Original Article

Role of fatty acid composites in the toxicity of titanium dioxide nanoparticles used in cosmetic products

JuOae Chang^{1,*}, Chang-Woo Lee^{2,*}, Helal Hussain Alsulimani¹, Jee Eun Choi¹,
Joo-Kyung Lee³, AhYoung Kim³, Bae Ho Park³, Jonghan Kim¹ and HeaYeon Lee^{1,4}

¹Department of Pharmaceutical Sciences, Northeastern University, Boston, MA, USA

²R&D Center, Boditech Med Inc., Chuncheon, South Korea

³Division of Quantum Phases and Devices, Department of Physics, Konkuk University, Seoul, South Korea

⁴Department of Nano-integrated Cogno-Mechatronics Engineering, Pusan National University, Pusan, South Korea

(Received January 22, 2016; Accepted May 24, 2016)

ABSTRACT — It has been recognized that the use of nanoparticles (NPs) in the cosmetic industry results in products with better efficacy and functionality. However, recent advances in molecular toxicology have revealed that NP exposure can promote cytotoxicity and oxidative damage, which has raised health concerns in the use of NPs in personal care products. Nevertheless, the mechanistic basis for the toxicity and safety of cosmetic NPs is poorly understood. The goal of the study was to determine the cytotoxicity and intracellular distribution of titanium dioxide (TiO₂) NPs containing fatty acid composites (palmitoleic acid, palmitic acid, stearic acid and oleic acid) commonly used in cosmetic products. Two types of cells, human fibroblast skin cells and adenocarcinoma lung cells, were exposed to either bare TiO₂ NPs or TiO₂ NPs mixed with fatty acids for up to 48 hr. NMR analysis confirmed that the fatty acid composites remained in the NPs after wash. The cytotoxicity of TiO₂ NPs was determined by cell viability measurement using quantitative confocal microscopy, and the localization of two different forms of TiO₂ NPs were assessed using electron spectroscopic imaging with transmission electron microscopy. TiO₂ NPs containing fatty acids posed significantly reduced cytotoxicity (80-88% decreases) than bare NPs in both cell types. Furthermore, there was less intracellular penetration of the NPs containing fatty acid composites compared with bare NPs. These results provide important insights into the role of fatty acids in protecting the cells from possible toxicity caused by NPs used in the production of cosmetic products.

Key words: Cosmetic, Cytotoxicity, Intracellular distribution, Nanosafety, Sunscreens

INTRODUCTION

Recently, nanomaterials have been extensively applied to cosmetic products due to several advantages. For example, titanium dioxide (TiO₂) nanoparticles (NPs) have been used as UV-blocking filters for their enhanced protective effect against UV-induced damages and their ability to improve texture and spreadability compared with other conventional metal oxide particles (Singh and Nanda, 2014). Moreover, sunscreens with micronized TiO₂ particles reduce risks of sunburn caused by UVB light, possibly through the induction of immune modulators (e.g. interleukin-10) by keratinocytes (van der Molen *et al.*, 1998). In addition to these benefits, less regulatory

oversight exists in the use of NPs in cosmetic products compared with other medical therapies, and the products can be sold to the consumers without prescription. Such freedom, convenience and benefits have made the use of NPs in cosmetics popular in the market. In 2005, the production of TiO₂ NPs was about 2,000 tons, which is worth \$70 million, and among these, 1,300 tons were used to make personal care products, such as sunscreens and cosmetics (Weir *et al.*, 2012).

In the plethora of personal care products that contain NPs, a large body of evidence has indicated that commonly used NPs, such as TiO₂ or zinc oxide, do not penetrate through the skin but remain on the cuticular surface of the skin, suggesting that they do

Correspondence: Jonghan Kim (E-mail: j.kim@neu.edu)
HeaYeon Lee (E-mail: he.lee@neu.edu)

*These authors equally contributed to this work.

not pose significant health risks (Sadrieh *et al.*, 2010; Monteiro-Riviere *et al.*, 2011; Filipe *et al.*, 2009). However, concerns remain about potential oxidative injury and DNA damage to the cells caused by direct exposure to NPs (Long *et al.*, 2007; Park *et al.*, 2008; Liu *et al.*, 2010). In addition to these two opposing views on using NPs in the production of personal care products, it has been known that various factors, such as composition and surface modification of NPs, can alter the toxicity of NPs (Kang *et al.*, 2015). Hence, it is important to understand if the components included in cosmetic products could influence the transport of NPs and consequently modify their toxic effects.

Abd Gani *et al.* (2014) demonstrated that TiO₂ particles coated with engkabang fat ester, which contains fatty acids commonly used in cosmetic products, enhanced UVA and UVB absorption. Although this study provides a mechanistic basis for the role of fatty acids in the beneficial effects of TiO₂ particles, the safety and toxicity of these composite particles have not been evaluated in cells or living systems. Also, the TiO₂ particles were not nano-sized and thereby failed to represent the most commonly used forms of TiO₂ particles found in personal care products. In addition, whether or not TiO₂ NPs in sunscreens could modify intracellular particle dissolution and consequently alter cytotoxicity is poorly understood. To examine the toxicity of TiO₂ NPs in cosmetics, we mixed the TiO₂ NPs with common fatty acids used as composites of cosmetics and compared their effects on cell viability with those of the bare TiO₂ NPs (B-NPs). We used four fatty acids (i.e. palmitoleic acid, oleic acid, palmitic acid and stearic acid) as fatty acid composites because they are commonly mixed in cosmetic products (Zielińska and Nowak, 2014; Andersen, 1987). Two types of cell culture systems, human fibroblast skin cells and human adenocarcinoma lung cells, were used to mimic most common exposure routes of the NPs used in cosmetics. Skin cells represent a primary site of cosmetic application in which exposure occurs through topical application of cosmetics. Lung cells portray exposure through inhalation of NPs that could occur through spray or powder formulations of the cosmetics (Nazarenko *et al.*, 2012), as well as occupational settings in the cosmetic-producing plants (CDC, 2011). We demonstrate that TiO₂ NPs in the presence of fatty acids exhibit decreased intracellular uptake of NPs and further protect cells from cytotoxicity caused by NP exposure.

MATERIALS AND METHODS

Cell culture

Human fibroblast skin (CCD-986SK) cells and adenocarcinoma lung (SK-LU-1) cells were provided by the Korean Cell Line Bank (Seoul, South Korea). The culture of cells was maintained in a phenol-red-free culture medium, Dulbecco's modified essential medium (Gibco, Grand Island, NY, USA), supplemented with 5% (v/v) fetal bovine serum (Gibco) and 1% (v/v) antibiotics (2 mM L-glutamine, 100 U/mL penicillin and 0.1 mg/mL streptomycin; Gibco). Cultured cells were kept at 37°C in a humidified 5% CO₂ incubator. Once the cells reached confluence, the culture medium was removed from the culture dish, and the cells were rinsed twice with sterile Dulbecco's phosphate-buffered saline (DPBS; Gibco). The confluent cell layers were enzymatically removed using trypsin/EDTA (Gibco) and resuspended in the culture medium.

Preparation of TiO₂ NPs

A stock solution of TiO₂ (0.5 M) was prepared by mixing titanium isopropoxide (TTIP) and triethanolamine (TEOA) with a molar ratio of TTIP:TEOA = 1:2, followed by the addition of distilled water. The stock solution (pH ~9.5) was then mixed with HClO₄ or NaOH solutions to adjust the pH of the solution to 10.5. Ethylenediamine (0.4 M) was further added as a shape controller. The solution was placed in an autoclave, aged at 100°C for 24 hr (first aging) and further aged at 140°C for 72 hr (second aging). The two-step aging process is essential to obtain uniform TiO₂ NPs because a hydrolyzed gel network forms during the first aging step, and the nucleation and growth of TiO₂ seeds occur during the second aging step. The gel matrix plays two decisive roles in the second aging to produce uniform particles: (i) as a reservoir of metal ions to lower the supersaturation and prevent extensive nucleation and (ii) as an anticoagulant that fixes the growing particles in the matrix. An opaque solution was obtained after the second aging process *via* transformation from the gel, and the resultant NPs dispersed in the solution were washed with NaOH (six times), HNO₃ (two times) and distilled water (four times) to remove residual organic compounds from their surfaces.

Preparation of nanoparticles coated with fatty acids

The TiO₂ B-NPs were initially placed into distilled water (particle concentration = 74.1 µg/mL) and dispersed. The initial suspension was dried and resuspended in distilled water to generate a B-NP stock suspension

at 3,000 µg/mL. Subsequently, the B-NP stock was diluted with distilled water or with fatty acids composites to produce B-NPs or CM-NPs, respectively. For CM-NPs, B-NPs were first mixed with 1% sepinov EMT 10 and stirred for 5 min, followed by an addition of fatty acids composites consisting of 5% jojoba oil (palmitoleic acid and palmitic acid), 14.9% emu oil (stearic acid and oleic acid), and 5% glycerin. After 15 min of stirring, a cream was formed. The final product was obtained after holding the cream at 4°C for 24 hr. This dilution step was repeated to produce lower concentrations of TiO₂ CM-NPs.

¹H-Nuclear magnetic resonance (¹H-NMR)

The presence of fatty acids on CM-NPs was determined by ¹H-NMR. The analysis was conducted using Bruker AVANCE 300 NMR spectrometer (Bruker, Billerica, MA, USA) using a well-cleaned 5 mm NMR tube. Both CM-NPs and B-NPs were suspended in ethanol and centrifuged at 13,000 rpm for 10 min to remove the supernatant. After repeated washes for five times, NPs were fully dried in the oven at 70°C. Dibromomethane was added as internal standard, deuterated chloroform (CDCl₃) as lock solvent and tetramethylsilane (TMS) as peak calibration reference.

Cell exposure to NPs

Both human fibroblast skin and adenocarcinoma lung cells were seeded at a density of 2.5×10^4 cells per well in 24-well plates. After one day (attachment phase), the cells were treated with B-NPs or CM-NPs at TiO₂ concentrations of 0, 1.95, 3.9, 7.81, 16.25, 31.25, 62.5, 125, 250, 500, and 1,000 µg/mL and incubated for 24 or 48 hr at 37°C.

Cytotoxicity assessment

After the exposure of cells to TiO₂ NPs, the supernatant was removed, and the cells were washed twice with DPBS. They were then subject to staining by propidium iodide (PI, cell-nonpermeable red fluorescent dye) and Live-Dye (cell-permeable green fluorescent dye) using a Live-Dead Cell Staining Kit (K501-100; BioVision Research Products, Milpitas, CA, USA). After staining, red and green fluorescence was observed under UV illumination using a confocal microscope (model C1; Nikon, Minato, Tokyo, Japan). Cells that appeared red under UV illumination were considered dead because PI is preferentially absorbed by dead cells. Green fluorescent cells were recognized as living because Live-Dye is preferentially absorbed by live cells. After counting the dead and living cells, the cytotoxicity at each TiO₂ concentration was assessed by the following calculation: dead

cells / (living cells + dead cells) × 100. The cytotoxicity results from different concentrations of NPs were further analysed using the hyperbolic effect model function (SigmaPlot 12.3; Systat, Chicago, IL, USA) to quantitatively characterize the maximal cell death effect ($E_{\max}$) and the concentration of TiO₂ at which 50% of cytotoxicity was achieved (EC_{50}).

Transmission electron microscopy

To characterize intracellular localization of TiO₂ NPs, cells exposed to the NPs were sampled for electron spectroscopic imaging (ESI) with transmission electron microscopy (TEM) as follows. The fresh samples were rinsed three times with DPBS and fixed for 30 min in modified Karnovsky fixative using glutaraldehyde (2.5% v/v) and paraformaldehyde (2% w/v) in a cacodylate buffer (0.1 M, pH 7.2-7.4) with calcium chloride (2.5 mM). The samples were then rinsed three times with a cacodylate wash buffer for 5 min. Next, they were fixed in osmic acid (2%) for 15 min and stained in a saturated aqueous solution of uranyl acetate for 15 min. The dehydration process was performed in a graded series of 30%, 50%, 70%, 95%, and twice in 100% ethanol, for 15 min each. Then the samples were infiltrated with a 50:50 mixture of 100% ethanol and Spurr's reagent. After the mixture was discarded during 1 hr, 100% Spurr's reagent was added to infiltrate the cells for another 1 hr. The samples were kept in a vacuum oven at 60°C overnight after the Spurr's reagent was changed. Finally, the samples were cut into light-gold-colored ultrathin sections with a microtome and stained on the grid with uranyl acetate and lead oxide (5 min each). After being washed with distilled water, the TEM cell samples were dried and then imaged under a Carl Zeiss (model LIBRA® 200; Carl, Zeiss, Oberkochen, Germany) energy-filtered TEM. The images were analyzed to characterize the penetration, distribution and specific locations of the TiO₂ NPs by ESI.

RESULTS

Fatty acids mixed with TiO₂ NPs remain on the NP surface

The CM-NPs were produced by mixing the TiO₂ B-NPs with fatty acids (Fig. 1a). NMR analysis demonstrated that the peaks of fatty acids were still observed in the CM-NPs after wash (Fig. 1b), indicating that most fatty acids remain on the surface of TiO₂ NPs. These results suggest a potential surface modification of NPs by fatty acid composites.

Fig. 1. Physicochemical properties of CM-NPs. (a) CM-NPs were generated by mixing TiO₂ NPs with palmitoleic acid, oleic acid, palmitic acid, and stearic acid. (b) Cosmetics mixture of fatty acids, washed CM-NPs, and washed B-NPs were analysed by ¹H-Nuclear Magnetic Resonance (¹H-NMR) using CDCl₃ as a lock solvent.

Fatty acid composites reduce NP-induced cytotoxicity in fibroblast skin cells

The fibroblast skin cells were exposed to B-NPs and CM-NPs at TiO₂ concentrations of 0–1,000 µg/mL for 24 hr or 48 hr and dead/living cell counts were analysed by confocal microscopy (Fig. 2). There were no dead cells observed in the control cells without exposure to NPs, whereas exposure to TiO₂ NPs at 1,000 µg/mL resulted in cell death (Fig. 2a). Of importance, more dead cells were found in cells exposed to TiO₂ B-NPs (85% dead cells) than those exposed to TiO₂ CM-NPs (54%) (Fig. 2a). These results indicate that fatty acid composites in NPs could protect skin cells from cytotoxicity induced by TiO₂ NP exposure.

We also characterized the dose-toxicity relationship of TiO₂ NPs by cell counting (Fig. 2b). The cytotoxicity of skin fibroblast cells exposed to B-NPs for 24 and 48 hr increased rapidly up to the NP concentration of 250 µg/mL, above which the rate slowed down. However, cells exposed to TiO₂ CM-NPs for 24 hr did not demonstrate appreciable changes in cytotoxicity at NP concentrations below 250 µg/mL. At low NP concentrations (below 62.5 µg/mL; Fig. 2b), < 10% of cells were dead when exposed to TiO₂ CM-NPs, whereas B-NPs killed > 30% of cells within 24 hr. As expected, the cytotoxicity of NPs was greater in 48-hr exposure compared with 24-hr exposure. Interestingly, the protective effect of CM-NPs on cytotoxicity still remained at low concentrations,

Fig. 2. Effect of fatty acid composites on cytotoxicity of TiO₂ NPs in fibroblast skin cells. (a) Cell viability was visualized by confocal microscope images after exposing fibroblast skin cells to TiO₂ B-NPs and CM-NPs for 24 hr to a TiO₂ concentration of 1,000 μ g/mL. Living and dead cells are visualized by green and red fluorescent proteins, respectively. Scale bar = 200 μ m. (b) Cytotoxicity after exposure to TiO₂ NPs was plotted over different TiO₂ concentrations (0–1,000 μ g/mL). The enlarged graph indicates the cytotoxicity results after exposure to 0–70 μ g/mL of TiO₂ concentrations.

but this effect was less prominent at high concentrations and longer exposure. Together, these results indicate that TiO₂ CM-NPs induce less cell death compared with the B-NPs, while this effect is less significant after prolonged exposure to NPs.

Table 1 provides a summary of the cytotoxicity parameters of TiO₂ NPs at concentrations of 0–1,000 μ g/mL in the skin cell culture. The EC₅₀ value (as TiO₂) of CM-NPs was almost doubled (219 μ g/mL) compared with that of B-NPs (66 μ g/mL) after 48-hr exposure, indicating

that the CM-NP toxicity is significantly decreased. When the cells were exposed to NPs for 24 hr, the difference in the EC₅₀ values were even more dramatic; CM-NPs were 8.5 times less toxic than B-NPs (i.e. 88% decrease in cytotoxicity). These data indicate that fatty acid composites are important in reducing TiO₂ NP-induced toxicity in human fibroblast cells.

Table 1. Cytotoxicity parameters of human fibroblast skin cells exposed to TiO₂ NPs.

TiO ₂ NPs	E _{max} , %	EC ₅₀ , µg/mL
B-NPs 24 hr	88.6 ± 5.4 (6.0)	125.1 ± 23.6 (18.8)
B-NPs 48 hr	85.4 ± 2.9 (3.4)	66.0 ± 8.0 (12.1)
CM-NPs 24 hr	116 ± 40.1 (34.7)	1062 ± 602 (56.6)
CM-NPs 48 hr	94.0 ± 4.0 (4.3)	219 ± 25.1 (11.5)

Data shown as means ± standard errors (% coefficient of variation). E_{max}, the maximum capacity of cytotoxicity; EC₅₀, the TiO₂ concentration at which a half of cells are dead (potency of cytotoxicity).

Fatty acids on TiO₂ NP surface protect adenocarcinoma lung cells from cell death

In addition to fibroblast cells, human adenocarcinoma lung cells (SK-LU-1) were exposed to various concentrations of NPs (Fig. 3). Exposure to B-NPs at a TiO₂ concentration of 1,000 µg/mL for 24 hr resulted in almost complete cell death (97% dead cells), as determined by confocal microscopy. In contrast, CM-NPs significantly reduced cell death (41%), similar to results from skin fibroblast cultures (Fig. 3a). While dead cell ratios after 24-hr and 48-hr exposure to B-NPs increased at the concentration of 16.25 µg/mL and reached a plateau value of

Fig. 3. Effect of fatty acid composites on cytotoxicity of TiO₂ NPs in adenocarcinoma lung cells. (a) Cell viability was visualized by confocal microscope images after exposing adenocarcinoma lung cells to TiO₂ B-NPs and CM-NPs for 24 hr to a TiO₂ concentration of 1,000 µg/mL. Scale bars = 200 µm. (b) Cytotoxicity after exposure to TiO₂ NPs was plotted over different TiO₂ concentrations (0–1,000 µg/mL). The enlarged graph indicates the cytotoxicity results after exposure to 0–70 µg/mL of TiO₂ concentrations.

90% (i.e. almost all cells were dead cells) at concentrations above 125 µg/mL, the cytotoxicity by CM-NPs after 24-hr and 48-hr exposure increased slowly to 20% and 38%, respectively (Fig. 3b). In contrast to skin fibroblast results, the cytotoxicity of CM-NPs after 48-hr exposure was significantly reduced compared with B-NPs, suggesting that the protective effect of fatty acid composites is greater in lung cells than in skin cells.

Table 2 shows quantitative measures of the cytotoxicity of B-NPs and CM-NPs in lung cells. The cytotoxicity in lung cells was reduced in CM-NPs compared with B-NPs. Interestingly, the E_{max} values were also decreased in cells exposed to CM-NPs. These results indicate that mixing NPs with fatty acids reduces the potency of NP-induced cytotoxicity (80% decrease in cytotoxicity or 4.9-fold increase in EC_{50} after 24-hr exposure; 1.6-fold increase in EC_{50} after 48-hr exposure).

Cosmetic composites attenuate intracellular uptake of TiO₂ NPs

TEM images were analyzed to identify TiO₂ B-NPs and CM-NPs within an individual fibroblast skin or adenocarcinoma lung cell. In addition, element analysis with ESI was necessary to observe precise intracellular localization of NPs. Both B-NPs and CM-NPs with a size of 20-30 nm TiO₂ (Fig. 4, a and b, respectively) entered the fibroblast skin cells after 24-hr exposure. However, the

Table 2. Cytotoxicity parameters of human adenocarcinoma lung cells exposed to TiO₂ NPs.

TiO ₂ NPs	E_{max} , %	EC_{50} , µg/mL
B-NPs 24 hr	104 ± 2.1 (2.0)	28.7 ± 2.5 (8.6)
B-NPs 48 hr	102 ± 2.0 (1.9)	15.3 ± 1.4 (9.1)
CM-NPs 24 hr	44.2 ± 2.7 (6.1)	141 ± 25.8 (18.3)
CM-NPs 48 hr	49.4 ± 2.8 (5.8)	24.1 ± 6.0 (25.0)

Data shown as means ± standard errors (% coefficient of variation). E_{max} , the maximum capacity of cytotoxicity; EC_{50} , the TiO₂ concentration at which a half of cells are dead (potency of cytotoxicity).

pattern of intracellular distribution of the two types of NPs was different: B-NPs (Fig. 4a') entered the cells in a greater extent than the CM-NPs (Fig. 4b'). Similar results were obtained in human adenocarcinoma lung cells exposed to B-NPs and CM-NPs for 24 hr (Fig. 4, c and d). In the B-NP-exposed lung cells, many nanomaterials were homogeneously distributed in a large area inside a cellular vesicle (Fig. 4c'). In contrast, CM-NPs appeared isolated as slightly dense groups in small areas of the cell (Fig. 4d'). Combined, these results indicate that mixing of TiO₂ NPs with fatty acid composites reduces intracellular uptake of toxic TiO₂ NPs in both types of cells.

Fig. 4. Intracellular localization of TiO₂ NPs in fibroblast skin cells and adenocarcinoma lung cells. ESI-TEM analysis was conducted using fibroblast skin cells [(a) and (b)] and adenocarcinoma lung cells [(c) and (d)] after exposure to TiO₂ B-NPs [(a) and (c)] and CM-NPs [(b) and (d)] for 24 hr. (a')-(d') correspond to enlarged images of square boxes in (a)-(d). Scale bars = 5 µm (a, b and d), 2 µm (c) and 1 µm (a'-d').

DISCUSSION

An increasing body of evidence has indicated that a direct exposure of cells to the NPs can increase the production of reactive oxygen species (ROS) and cause oxidative injury and DNA damage (Park *et al.*, 2008; Long *et al.*, 2007; Liu *et al.*, 2010; Shukla *et al.*, 2011; Heng *et al.*, 2010; Sharma *et al.*, 2009). Although the multilayer skin system prevents NPs from entering into the deep skin layer (Xie *et al.*, 2015; Schilling *et al.*, 2010; Newman *et al.*, 2009; Sadrieh *et al.*, 2010; Monteiro-Riviere *et al.*, 2011; Filipe *et al.*, 2009), surface modification of NPs that are commonly used in cosmetics, such as TiO₂ or zinc oxide, could alter the transport of NPs and thereby alter the cytotoxicity of NPs. Abd Gani *et al.* (2014) showed that coating TiO₂ particles with fatty acids commonly used in cosmetics can increase UV absorption, but have not demonstrated the efficacy of “nanosized” particles. Leung *et al.* (2012) observed that the antibacterial activity is enhanced as a result of surface modification of zinc oxide NPs. However, neither study examined the effect of surface modification of NPs in cell toxicity. In particular, Serpone *et al.* (2001) suggested surface modification as a method to reduce NP-induced DNA damage, but the idea has not been tested *in vitro* or *in vivo*. We here show that TiO₂ NPs in themselves possess considerable cytotoxicity when exposed directly to skin and lung cells, but fatty acid composites typically found in cosmetic products significantly reduce NP-associated cytotoxicity. To our knowledge, this is the first study to directly examine a protective role of fatty acid components in NP-induced cell toxicity.

Our results demonstrated decreased penetration and increased cell viability of the CM-NPs compared with the B-NPs, suggesting that the changes in NP kinetics likely alter the cytotoxicity of NP exposure. Several physicochemical and physiological factors influence the uptake of NPs (Kang *et al.*, 2015). In particular, surface modification can alter the extent and rate of cell entrance of NPs. For example, positive charges on the NPs increase electrostatic interaction with the negatively-charged membrane, thereby promoting cellular uptake (Salatin *et al.*, 2015). In some instances, surface modification of NPs affects not only intracellular uptake, but also subcellular distribution inside the cell. Nativo *et al.* (2008) illustrated that gold NPs that are chemically modified with membrane-penetrating peptide sequences are able to escape from lysosomal digestion inside the cell and direct NPs to the nucleus. Importantly, Abd Gani *et al.* (2014) demonstrated that engkabang oil-coated TiO₂ particles increase zeta potential without changing overall negative charge

of the particles. Higher zeta potentials suggest increased repelling force among particles, thereby forming fewer agglomerates. Since the agglomerates are more easily taken up by the cell *via* endocytic transport than individual NPs (Andersson *et al.*, 2011), our results of reduced penetration of the CM-NPs could be explained by decreased degree of agglomeration, likely due to fatty acid-associated surface modification. Future studies should be carried out to understand possible changes in the charge, subcellular distribution, zeta potential and agglomerate formation of TiO₂ NPs coated with fatty acids.

In the current study, we characterized the cytotoxicity and extent of intracellular localization of TiO₂ NPs mixed with fatty acid composites compared with bare TiO₂ NPs as a control source material. Although our results indicate that fatty acids significantly decreased the cytotoxicity of NPs, our study is limited to *in vitro* findings within 48 hr. Our results demonstrate that cells exposed to CM-NPs for 48 hr caused more damage to cells than the 24-hr exposure, indicating that prolonged exposure increases cytotoxicity. Interestingly, in fibroblast skin cells the 48-hr exposure to CM-NPs produced similar results to those from 24-hr exposure to B-NPs. One explanation could be a possible removal of fatty acids from the TiO₂ CM-NPs with prolonged incubation, which consequently alters intracellular distribution and toxicity of NPs. A characterization of fatty acid contents in the remaining TiO₂ CM-NPs after 24- to 48-hr incubation would address this question. In addition, a future study is necessary to explore the long-term effect of fatty acids on TiO₂ NP-induced toxicity to represent more realistic subchronic or chronic exposure. Moreover, it is important to verify the protective role of fatty acid composites against adverse effects of TiO₂ NPs by other types of toxicity measures, such as the comet assay (Bhabra *et al.*, 2009; Shukla *et al.*, 2013). Also, it remains to be examined whether or not these beneficial effects of surface modification also exist *in vivo*. These studies will provide an important insight into the establishment of safety and toxicity profiles in the use of NPs in cosmetics that are often used over a long period of time.

Several other approaches will help to better understand the molecular mechanisms of NP-mediated cytotoxicity and further confirm the utility and safety of the use of CM-NPs. Xiong *et al.* (2013) revealed that TiO₂ NPs enhance the generation of ROS, plasma membrane damage and depolarization of mitochondria by high throughput screening and ELISA. Whether or not CM-NPs cause less DNA damage or oxidative stress should be rigorously tested by specific assays that assess nuclear and mitochondrial damage as well as ROS production. Related to

this idea, it is possible that low doses of NPs with prolonged exposure may not elicit direct, visible cytotoxicity, but could be sufficient to generate ROS. This is an important question since emerging evidence has suggested that elevated ROS levels are closely associated with the process of aging and progressive impairment in cellular function (Genova *et al.*, 2004; Schöneich, 1999; Circu and Aw, 2010; Seifried *et al.*, 2007). Furthermore, the cytotoxicity of soluble metals resulting from intracellular dissolution of metal NPs should be rigorously examined. Numerous studies have demonstrated biochemical alterations and behavioural dysfunction in animals and humans caused by exposure to various metals (Chang *et al.*, 2014; Ye and Kim, 2015a, 2015b; Menon *et al.*, 2016). Measuring the soluble metal concentrations of NP-exposed cells and cell culture media would provide important information about the potential effect of NP dissolution on metal toxicity. Also, these studies can benefit from more relevant cell culture models. For example, Bhabra *et al.* (2009) utilized a multilayer cell system to replicate cellular barriers in human tissues and demonstrated DNA damage by cobalt-chromium NPs to the deepest cell layer below the barrier even without direct contact, suggesting ATP as a signaling molecule that communicates the DNA damage to the lowest layer of cells. In addition, it is important to identify which of the four fatty acids used in current study provide(s) most significant protective effect from cytotoxicity. Likewise, the effect of cosmetic components other than fatty acids on cell viability remains to be characterized. These efforts will increase our understanding about the toxicity of metal oxide NPs not only in the use of personal care products in everyday lives, but in other medical diagnostic and therapeutic applications.

ACKNOWLEDGMENTS

This work was supported in part by the Ministry of Science, ICT and Future Planning (MSIP) (No. 2014-052607 for H.Y.L.; No. 2013R1A3A2042120 for J.K.L., A.Y.K., and B.H.P.). The authors are grateful to Dr. Kyuhong Lee (KIT) for help during NMR analysis.

Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

- Abd Gani, S.S., Basri, M., Kassim, A., Raja Abdul Rahman, R.N.Z., Salleh, A.B. and Ismail, Z. (2014): Characterization of encapsulated titanium dioxide using engkabang fat esters for cosmeceutical purposes. *Int. J. Pharm. Chem. Biol. Sci.*, **4**, 725-737.
- Andersen, F. (1987): Final Report on the Safety Assessment of Oleic Acid, Lauric Acid, Palmitic Acid, Myristic Acid, and Stearic Acid. *J. Am. Coll. Toxicol.*, **6**, 321-401.
- Andersson, P.O., Lejon, C., Ekstrand-Hammarström, B., Akfur, C., Ahlinder, L., Bucht, A. and Osterlund, L. (2011): Polymorph- and size-dependent uptake and toxicity of TiO₂ nanoparticles in living lung epithelial cells. *Small*, **7**, 514-523.
- Bhabra, G., Sood, A., Fisher, B., Cartwright, L., Saunders, M., Evans, W.H., Surprenant, A., Lopez-Castejon, G., Mann, S., Davis, S.A., Hails, L.A., Ingham, E., Verkade, P., Lane, J., Heesom, K., Newson, R. and Case, C.P. (2009): Nanoparticles can cause DNA damage across a cellular barrier. *Nat. Nanotechnol.*, **4**, 876-883.
- CDC (2011): Occupational Exposure to Titanium Dioxide. *Current Intelligence Bulletin* 63.
- Chang, J., Kueon, C. and Kim, J. (2014): Influence of lead on repetitive behavior and dopamine metabolism in a mouse model of iron overload. *Toxicol. Res.*, **30**, 267-276.
- Circu, M.L. and Aw, T.Y. (2010): Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radic. Biol. Med.*, **48**, 749-762.
- Filipe, P., Silva, J.N., Silva, R., Cirne de Castro, J.L., Marques Gomes, M., Alves, L.C., Santus, R. and Pinheiro, T. (2009): Stratum corneum is an effective barrier to TiO₂ and ZnO nanoparticle percutaneous absorption. *Skin Pharmacol. Physiol.*, **22**, 266-275.
- Genova, M.L., Pich, M.M., Bernacchia, A., Bianchi, C., Biondi, A., Bovina, C., Falasca, A.I., Formiggini, G., Castelli, G.P. and Lenaz, G. (2004): The mitochondrial production of reactive oxygen species in relation to aging and pathology. *Ann. N Y Acad. Sci.*, **1011**, 86-100.
- Heng, B.C., Zhao, X., Xiong, S., Ng, K.W., Boey, F.Y. and Loo, J.S. (2010): Toxicity of zinc oxide (ZnO) nanoparticles on human bronchial epithelial cells (BEAS-2B) is accentuated by oxidative stress. *Food Chem. Toxicol.*, **48**, 1762-1766.
- Kang, H., Mintri, S., Menon, A.V., Lee, H.Y., Choi, H.S. and Kim, J. (2015): Pharmacokinetics, pharmacodynamics and toxicology of theranostic nanoparticles. *Nanoscale*, **7**, 18848-18862.
- Leung, Y.H., Chan, C.M., Ng, A.M., Chan, H.T., Chiang, M.W., Djurišić, A.B., Ng, Y.H., Jim, W.Y., Guo, M.Y., Leung, F.C., Chan, W.K. and Au, D.T. (2012): Antibacterial activity of ZnO nanoparticles with a modified surface under ambient illumination. *Nanotechnology*, **23**, 475703.
- Liu, S., Xu, L., Zhang, T., Ren, G. and Yang, Z. (2010): Oxidative stress and apoptosis induced by nanosized titanium dioxide in PC12 cells. *Toxicology*, **267**, 172-177.
- Long, T.C., Tajuba, J., Sama, P., Saleh, N., Swartz, C., Parker, J., Hester, S., Lowry, G.V. and Veronesi, B. (2007): Nanosize titanium dioxide stimulates reactive oxygen species in brain microglia and damages neurons *in vitro*. *Environ. Health Perspect.*, **115**, 1631-1637.
- Menon, A.V., Chang, J. and Kim, J. (2016): Mechanisms of divalent metal toxicity in affective disorders. *Toxicology*, **339**, 58-72.
- Monteiro-Riviere, N.A., Wiench, K., Landsiedel, R., Schulte, S., Inman, A.O. and Riviere, J.E. (2011): Safety evaluation of sunscreen formulations containing titanium dioxide and zinc oxide nanoparticles in UVB sunburned skin: an *in vitro* and *in vivo* study. *Toxicol. Sci.*, **123**, 264-280.
- Nativo, P., Prior, I.A. and Brust, M. (2008): Uptake and intracellular fate of surface-modified gold nanoparticles. *ACS Nano*, **2**, 1639-1644.
- Nazarenko, Y., Zhen, H., Han, T., Liyo, P.J. and Mainelis, G. (2012): Nanomaterial inhalation exposure from nanotechnology-based

- cosmetic powders: a quantitative assessment. *J. Nanopart. Res.*, **14**.
- Newman, M.D., Stotland, M. and Ellis, J.I. (2009): The safety of nanosized particles in titanium dioxide- and zinc oxide-based sunscreens. *J. Am. Acad. Dermatol.*, **61**, 685-692.
- Park, E.J., Yi, J., Chung, K.H., Ryu, D.Y., Choi, J. and Park, K. (2008): Oxidative stress and apoptosis induced by titanium dioxide nanoparticles in cultured BEAS-2B cells. *Toxicol. Lett.*, **180**, 222-229.
- Sadrieh, N., Wokovich, A.M., Gopee, N.V., Zheng, J., Haines, D., Parmiter, D., Siitonen, P.H., Cozart, C.R., Patri, A.K., McNeil, S.E., Howard, P.C., Doub, W.H. and Buhse, L.F. (2010): Lack of significant dermal penetration of titanium dioxide from sunscreen formulations containing nano- and submicron-size TiO₂ particles. *Toxicol. Sci.*, **115**, 156-166.
- Salatin, S., Maleki Dizaj, S. and Yari Khosroushahi, A. (2015): Effect of the surface modification, size, and shape on cellular uptake of nanoparticles. *Cell Biol. Int.*, **39**, 881-890.
- Schilling, K., Bradford, B., Castelli, D., Dufour, E., Nash, J.F., Pape, W., Schulte, S., Tooley, I., van den Bosch, J. and Schellau, F. (2010): Human safety review of "nano" titanium dioxide and zinc oxide. *Photochem. Photobiol. Sci.*, **9**, 495-509.
- Schöneich, C. (1999): Reactive oxygen species and biological aging: a mechanistic approach. *Exp. Gerontol.*, **34**, 19-34.
- Seifried, H.E., Anderson, D.E., Fisher, E.I. and Milner, J.A. (2007): A review of the interaction among dietary antioxidants and reactive oxygen species. *J. Nutr. Biochem.*, **18**, 567-579.
- Serpone, N., Salinaro, A. and Emeline, A. (2001): Deleterious Effects of Sunscreen Titanium Dioxide Nanoparticles on DNA. Efforts to Limit DNA Damage by Particle Surface Modification. *Proc. SPIE*, **4258**, 86-98.
- Sharma, V., Shukla, R.K., Saxena, N., Parmar, D., Das, M. and Dhawan, A. (2009): DNA damaging potential of zinc oxide nanoparticles in human epidermal cells. *Toxicol. Lett.*, **185**, 211-218.
- Shukla, R.K., Kumar, A., Gurbani, D., Pandey, A.K., Singh, S. and Dhawan, A. (2013): TiO₂ nanoparticles induce oxidative DNA damage and apoptosis in human liver cells. *Nanotoxicology*, **7**, 48-60.
- Shukla, R.K., Sharma, V., Pandey, A.K., Singh, S., Sultana, S. and Dhawan, A. (2011): ROS-mediated genotoxicity induced by titanium dioxide nanoparticles in human epidermal cells. *Toxicol. In Vitro*, **25**, 231-241.
- Singh, P. and Nanda, A. (2014): Enhanced sun protection of nanosized metal oxide particles over conventional metal oxide particles: an *in vitro* comparative study. *Int. J. Cosmet. Sci.*, **36**, 273-283.
- van der Molen, R.G., Hurks, H.M., Out-Luiting, C., Spies, F., van't Noordende, J.M., Koerten, H.K. and Mommaas, A.M. (1998): Efficacy of micronized titanium dioxide-containing compounds in protection against UVB-induced immunosuppression in humans *in vivo*. *J. Photochem. Photobiol. B*, **44**, 143-150.
- Weir, A., Westerhoff, P., Fabricius, L., Hristovski, K. and von Goetz, N. (2012): Titanium dioxide nanoparticles in food and personal care products. *Environ. Sci. Technol.*, **46**, 2242-2250.
- Xie, G., Lu, W. and Lu, D. (2015): Penetration of titanium dioxide nanoparticles through slightly damaged skin *in vitro* and *in vivo*. *J. Appl. Biomater. Funct. Mater.*, **13**, e356-e361.
- Xiong, S., George, S., Yu, H., Damoiseaux, R., France, B., Ng, K.W. and Loo, J.S. (2013): Size influences the cytotoxicity of poly (lactic-co-glycolic acid) (PLGA) and titanium dioxide (TiO₂) nanoparticles. *Arch. Toxicol.*, **87**, 1075-1086.
- Ye, Q., Kim, J. (2015a): Effect of olfactory manganese exposure on anxiety-related behavior in a mouse model of iron overload hemochromatosis. *Environ. Toxicol. Pharmacol.*, **30**, 333-341.
- Ye, Q., Kim, J. (2015b): Loss of hfe function reverses impaired recognition memory caused by olfactory manganese exposure in mice. *Toxicol. Res.*, **31**, 17-23.
- Zielińska, A. and Nowak, I. (2014): Fatty acids in vegetable oils and their importance in cosmetic industry. *CHEMIK*, **68**, 103-110.