlined in this review in the in vitro and animal studies, respectively. The data from the reviewed in vitro experiments should be interpreted with caution given that the findings from studies conducted in cell lines are of limited value in assessing the potential toxicity NPs pose to humans from topically applied sunscreens. Similarly, the limitations of the reviewed animal studies, where NPs were administered at relatively high concentrations through
exposure routes that are not relevant in the context of sunscreen use and at high frequency, should also be acknowledged. Given the majority of studies found no evidence of skin penetration of NPs when applied dermally, it is highly unlikely that the high systemic NP concentrations attained in these experimental animals would be achieved in people, even if accidental intake occurred via these non-dermal routes. Therefore, any deductions made regarding the safety of topically applied sunscreens in humans by extrapolating these findings in animals to humans, are of limited value.

It is also crucial to emphasize that NPs present in sunscreens are modified to reduce their potential to generate ROS, which largely mediate NP-induced cytotoxicity and genotoxicity. During the manufacturing process the surface of the NPs may be coated to reduce the formation of ROS, even after UV exposure. Sunscreens may also contain antioxidants in order to neutralise ROS generated by NPs. Additionally, endogenous protective mechanisms, such as antioxidant activity mediated by a range of intracellular enzymes and factors, will likely protect against the damaging effects of oxidative stress generated by any exposure to nanoparticles. Minimal dermal penetration of NPs is likely to be adequately counteracted by these natural cellular defences.

In conclusion, on current evidence, neither TiO₂ nor ZnO NPs are likely to cause harm when used as ingredients in sunscreens. The current state of knowledge strongly indicates that the minor risks potentially associated with NPs in sunscreens are vastly outweighed by the benefits that NP-containing sunscreens afford against skin damage and, importantly, skin cancer.

6. Bibliography


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Position of Titanium Dioxide Stewardship Council:


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# Version history

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