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REVIEW ARTICLE

Safety of titanium dioxide nanoparticles in cosmetics

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Abstract

Titanium dioxide (TiO_2) is widely used in a variety of products including cosmetics. TiO_2 in its nanoparticle form (nano-TiO_2) is now the only form used as an ultraviolet (UV) filter in sunscreens, but also in some day creams, foundations and lip balms. While its efficacy as a UV filter is proven in the prevention of skin cancers and sunburns, some concerns have been raised about its safety. Indeed, considering its small size, nano-TiO_2 is suspected to penetrate dermal, respiratory or gastrointestinal barriers, disseminate in the body and therefore constitute a potential risk to the consumer. At the skin level, most studies performed in humans or animals showed that nano-TiO_2 did not penetrate beyond the outer layers of *stratum corneum* to viable cells and did not reach the general circulation, either in healthy or in compromised skin. The Scientific Committee on Consumer Safety (SCCS) considers nano-TiO_2 as a non-sensitizer and as mild- or non-irritant to skin and concludes in no evidence of carcinogenicity (supported by the European Chemicals Agency), mutagenicity or reproductive toxicity after dermal exposure to nano-TiO_2. According to the SCCS, nano-TiO_2 from sunscreens does not present any health risk when applied on the skin at a concentration up to 25%. However, the SCCS does not recommend the use of nano-TiO_2 in formulations that may lead to exposure of the consumer's lungs by inhalation (sprayable products and powders). Indeed, even if human data are sparse and inconsistent, lung inflammation was reported in animals. In 2016, the EU Cosmetic Regulation made nano-TiO_2 as an authorized UV filter, except in products that could lead to exposure of the lungs. After oral exposure, nano-TiO_2 absorption and toxicity are limited. The incidental oral exposure to nano-TiO_2 contained in lip balms is thus not expected to induce adverse health effects.
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Conflict of interest

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Introduction

Titanium dioxide (TiO_2) is widely used in a variety of products including paints, cosmetics, orthodontic composites and food. As a food additive, it is usually used as anticaking or whitening agent or to enhance the colour and sheen of food.[1–5] In cosmetics, TiO_2 may be used either as a white pigment in its microcrystalline form only[6] or as inorganic ultraviolet (UV) filter, primarily in sunscreens, but also in some day creams, foundations and lip balms, to provide protection against the known carcinogenic effects of UV radiation.[6] TiO_2 as a UV filter was used in its microparticulate form in the first marketed sunscreens, but formulated as such, it was difficult to apply and left a white residue after application.[5] The introduction in the 1980s of colourless, ultrafine particles of TiO_2 ranging from 1 to 150 nm in size reduced these unfavourable characteristics while maintaining the sunscreens' photoprotective capability against both UVA and UVB. TiO_2 in its nanoparticle form (nano-TiO_2) is now the only form used as a UV filter.

While nano-TiO_2 has proven its efficacy as UV filter in the prevention of skin cancers and sunburns, some concerns have been raised about its safety.[7] First, nano-TiO_2 is photoreactive with a resulting increase in reactive oxygen species (ROS) known to be implicated in cellular damage.[8] This issue has been solved by coating nanoparticles with alumina or silica, to quench the production of ROS. In addition, as coating improves the dispersion of TiO_2 nanoparticles and their compatibility with other

ingredients within sunscreen formulations, nano-TiO_2 is always used in its coated form in cosmetics.

A second important concern was that considering its size in the nano range, nano-TiO_2 is suspected to penetrate dermal, respiratory or gastrointestinal barriers, disseminate in the body and therefore to constitute a potential risk to the consumer.[9]

The first scientific opinion on the safety of TiO_2 as a UV filter at a maximum of 25% in cosmetic products was adopted in 2000 by the SCCNFP.[10] However, as this opinion related to TiO_2 irrespective of its particle size, the Scientific Committee on Consumer Safety (SCCS) reviewed the safety of nano-TiO_2, taking into account abnormal skin conditions and the possible impact of mechanical effects on skin penetration.[11] The SCCS concluded in 2014 that 'based on the currently available scientific evidence which shows an overall lack of dermal absorption of TiO_2 nanoparticles', the use of nano-TiO_2 at a concentration up to 25% as a UV filter in sunscreens could be 'considered to not pose any risk of adverse effects in humans after application on healthy, intact or sunburnt skin'.

Although sunscreens and other cosmetics providing UV protection are used through skin application, they can be available as sprayable products, which may also expose consumer lungs to nano-TiO_2 by inhalation.[12] As the SCCS opinion dealt only with dermal applications of nano-TiO_2, the SCCS published another opinion not recommending the use of nano-TiO_2 in spray applications that could lead to exposure of the lungs to nano-TiO_2 by inhalation.[13] Following this opinion, the EU Cosmetic Regulation made nano-TiO_2 an authorized UV filter, except in spray products.[14] The International Agency for Research on Cancer (IARC) has classified TiO_2, in the bulk form, as a possible carcinogen for humans (Group 2B) when inhaled, based on evidence in experimental animals. In addition, in their last opinion published in 2018, the SCCS has concluded that the information was insufficient to allow assessment of the safety of use of nano-TiO_2 in spray applications that could lead to exposure of the lungs.[12]

Finally, as some manufacturers can also use nano-TiO_2 in UV-protecting lip balms that may be incidentally ingested, the potential harmful effects of nano-TiO_2 used in cosmetics should also be considered in the context of oral ingestion.[15]

The objective of the present document is to review safety data concerning nano-TiO_2 in cosmetic products to provide UV protection, based on data available in the SCCS and ANSES opinions and data available in the scientific literature since those opinions were published.

Methods

The SCCS recently published several opinions related to the use of nano-TiO_2 as a UV filter.[11–13] Furthermore, the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) recently published a collective expert appraisal report which summarized toxicological data of nano-TiO_2 after inhalation exposure.[16] These data are used in the current review. In addition, to retrieve updated relevant articles, a systematic search of the safety data related to skin exposure published from 1 January 2014 to 31 January 2019 was performed in the PubMed database, by using the terms 'titanium dioxide' AND 'skin' OR 'penetration' OR 'absorption'. The articles were screened by two reviewers based on titles and abstracts; only those dealing with the safety of nano-TiO_2 were selected.

Nano-TiO_2 types and physicochemical characteristics

TiO_2 particles ranging from 200 to 400 nm are mostly used to whiten or opacify many consumer products (e.g. paints, papers, toothpastes, sunscreens).[17] Nano-TiO_2 that range from 1 to 100 nm is used in particular as an automotive catalytic converter and UV protection agent, promoting either dispersion or resistance to photoactivity.[17] The surface of nano-TiO_2 can be modified by inorganic metal oxides (e.g. alumina and amorphous silica) and organic molecules (e.g. polyols and dimethicone) according to its future usage. Several types of nano-TiO_2 can therefore be produced with different physicochemical characteristics such as the crystal structure (i.e. anatase and rutile phases), shape (nanotubes, nanowires and nanosphere), particle size, surface area and surface modification (e.g. surface treatment or coating).[18] Depending on these characteristics, each nano-TiO_2 type will be treated specifically in the human body and has its own toxicity profile.[17] The forms of nano-TiO_2 used in sunscreens are mostly the rutile crystal structure or a rutile/anatase combination, rarely the anatase structure only.[11] It should be pointed out that many toxicological studies of nano-TiO_2 use AEROXIDE® P25 (Evonik, Essen, Germany), consisting mostly of nano-TiO_2 <25 nm under their anatase form (80–90%), as their object of research.[19] However, P25 is generally used in catalytic and photocatalytic industrial purposes but not in cosmetics. Furthermore, P25 nano-TiO_2 is not coated to reduce photoactivity, whereas nano-TiO_2 used in sunscreens has surface modification like coating and consists mainly in the less photoactive rutile type. The significance of the results of P25-based studies for risk assessment of nano-TiO_2 use in sunscreens may be therefore questionable.[19]

Absorption and distribution

Dermal exposure

Dermal/percutaneous absorption in healthy skin More than 20 studies dealing with dermal penetration of nano-TiO_2 in healthy skin, performed *in vitro*, *ex vivo* or *in vivo* either in animals or in humans, were analysed in detail by the SCCS in 2013–2014.[11] These studies reflected 'real life' by using sunscreen formulations containing TiO_2. According to most of them, nano-TiO_2 generally stays on the skin after application of a sunscreen formulation; only a small proportion of the nanoparticles are likely to penetrate deeper in the *stratum corneum*, and they do not reach the viable epidermis or dermis cells.[11] Only 2 studies suggested a

cutaneous penetration of nano-TiO_2 into the *stratum granulosum* when using human foreskin grafts transplanted onto SCID mice[20] or in the dermis of minipigs.[21] However, in the latter, only an insignificant amount of scattered and isolated nanoparticles was detected by electronic microscopy. Furthermore, considering that pigskin was shown to be up to 4 times more permeable than human skin,[22] it is difficult to extrapolate this effect in humans *in vivo*. Moreover, several studies demonstrated that nano-TiO_2 does not penetrate beyond the *stratum corneum* of pigskin when coated with cetyl phosphate, manganese dioxide or trimethoxycaprylylsilane.[15]

The limited nano-TiO_2 skin penetration to the *stratum corneum* has been mostly confirmed by the updated literature, including a more recent individual study performed both *in vitro* and *in vivo* in rats[23] and studies reported by the Australian Therapeutic Goods Administration (TGA), a part of the government health department, in their updated scientific review report concerning the safety of TiO_2 and ZnO nanoparticles in sunscreens in 2016.[24] The three studies reported by the Australian TGA that were published after the SCCS opinion were performed *in vitro*[25] or *in vivo* in humans.[26,27] The study performed *in vitro* and one of the studies performed *in vivo* in six subjects[26] confirmed the limited nano-TiO_2 skin penetration, which was not associated with diffusion into viable cells. However, the other studies performed *in vivo* in humans, which assessed repeated nano-TiO_2 dermal exposure in two subjects, did not confirm these results.[27] Indeed, 7 days after application of a commercial sunscreen containing nano-TiO_2 (2 mg/cm^2 over a total skin area of 600 cm^2) six times a day, nano-TiO_2 was detected beyond the *stratum corneum*, into viable cells in the epidermis, with a transmission electron microscope equipped with an EDX.[27] Data on *in vivo* dermal/percutaneous absorption in human skin are presented in Table 1.

Dermal/percutaneous absorption in compromised skin Five studies performed in mice ($N = 1$), pigs ($N = 2$) or humans ($N = 2$) analysed in detail by the SCCS demonstrated that nano-TiO_2 contained in a sunscreen formulation did not penetrate compromised skin, either stripped/dermabraded, sunburnt (simulated with UVB radiations) or psoriatic.[11] Even if nano-TiO_2 penetrated into deeper areas of the *stratum corneum* in psoriatic skin than in healthy skin, they did not reach living cells in either psoriatic or healthy skin[11] (Table 1).

Two out of the three studies published after the SCCS opinion, and assessing dermal/percutaneous absorption in compromised skin, confirmed these results. The study by Xie *et al.*,[23] performed in rats, showed that nano-TiO_2 did not penetrate the *stratum corneum* in skin either intact or slightly damaged with 2% sodium lauryl sulphate (SLS) solution, both *in vitro* and *in vivo*. Moreover, in the study by Crosera *et al.*,[25] performed on human skin *in vitro* by using static diffusion cells, nano-TiO_2 was only detected in the epidermis of both healthy and needle-abraded skin samples after a 24-h exposure to a sonicated suspension of nano-TiO_2 (606 μg/cm²; Table 1). Of note, in that study, the total amount of nano-TiO_2 was similar in both healthy and needle-abraded skin, indicating that lesions did not increase permeation. In the third study, performed *in vivo* in humans and described above, nano-TiO_2 was detected in viable cells in the epidermis, beyond the *stratum corneum*, in sunburnt skin simulated with UVB radiations, 0.4 J/cm^2[27] (Table 1). However, these results should be considered with caution as only one type of sunscreen was tested in only two volunteers.

Distribution after dermal exposure A study assessed the nano-TiO_2 distribution after topical application to the dorsal skin of hairless rats for 56 days.[28] Nano-TiO_2 was detected in the *stratum corneum* layer of the epidermis and follicular epithelium, but not in the viable skin areas. No titanium was detected in internal organs by inductively coupled plasma mass spectroscopy. However, the concentration of titanium was higher in the lung samples of rats treated with nano-TiO_2 than in the lung samples of control rats. This was probably due to the inhalation of nano-TiO_2.[28] A long-term study showed a small increase in titanium level in the liver tissue of hairless mice exposed to topical applications of sunscreen containing nano-TiO_2 once a week for 36 weeks.[29] This increase was higher in comparison with that observed in untreated mice, but similar to that observed in mice receiving UV radiation after sunscreen application. The authors concluded that this increase was possibly due to oral absorption of residual TiO_2 after washing. Moreover, these results suggest that the dermal permeability of nano-TiO_2 is not enhanced by UV radiation.[29]

In conclusion, almost all *in vitro*, *in vivo* and *ex vivo* studies, performed in humans or animals, showed that nano-TiO_2 penetration was largely limited to the *stratum corneum*. With the exception of one study, nano-TiO_2 did not penetrate into the skin beyond the surface layers to viable cells and did not reach the general circulation, either in healthy or in compromised skin. According to studies performed in rodents, nano-TiO_2 distribution after dermal exposure is very limited and probably due to inhalation or oral exposure.

In 2014, the SCCS[11] concluded that nano-TiO_2 at a concentration up to 25% as a UV filter in sunscreens can be considered not to pose any risk of adverse effects in humans after application on healthy, intact or sunburnt skin. Results published afterwards support the SCCS conclusions.

Inhalation exposure

Absorption after inhalation exposure In 2015, the SCCS[13] indicated that considering the size of nanoparticles, there are concerns about whether inhaled airborne nanoparticles are safe, particularly from spray products that could lead to exposure of the consumer's lungs to nano-TiO_2 by inhalation.

Due to their size, inhaled nanoparticles are mainly found in the upper airways (nose, mouth, pharynx, larynx and trachea),

Table 1 *In vivo* nano-TiO_2 dermal/percutaneous absorption in human skin'

Reference	Subject type	Product type	Dose	Zone	Application duration	Sample analysed	Analytical method	Main findings
Mavon *et al.*[134]	3 adults 3F	Water-in-oil emulsions containing 3% ultrafine coated (trimethyloctylsilane) TiO_2	2 mg/cm^2 formulation, i.e. 60 μg/cm^2 TiO_2	Upper arm (10 cm^2)	5 h	Punch biopsies (6 mm) made consecutively after 1, 8 and 15 tape strippings	Colorimetric assay, spectrophotometry TEM + PIXE	Recovery of 93% of the TiO_2 dose in the 15 tape strippings (most in the first 3) Localization of the remaining 7% in the furrows and in the opened infundibulum No penetration into the viable skin tissue.
Filipe *et al.*[135]	Adults (25–65 y)	Sunscreens† containing coated (Al_2O_3 and SiO_2) nano-TiO_2	0.5–1.0 mg/cm^2	Sacral region Buttocks (25 cm^2)		Punch biopsies (3 mm)	STIM + PIXE	Overall, nano-TiO_2 penetration to the outer layers of *stratum corneum*, but not to the viable epidermis
	Normal skin (N = 9)	A, B, C†			2 h			Similar nano-TiO_2 (A, B and C) penetration profiles
	Stripped skin‡ (N = 10)	A, B, C†			48 h under occlusion			Negligible adhesion of the sunscreen formulation
	Psoriatic skin (N = 4)	A†			48 h			Titanium distribution often non-uniform: deposit in some 'hot-spots' at the outer layers of stratum corneum, partly in the hair follicle infundibulum
Coelho *et al.*[26]	6 adults 4F/2M (av 37 y)	Sunscreen containing nano-TiO_2	2 mg/cm^2	Lower back (25 cm^2)	Once daily for 3 (N = 2) or 8 days (N = 4)	Shave biopsies, 1 day after the last sunscreen application	TEM + SEM-EDX	<30 nano-TiO_2 or aggregates, mainly in the dermis surrounding the hair follicle
Naess *et al.*[27]	2 adults 2M	Sunscreen containing nano-TiO_2 ± UVB (0.4 J/cm^2)§	2 mg/cm^2	Back (600 cm^2)	6 times/day for 7 days	Punch biopsies (2.5 mm) before the first and after the last sunscreen application/ 48 h and 7 days after UVB exposure	TEM-EDX	1–10 nano-TiO_2 (10–>100 nm) in 3–4 sections of 200 μm × 60 μm Nano-TiO_2 located in the cytoplasm of cells in the *stratum granulosum* and *stratum spinosum* No cell damage near the intracellular nano-TiO_2

†Three sunscreen formulations: A, contained only TiO_2; B, contained TiO_2 + ZnO; C, contained coated rutile TiO_2. ‡Removal of parts of the outer layers of the *stratum corneum* by tape stripping (≥15 strips) before sunscreen application. §Inducing erythema resembling sunburned skin.

Abbreviations: av, average; EDX, energy-dispersive X-ray spectroscopy; F, female subject; M, male subject; nano-TiO_2, nanoparticles of titanium dioxide; PIXE, particle-induced X-ray emission; SEM, scanning electron microscopy; STIM, scanning transmission ion microscopy; TEM, transmission electron microscopy; UVB, ultraviolet B; y, years old.

but they can also reach the deeper lungs and deposit in alveoli. In general, cough and mucociliary clearance quickly remove particles from most upper airway areas ($t_{1/2}$ in healthy humans: 2–4 h), while in the lung periphery alveolar macrophages slowly clear particles.[30] Of note, it is estimated that about 10% of insoluble particles remain in human lungs due to the very slow clearance rate.[30]

Distribution after inhalation exposure Studies assessing the distribution of nano-TiO_2 after inhalation exposure and analysed in the ANSES report[16] were performed in rodents, mainly in rats using mostly P25 nano-TiO_2 which are not utilized in cosmetic applications. Therefore, the results of P25-based studies may not be transferable to the nano-TiO_2 forms used in sunscreens.[19]

In the lungs of female Wistar rats, the presence of nano-TiO_2 was reported in alveolar macrophages and, to a lesser extent, in pneumocytes.[31] In the absence of pulmonary overload, the exposure duration does not seem to impact either the lung distribution of nano-TiO_2 or its half-life,[32,33] estimated at 2 months.[34] Nano-TiO_2 may translocate to other organs to a limited extent. In several studies, nanoparticles were detected in the liver, heart, kidneys, pancreas, spleen, brain or blood after inhalation and translocation through the lung barrier.[31,35–38] Nevertheless, this phenomenon does not appear to be predominant as the translocation rate is slower than the lung clearance rate.[39]

In conclusion, inhaled nanoparticles can be found in the lungs. Inhaled nano-TiO_2 is capable of diffusing across the lung barrier and translocating throughout the body even if this phenomenon seems to be limited.

Oral exposure

Absorption after oral exposure As the ingredients used in lip balms may be incidentally ingested, it is necessary to consider the potential ability of nano-TiO_2 to penetrate oral and gastrointestinal mucosa. Currently available data were retrieved from studies performed in pigs, rats and humans.

Using an *ex vivo* model of porcine oral mucosa, nano-TiO_2 was shown to rapidly interact with the mucous layer, penetrate the oral epithelium and impact on the physiological homeostasis of buccal/sublingual cells in the oral cavity.[40] Three studies performed *in vivo* in rats showed that oral administration of nano-TiO_2 either led to extremely low systemic absorption of nano-TiO_2 from the gastrointestinal tract[35,41] or did not lead to significant nano-TiO_2 absorption.[42] The nano-TiO_2 dose absorbed across the intestinal barrier was estimated to be about 0.6%, 0.2% and 0.05% of the administered dose only, respectively, 1 h, 4 h and 7 days after administration.[35] In humans, a 3D organotypic human buccal mucosa model was used to access nano-TiO_2 penetration *in vitro*. Nano-TiO_2 penetrated the reconstituted human normal buccal epithelium, with most of the particles remaining in the upper third of the epithelial tissue.[43] Another study assessed gastrointestinal absorption of nano-TiO_2 *in vivo*: a single dose of nano-TiO_2 (5 mg/kg bw), dispersed in water, was administered to nine subjects. Only negligible absorption of nano-TiO_2 via the gastrointestinal tract was observed after 2, 4, 24 and 48 h.[44]

Currently available data thus showed nano-TiO_2 penetration through *in vitro*/*ex vivo* models of oral mucosa, but negligible nano-TiO_2 absorption, if any, via the gastrointestinal tract after oral exposure to nano-TiO_2 *in vivo*, either in rats or in humans.

Distribution after oral exposure Two studies performed in rodents were analysed in a study report from INERIS (French National Institute for Industrial Environment and Risks).[45] The study performed in mice showed that 2 weeks after a single administration of nano-TiO_2 (25 and 80 nm, column/spindle shape, 5 g/kg bw, gavage), particles mainly accumulated in the liver, spleen, kidneys and lungs.[46] The very high nano-TiO_2 dose used in this study is not representative of human exposure.[47] In contrast, the study performed in rats did not show any significant increase of titanium in liver, spleen, kidney and even brain in comparison with the vehicle control group, and no dose–response relationship was observed after nano-TiO_2 (264.4, 520.8 and 1041.5 mg/kg bw/day) was orally administered daily for 13 weeks.[41] However, a more recent study using radiolabelled nano-TiO_2 showed nano-TiO_2 distribution in rat liver, lungs, kidneys, brain, spleen, uterus and skeleton 7 days after administration of a single dose of nano-TiO_2 (about 40 μg/kg bw), even if the estimated absorbed dose was low (0.09–0.98 ng/g depending on the organ).[35] These results suggested that upon repeated long-term oral exposure, nano-TiO_2 may accumulate in specific organs and thereby present a risk in humans who are orally exposed to nano-TiO_2.

In conclusion, following oral intake, nano-TiO_2 can potentially permeate the gastrointestinal lining but to a limited extent.

Toxicity

Cytotoxicity

Skin cells Most *in vitro* studies used the human keratinocyte HaCaT cell line to assess nano-TiO_2 skin cytotoxicity.[24,32] Two studies analysed by the TGA reported decreased cell viability of HaCaT cells after *in vitro* exposure to nano-TiO_2.[25,48] Doses varied from 0.007 to 50 μg/cm^2 or from 1 to 100 μg/mL and the exposure duration from 24 h to 7 days. When several nano-TiO_2 concentrations were tested, a dose-dependent effect was observed. On the contrary, five studies reported no effect of nano-TiO_2 (0.1–25 μg/cm^2 or 1–100 μg/mL) on HaCaT cell viability after 2–24 h of exposure,[49–53] but one of them showed a dose-dependent increase in apoptosis.[52] Data on nano-TiO_2 cytotoxicity assessed in human skin cells are presented in Table 2. Except for the study by Crosera *et al.*, all these studies assessed ROS formation and all of them showed that nano-TiO_2

induced ROS and suggested that these components would be responsible of nano-TiO_2 cytotoxicity. ROS induction in HaCaT cells was shown to be enhanced by UVA[50] and UVB[49] irradiation, but not by UVC irradiation,[52] thus demonstrating phototoxicity of nano-TiO_2 to human skin keratinocytes. Interestingly, after UVA irradiation, either less or no phototoxicity was observed in HaCaT cells with the rutile form of nano-TiO_2 in comparison with the anatase form.[50,51] Of note, no phototoxicity was observed with the anatase form of nano-TiO_2 in the EpiDerm™ 3D skin model51 In contrast to the HaCaT cell line that consists of human immortalized keratinocytes, the EpiDerm™ 3D model is a reconstructed human epidermis with normal human-derived epidermal keratinocytes that is expected to provide a more integrated response.

In 2013–2014, the SCCS indicated that surface coating of nano-TiO_2 was very important to reduce its phototoxicity.[11]

Table 2 Nano-TiO_2 cytotoxicity assessed in the HaCaT human keratinocyte cell line'

References	Nano-TiO_2 type	Dose	Exposure time	Assay	Cytotoxic effect/ Reduced cell viability	Effective concentration
Rancan *et al.*[49]	Anatase (10 ± 2 nm), uncoated (gift)	10–500 μg/mL	2 h	XTT†	NO	NA
Yin *et al.*[50]	Anatase (<25 nm and 325 mesh), rutile (<100 nm; Sigma) and P25 (anatase/rutile mixture, 86%/ 14%; Degussa)	50 and 100 μg/mL (sonicated)	4 h	MTS‡	NO	NA
Horie *et al.*[51]	Anatase (Ishihara Sangyo Kaisha Ltd.; Tayca Corporation) and rutile (Tayca Corporation)	100 μg/mL (sonicated)	6, 24 h	WST-1† and LDH§	NO	NA
Tucci *et al.*[53]	Anatase (Sigma)	5, 50 and 100 μg/ mL (sonicated)	24 h	PI¶	NO††	NA
Wright *et al.*[52]	Anatase H_2TiO_7 (12 nm; gift)	0.1, 1, 10 and 25 μg/cm^2 (sonicated)	24 h	MTT‡‡	NO	NA
			12 h, 24 h	Hoechst 33342§§	YES, CC-dependent increase in apoptosis¶¶	0.1–10 μg/cm^2 (about 40–65% at 12 h/50–80% at 24 h)
Crosera *et al.*[25]	Anatase/rutile mixture, 90%/ <10%††† (Sigma Aldrich)	0.007–50 μg/cm^2 (sonicated)	24 h, 48 h, 7 days	MTT‡‡	YES, very low, CC-dependent, ET-independent	Min = 5.5 μg/cm^2 EC_{50} = 44 μg/cm^2 (95% CL: 31–62 μg/ cm^2; 7 days)
				Alamar Blue®‡‡	YES, slightly higher vs. MTT assay, CC-dependent, ET-dependent	Min = 0.6 μg/cm^2 EC_{50} = 1.9 μg/cm^2 (95% CL = 1.3–2.7 μg/cm^2; 7 days, highest effect).
			7 days	PI¶	YES, CC-dependent	Min = 5.5 μg/cm^2 EC_{50} = 38 μg/cm^2 (95% CL = 31–47 μg/cm^2)
Gao *et al.*[48]	P25, anatase/ rutile mixture (5–6 nm; Degussa)	1–100 μg/mL	24 h	MTT‡‡	YES, CC-dependent	Min = 0.5 μg/mL Max = 100 μg/mL (77%)

†Mitochondrial activity. ‡Activity of (mainly mitochondrial) dehydrogenases. §Cell membrane damage (release of cytosolic LDH). ¶Index of necrotic or late apoptotic cell death. ††No significant differences neither in cell death nor in cell cycle profile vs. control cells. ‡‡Cellular viability. §§Apoptosis. ¶¶No effect with the 25 μg/cm^2 dose. †††Nano-TiO_2 size distribution centred on the value of 38 nm.

Abbreviations: CC, concentration; CL, confidence limit; EC_{50}, half-maximal effective concentration; ET, exposure time; LDH, lactate dehydrogenase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4sulfophenyl)-2H-tetrazolium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NA, not applicable; PI, propidium iodide; WST-1, 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium; XTT, 2,3-Bis-(2-methoxy 4-nitro-5-sulfophenyl)-2H-tetrazolium5-carboxanilide salt.

Lung cells Four studies analysed in the review by Zhang *et al.*[32] used the human lung cancer A549 cell line to assess nano-TiO_2 inhalation or pulmonary cytotoxicity *in vitro*. All of them showed that nano-TiO_2 induced oxidative stress and/or apoptosis.[32]

In conclusion, cytotoxicity of nano-TiO_2 seems to be mediated by ROS production and enhanced by UVA or UVB irradiation *in vitro*. Interestingly, less or no phototoxicity was observed in a human keratinocyte cell line with the rutile form of nano-TiO_2 in comparison with the anatase form, and no phototoxicity was observed with the anatase form in a 3D human skin model. Surface coating of nano-TiO_2 reduces its phototoxicity. It should be noted that P25 nano-TiO_2 is uncoated and generally used commercially for catalytic reactions and not for cosmetic applications.

Dermal toxicity

No studies relevant for the assessment of acute dermal toxicity of nano-TiO_2 are available.[11]

Concerning skin sensitization, results of the three studies performed in guinea pigs and analysed by the SCCS showed that nano-TiO_2 was a non-sensitizer.[11] These results were confirmed by more recent studies reported by the TGA.[24] Indeed, two studies performed in mice showed no skin sensitization after dermal application of nano-TiO_2 on the ears for 3 days.[1,54] However, nano-TiO_2 was shown to increase dermal sensitization induced by 2,4-dinitrochlorobenzene.[54]

Concerning skin irritation, the studies analysed by the SCCS provided only limited relevant information.[11] From the seven studies performed in guinea pigs ($N = 1$) or rabbits ($N = 6$), only four performed in rabbits were relevant. Indeed, as the TiO_2 particle size was not specified in the three other studies, the 'nano' size could not be assured. Nano-TiO_2 of anatase/rutile types coated with trimethoxy-*n*-octyl-silane was used in two studies, and the results were not consistent: neither erythema nor oedema were observed in one study, while very slight erythema and oedema were observed 1 day after skin patch application in the other study. The two other studies, in which the proportion of nano-TiO_2 was not specified, evaluated 5-day repeat applications and showed slight irritation (mean irritation scores: 0.13–1.92). In one more recent study reported by the TGA,[24] neither erythema nor oedema was observed in rabbits after dermal exposure to 0.5 g of nano-TiO_2 for 4 h.[55] In the same study, and no skin irritation was observed using a 3D human skin model (KeraSkin™; Modern Cell & Tissue Technology, Seoul, Korea) after application of nano-TiO_2 at a final concentration of 25% (w/v).[55] Likewise, no signs of dermal irritation were observed after exposure of another human 3D skin model derived from epidermal keratinocytes (EpiDerm™) to 1 mg/mL of four nano-TiO_2.[56]

In conclusion, nano-TiO_2 is considered as mild- or non-irritant to skin.[11]

Inhalation exposure

Data reported below come from the ANSES report.[16] Most of the data focus on studies performed with the P25 form, which consists of nano-TiO_2 only (anatase: 80–90%/rutile: 20–10%), generally used in several catalytic and photocatalytic industrial applications and not in cosmetic applications.

Acute toxicity *Pulmonary effects.* Six acute toxicity studies, either performed in mice ($N = 4$)[57–60] or in rats ($N = 2$),[61,62] assessed pulmonary effects after nano-TiO_2 inhalation. Of them, one reported irritation[60] and three reported mild or moderate pulmonary inflammation, with or without histopathological changes.[58,60,61]

Microvascular effects. Six studies, all performed in rats by the same research team, investigated the effects of nano-TiO_2 on the microvascular system by assessing arteriolar responsiveness.[63–68] In these studies, acute inhalation of nano-TiO_2 (P25, primary particle size 21 nm, 1.5–20 mg/m^3 for 4–12 h to achieve a pulmonary deposition of 4–90 μg) impaired vasodilation in the systemic microcirculation (arterioles of the spinotrapezius muscle,[63,64] subepicardial arterioles,[65] coronary arterioles[66] and uterine arterioles[68]). This alteration was due to endothelial dysfunction mediated by the production of free radicals, thus reducing the bioavailability of nitric oxide.[64,66,67]

Repeated dose toxicity *Animal data. Pulmonary effects*—Five repeated inhalation toxicity studies using multiple nano-TiO_2 concentrations (from 0.5 to 10.0 mg/m^3[69] or 2 to 50 mg/m^3[70] for 5 days or from 0.5 to 1.84 mg/m^3[34] or 2.5 to 10.0 mg/m^3[71] for 4 weeks or from 0.5 to 10.0 mg/m^3 for 13 weeks[33]) showed pulmonary inflammation either in mice or in rats, but not in hamsters. Lung histopathological changes were highlighted in rats.[33,34,70] Moreover, hypertrophy/hyperplasia of the bronchi and bronchioles[70] or preneoplastic effects such as metaplasia[33] were also observed in rats exposed to the highest nano-TiO_2 concentration (10 mg/m^3 for 13 weeks or 50 mg/m^3 for 5 days, respectively). Other studies conducted using a single concentration confirmed these results, qualitatively or quantitatively.[31,72] Results observed in rats only can be due to lung overload, a phenomenon that results from impairment of lung clearance. It seems to be specific to rats exposed to poorly low-toxicity particles like TiO_2.[73]

Cardiovascular effects—Five repeated inhalation toxicity studies or instillation studies performed in mice ($N = 4$)[74–77] or rats ($N = 1$)[78] were analysed. In mice, repeated long-term exposure to nano-TiO_2 (1.25, 2.5 or 5 mg/kg bw for 9 months) was associated with atherosclerosis.[75] Another study showed increased plasma levels of serum amyloid A (SAA, a known risk factor for cardiovascular diseases that accelerates

atherosclerotic plaque development) in pregnant mice exposed to 42 mg/m^3 of nano-TiO_2 for 11 days.[74] Two studies performed in ApoE knock-out mice, an atherosclerosis-susceptible animal model, resulted in conflicting results: on the one hand, exposure to nano-TiO_2 (0.5 mg, 2.5 mg and 5 mg/kg bw/week for 6 weeks) induced endothelial and lipid metabolism dysfunction, contributing to atherosclerosis progression,[77] and on the other hand, exposure to nano-TiO_2 (0.5 mg/kg bw/week for 4 weeks) was associated with modest plaque progression and was not associated with either inflammation or vasodilatory dysfunction.[76] In pregnant rats, microvascular dysfunction was observed after exposition to about 11 mg/m^3/h, 5 h/day for 7–9 days. Of note, high doses of nano-TiO_2 (10–42 mg/m^3) were used in most of these studies.

Effects on the immune system—The effects of nano-TiO_2 on the immune system were evaluated in many studies. Some showed disturbance of the immune system in rats (e.g. increased $CD4^+$/$CD8^+$ ratio,[79] increase in NK cells number[80] and activity[81]), but it seems difficult to conclude with respect to the immunotoxicity of nano-TiO_2 due to the variability of protocols and exposure routes (aerosolized, inhalation, intranasal exposure and nose-only application).

Neurotoxicity—The eight analysed studies performed in mice[82–89] showed various effects of nano-TiO_2 on the nervous system: histological changes in the hippocampus and cerebral cortex,[82–84,87] proliferation of glial cells, necrosis, signs of cell degeneration,[86,87] as well as dysregulation of genes related to oxidative stress.[85,86] Moreover, nano-TiO_2 impaired spatial recognition memory in mice.[87,88] Nano-TiO_2 toxicity on the brain, especially on the hippocampus, seems to be dose-dependent.[87,88]

In rats, the study by Horvath *et al.*[90] showed a significant slow-down of sensory evoked potentials and tail nerve action potential, and the study by Disdier *et al.*[91] evidenced a decreased expression of a neuronal activity marker (synaptophysin), exacerbated in older rats even if TiO_2 nanoparticles were not detected in the brain.

Liver toxicity—Liver toxicity was investigated in two studies which did not report the same results. No liver toxicity was observed in a transcriptomic analysis after a 10-day inhalation challenge with 42 mg/m^3 of nano-TiO_2 in mice,[72] while oedema and cytoplasmic loss of hepatic cells were observed after instillation exposure to 0.5, 4 and 32 mg/kg bw of nano-TiO_2 for 4 weeks in rats.[79]

Kidney toxicity—In mice, histopathological changes including tubular dilatation and necrosis, as well as increased oxidative stress and alterations in renal function markers, were reported after instillation of nano-TiO_2 (0.5 mg/week for 4 weeks) in the only study that assessed kidney toxicity.[92]

Human data. In humans, eight studies assessed nano-TiO_2 toxicological effects on workers exposed to nano-TiO_2 by inhalation.[93–100] Results suggested possible pulmonary and cardiovascular effects. Nevertheless, no causal link between TiO_2 inhalation exposure and the observed effects could be established in these studies.

In conclusion, several studies performed in rodents showed nano-TiO_2 toxicity at several levels (pulmonary inflammation, cardiovascular effects and neurotoxicity), mainly using high doses of nano-TiO_2 far exceeding human exposures, including cases of occupational exposure. Moreover, results observed in rats at the pulmonary level can be due to lung overload. Results concerning liver and the immune system were inconsistent, and only one study dealt with kidney toxicity. No conclusion can be drawn in humans as no causal link could be established between TiO_2 inhalation exposure and the possible pulmonary and cardiovascular observed effects, in addition to several biases that limit the interpretation of some studies.

Oral exposure

Acute toxicity Acute toxicity was shown in a study performed in female mice exposed to a very high nano-TiO_2 dose (5 g/kg bw, gavage): increase in relative liver weight in comparison with the control group, hepatic inflammatory response, slight histopathological alterations of the liver and kidneys, and increased levels of enzymatic biomarkers of cardiac lesions.[46] Otherwise, studies performed in rodents usually show low oral acute toxicity of nano-TiO_2 with lethal dose $(LD)_{50}$ values higher than 2150 mg/kg bw or even 5000 mg/kg bw.[11,45,101]

Repeated dose toxicity Some rodent studies showed nano-TiO_2 toxicity at several levels: immune system,[102] central nervous system,[88,103] kidneys,[104] liver,[105] spleen[106] and fertility.[107,108]

In rats, nano-TiO_2 (10 mg/kg bw/day, 7 days, gavage) was shown to increase dendritic cells frequency in Peyer's patches but not in the spleen. No intestinal inflammation was reported, and no (*in vivo*) or limited (*in vitro*) effects were observed on Treg and Th cell subsets.[102] All other studies were performed in mice. Nano-TiO_2 (0.5, 10 and 50 mg/kg bw/day for 60 days or 2.5, 5 and 10 mg/kg bw/day for 90 days, respectively) impaired neurofunction and spatial recognition memory behaviour.[88,103] Kidney toxicity was also evidenced after intragastric administration of nano-TiO_2 (2.5, 5 and 10 mg/kg bw/day for 90 days), with an inflammatory response and cell necrosis.[104] In the liver, histopathological changes were observed after oral administration of 250 mg/kg bw/day of nano-TiO_2 for 30 days; no effect was observed with both lower tested doses (62.5 and 125 mg/kg

bw/day). Moreover, dose-dependent increased enzymatic activities were observed in the 125 and 250 mg/kg bw/day groups.[105] Nevertheless, those high doses do not reflect the possible human exposure. In another study, splenic damage was observed with lower nano-TiO_2 doses (10 mg/kg bw/day for 15, 30, 45, 60, 75 or 90 days, gavage), with time-dependent inflammation and cell necrosis.[106] Two 90-day repeated exposure studies evaluated the effects of nano-TiO_2 (2.5, 5 and 10 mg/kg bw/day, gavage) on fertility in mice.[107,108] In females, dose-dependent decreased fertility (mating rate, pregnancy rate and number of newborns), ovarian inflammation and follicular atresia were reported.[107] In males, testicular lesions, sperm malformations and altered serum sex hormone levels were observed.[108]

In conclusion, studies performed in rodents showed low oral acute toxicity of nano-TiO_2 except one study using very high doses. Repeated dose studies showed nano-TiO_2 toxicity at various levels (central nervous system, kidney, spleen and gametes), but the doses used were far higher than those to which humans can be exposed in the context of an incidental oral exposure through cosmetic use.

Mutagenicity/genotoxicity

The genotoxic potency of nano-TiO_2, assessed both *in vitro* (cells, tissues) and *in vivo* (rodents) was largely reported in many reviews.[11,16,24] Various forms of nano-TiO_2, with different shape, size, coating, surface reactivity, charge and crystallinity, were used, and the results of all these studies are inconsistent. Some of them demonstrated that nano-TiO_2 could cause DNA damage and that the genotoxic effect would be due to a secondary mechanism of action involving free radicals.[16] Of note, free radical production is limited in sunscreens due to nano-TiO_2 coating and the potential presence of antioxidants.[24] Moreover, many studies showed that nano-TiO_2 did not reach viable skin cells after topical application, and genotoxic effects were only observed with high concentrations of nano-TiO_2 after oral or inhalation exposure in animals. Consequently, nano-TiO_2 can be considered as a weak genotoxic agent, as do national and international governmental organizations (ANSES, IARC, NIOSH and OECD).

Therefore, nano-TiO_2 in the form and size used in cosmetics is unlikely to be genotoxic.

Carcinogenicity

Dermal exposure Three studies performed in mice ($N = 1$), rats ($N = 1$) or both ($N = 1$) were evaluated in detail by the SCCS in 2013–2014.[11] In mice, no carcinogenic promoter activity was observed with uncoated nano-TiO_2 in both studies.[109,110] Likewise, no carcinogenic promoter activity was observed with alumina-coated or stearic acid-coated nano-TiO_2. However, an increase in the number of tumours was found among mice treated with silica-coated nano-TiO_2.[110] Nevertheless, as this increase was not significant and positive controls were lacking, no conclusion could be drawn. In rats, no conclusion could be drawn from both studies due to the absence of any positive controls and the lack of experience with the models used.[110,111]

These results on carcinogenicity through dermal exposure are therefore inconclusive. However, as there is no cutaneous penetration beyond the surface layers, there is no systemic risk. The Committee for Risk Assessment [RAC, European Chemicals Agency (ECHA)] considers that there is no experimental evidence for TiO_2 carcinogenicity for the dermal route.[112]

Inhalation exposure Only one study performed in 1995 investigated the carcinogenic potential of nano-TiO_2 after inhalation exposure in animals. The results showed an increase in the incidence of lung tumours in rats but not in mice exposed to repeated doses of nano-TiO_2 (7.2 mg/m^3 for 4 months followed by 14.8 mg/m^3 for 4 months and 9.4 mg/m^3 for 5.5 months [mice] or 16 months [rats]).[113] Among three studies investigating the carcinogenic potential of nano-TiO_2 after instillation exposure,[114–116] only one confirmed the nano-TiO_2 promotor potential.[116]

In humans, a potential relationship between exposure to TiO_2 and the occurrence of cancers was assessed in seven epidemiological studies.[117–123] An increase in death due to lung cancer was reported in most of these studies, although no causal relationship could be established.

We can conclude from the study of Heinrich *et al.*[113] that nano-TiO_2 (P25 as material tested) is a lung carcinogen in rats at a concentration resulting in pulmonary inflammation and altered clearance. This is consistent with the previous nano-TiO_2 classification as suspected/possible carcinogen in humans by other organizations [IARC, NIOSH and RAC (ECHA)]. Nevertheless, results obtained with the P25 form of nano-TiO_2 cannot be extrapolated to other forms of nano-TiO_2, and the concentrations used greatly exceed maximum human exposure.

Oral exposure The few available data do not seem to indicate any nano-TiO_2 carcinogenic promoter activity after oral exposure.[45] The Committee for Risk Assessment (RAC) also considers that there is no experimental evidence for TiO_2 carcinogenicity for the oral route.[112]

Reproductive toxicity

Dermal exposure According to the SCCS, there is no relevant study on reproductive toxicity after dermal exposure to nano-TiO_2.[11,15]

Inhalation exposure Nine studies, performed in mice ($N = 4$)[124–127] or rats ($N = 5$),[78,128–131] suggest a possible effect of pre- or peri-natal inhalation exposure to nano-TiO_2. In mice, lung inflammation was reported in the gestating females,[124] and

moderate neurobehavioural changes[124] as well as gene expression in female liver were reported in the offspring.[127] In the F1 generation, a trend in reduced sperm counts was also observed.[126] However, sex ratio or viability did not seem to be impaired. In rats, a decrease in the litters' height and weight was reported after inhalation exposure of gestating females to 10 mg/m^3 of nano-TiO_2 for 11 days. However, this was not the case when gestating females were exposed for 7 or 8 days. Microvascular and cardiac changes,[78,128,131] and effects on cognitive and behavioural functions[130] were observed in the offspring.

Oral exposure Both studies reported hereafter were performed in rats. Abnormal lung development with macrophage infiltration was reported in neonates at term, i.e. 9 days after the last nano-TiO_2 dose administered to pregnant females (200 mg/kg bw/day, gavage from the 6th to the 12th day of gestation).[132] Neurotoxic effects of nano-TiO_2 were also reported: reduced cell proliferation in the hippocampus of the neonates and impaired learning and memory in offspring aged 60 days were observed after administration of nano-TiO_2 to pregnant females (100 mg/kg bw/day, gavage from the 2nd to the 21st day of gestation).[133]

Conclusion

According to the information reported in this review, nano-TiO_2 is considered as a non-sensitizer and as mild- or non-irritant to skin. Moreover, there is no evidence of carcinogenicity, mutagenicity or reproductive toxicity after dermal exposure to nano-TiO_2. Nano-TiO_2 exhibits *in vitro* cytotoxicity, apparently mediated by ROS production and enhanced by UVA or UVB irradiation. However, no cytotoxic effect was reported using a 3D human skin model, and nano-TiO_2 used in cosmetics is usually coated to decrease ROS production. Above all, as nano-TiO_2 does not seem to penetrate the skin beyond the surface layers to viable cells and does not reach the general circulation after application to either healthy or compromised skin, nano-TiO_2 from sunscreens does not appear to present any health risks when applied on the skin at a concentration up to 25%.

However, the SCCS does not recommend the use of nano-TiO_2 in formulations that may lead to exposure of the consumer's lungs by inhalation, i.e. sprayable products and powders. Indeed, even if human data are sparse and inconsistent, lung inflammation was reported in animals.

After oral exposure, nano-TiO_2 absorption and toxicity seem to be limited. The incidental oral exposure to nano-TiO_2 contained in lip balms is thus not expected to induce adverse health effects.

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