RESEARCH ARTICLE

Comparative Study of Toxic Effects of Anatase and Rutile Type Nanosized Titanium Dioxide Particles in vivo and in vitro

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Abstract

Two types of nanosized titanium dioxide, anatase (anTiO₂) and rutile (rnTiO₂), are widely used in industry, commercial products and biosystems. TiO₂ has been evaluated as a Group 2B carcinogen. Previous reports indicated that anTiO₂ is less toxic than rnTiO₂, however, under ultraviolet irradiation anTiO₂ is more toxic than rnTiO₂ in vitro because of differences in their crystal structures. In the present study, we compared the in vivo and in vitro toxic effects induced by anTiO₂ and rnTiO₂. Female SD rats were treated with 500 µg/ml of anTiO₂ or rnTiO₂ suspensions by intra-pulmonary spraying 8 times over a two week period. In the lung, treatment with anTiO₂ or rnTiO₂ increased alveolar macrophage numbers and levels of 8-hydroxydeoxyguanosine (8-OHdG); these increases tended to be lower in the anTiO₂ treated group compared to the rnTiO₂ treated group. Expression of MIP1α mRNA and protein in lung tissues treated with anTiO₂ and rnTiO₂ was also significantly up-regulated, with MIP1α mRNA and protein expression significantly lower in the anTiO₂ group than in the rnTiO₂ group. In cell culture of primary alveolar macrophages (PAM) treated with anTiO₂ and rnTiO₂, expression of MIP1α mRNA in the PM and protein in the culture media was significantly higher than in control cultures. Similarly to the in vivo results, MIP1α mRNA and protein expression was significantly lower in the anTiO₂ treated cultures compared to the rnTiO₂ treated cultures. Furthermore, conditioned cell culture media from PAM cultures treated with anTiO₂ had less effect on A549 cell proliferation compared to conditioned media from cultures treated with rnTiO₂. However, no significant difference was found in the toxicological effects on cell viability of ultraviolet irradiated anTiO₂ and rnTiO₂. In conclusion, our results indicate that anTiO₂ is less potent in induction of alveolar macrophage infiltration, 8-OHdG and MIP1α expression in the lung, and growth stimulation of A549 cells in vitro than rnTiO₂.  

Keywords: Nanosized titanium dioxide - anatase - rutile - lung toxicity - MIP1α

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Introduction

There are three mineral forms of natural titanium dioxide particles: rutile, anatase and brookite. Engineered anatase and rutile nanosized titanium dioxide particles (anTiO₂ and rnTiO₂) are being manufactured in large quantities worldwide and applied in many fields including material industry, electronic industry, commercial products and biosystems. Due to differences in crystal structure, anTiO₂ has better photocatalytic activity than rnTiO₂ (Kakinoki et al., 2004). Accordingly, anTiO₂ is mainly used in paints, such as surface painting of the walls and windows of buildings and vehicles, and photocatalytic systems, while rnTiO₂ is preferentially used in cosmetics, sunscreen and food additives.

Large quantity production and widespread application of nTiO₂ have given rise to concern about its health and environmental effects. Anatase and rutile type titanium dioxide particles, nanosized and larger, are evaluated as Group 2B carcinogens (possibly carcinogenic to humans) by WHO/International Agency for Research on Cancer (IARC, 2010), based on 2-year animal aerosol inhalation studies (Mohr et al., 2006). Pulmonary exposure to rnTiO₂ promotes DHPN-induced lung carcinogenesis in rats, and the promotion effect is possibly associated with rnTiO₂ burdened alveolar macrophage derived macrophage inflammatory protein 1 alpha (MIP1α), which acts as a growth factor to stimulate the proliferation of human lung adenocarcinoma cells (A549) in vitro (Xu et al., 2010). Dermal application of anTiO₂ has been shown to cause significant increases in the level of superoxide dismutase and malondialdehyde in hairless mice (Wu et al., 2009). Size and photoactivation affect the in vitro toxicity of anTiO₂ and rnTiO₂. anTiO₂ (10 and 20 nm) induces

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oxidative DNA damage, lipid peroxidation and micronuclei formation, and increases hydrogen peroxide and nitric oxide production in BEAS-2B cells, a human bronchial epithelial cell line, but anTiO$_2$ 200 nm particles do not (Gurr et al., 2005). In contrast, both nano-sized and 200nm rutile-type (ruTiO$_2$) are toxic in vitro (Gurr et al., 2005; Sayes et al., 2006). On the other hand, under ultraviolet irradiation, anTiO$_2$ is 100 times more toxic to human dermal fibroblasts and A549 cells than rutile-type, and is more potent than rutile-type in the induction of lactate dehydrogenase release, reactive oxygen species production and interleukin 8 secretion (Sayes et al., 2006). Experimental data demonstrating differences in the toxic effects of anTiO$_2$ and rutile-type, in vivo, however, are still lacking.

Respiratory exposure to nTiO$_2$ particles can occur both at the workplace, e.g., in manufacturing and packing sites, and outside the workplace during their use (Maynard et al., 2006; Schulte et al., 2008). In the present study, we delivered anTiO$_2$ and rutile-type to the rat lung by trans-tracheal intra-pulmonary spraying (TIPS) and compared lung inflammation and several toxicological parameters induced by anTiO$_2$ and rutile-type. The results indicated that obvious lung inflammatory lesions were not observed in the rats, and anTiO$_2$ or rutile-type particles were phagocytosed by alveolar macrophages. Analysis of alveolar macrophage induction, 8-OHdG level in the lung, and MIP1α expression both in vivo and in vitro in the lung and in vitro in PAM indicated that anTiO$_2$ elicited lower levels of biological responses than rutile-type. Long-term toxic effects of anTiO$_2$ and rutile-type still need to be clarified.

**Materials and Methods**

**Preparation and characterization of nTiO$_2$ suspension**

Nanoparticle TiO$_2$ particles (anatase type without coating, primary size 25 nm and rutile type without coating, primary size 20 nm) were provided by Japan Cosmetic Association, Tokyo, Japan. Both anTiO$_2$ and rutile-type particles were suspended in saline at 500 μg/ml and then autoclaved. The suspensions were sonicated for 20 min shortly before use to prevent aggregate formation.

Characterization of nTiO$_2$ was conducted as follows: The shapes of nTiO$_2$ in suspension were imaged by transmission electron microscope (TEM) and scanning electron microscopy (SEM). Element analysis was performed by a JEM-1010 transmission electron microscope (JEOL Co., Tokyo, Japan), and the photos were then analyzed by a JEM-1010 transmission electron microscope (JEOL Co., Tokyo, Japan) equipped with an X-ray microanalyzer (EDAX, Tokyo, Japan). Over 1000 particles of anTiO$_2$ and rutile-type were measured.

**Animals**

Female Sprague-Dawley rats (SD rats) were purchased from CLEA Japan Co., Ltd (Tokyo, Japan). The animals were housed in the animal center of Nagoya City University Medical School, maintained on a 12 hour light-dark cycle and received oriental MF basal diet (Oriental Yeast Co., Tokyo, Japan) and water ad lib. The research was conducted according to the Guidelines for the Care and Use of Laboratory Animals of Nagoya City University Medical School and the experimental protocol was approved by the Institutional Animal Care and Use Committee (H22M-19).

**Trans-tracheal intra-pulmonary spraying (TIPS) protocol**

Three groups of 6 female SD rats (Group 1, saline; Group 2, anTiO$_2$; and Group 3, rutile-type) aged 9 weeks were administered to Groups 2 and 3 was 2.0 mg per rat. Six hours after the last spraying, the animals were killed and the whole lung was excised and divided into two parts; the left lung was cut into pieces and immediately frozen at -80°C and used for biochemical analysis, and the right lung was fixed in 4% paraformaldehyde solution in phosphate-buffered saline (PBS) adjusted to pH 7.3 and processed for immunohistochemical, light microscopic and transmission electron microscopic (TEM) examinations.

**Light microscopy and transmission electron microscopy**

Hematoxylin and eosin (H&E) stained sections were used for pathological observation. The number of alveolar macrophages in H&E lung tissue slides was counted and expressed as number per mm$^2$.

Slides were observed under light microscopic observation, the corresponding area in the paraffin block was cut out, deparaffinized and embedded in epoxy resin and processed for TEM and titanium element analysis with a JEM-1010 transmission electron microscope (JEOL Co. Ltd, Tokyo, Japan) equipped with an X-ray microanalyzer (EDAX, Tokyo, Japan).

**Analysis of 8-hydroxydeoxyguanosine levels**

For the analysis of 8-hydroxydeoxyguanosine (8-OHdG) levels, genomic DNA was isolated from a piece of the left lung with a DNA Extractor WB kit (Wako Chemicals Co., Ltd). 8-OHdG levels were determined with an 8-OHdG ELISA Check kit (Japan Institute for Control of Aging, Shizuoka, Japan).

**RNA isolation, cDNA synthesis and RT-PCR analysis of gene expression**

Pieces of the left lungs (50-100 mg) were thawed, rinsed 3 times with ice cold PBS, and total RNA was isolated using 1 ml Trizol Reagent (Invitrogen, Karlsruhe, Germany). For reverse transcription PCR (RT-PCR) and real-time PCR, first strand cDNA synthesis from 2 μg of total RNA was performed using SuperScript™ III First-Strand Synthesis
System (Invitrogen of Life Technologies, CA) according to the manufacturer’s instructions. PCR primers for rat MIP1α were 5’-TTTGTAGACCCAGCCCTTT -3’ (forward) and 5’-CTCAAGCCCCCTGCTCTACAC-3’ (reverse), and the product size was 191bp. b-actin was used as internal control and the primers were 5’- AGCCATGTACGTCAGCCTCC-3’ (forward) and 5’-CCTTCACGTGTGGTTGGTGA-3’, and the product size was 228 bp. RT-PCR was conducted using an iCycler (BioRad Life Sciences, CA) as follows: 95°C 20 sec, 60°C 20 sec, 72°C 30sec, 30 cycles for MIP1α; and 95°C 20 sec, 60°C 20 sec, 72°C 30sec, 15 cycles for b-actin. Real-time PCR analysis of MIP1α gene expression was performed with a 7300 Real Time PCR System (Applied Biosystem, CA) using Power SYBR Green PCR Master Mix (Applied Biosystem, CA) according to the manufacturer’s instructions. b-actin gene was used as the normalizing reference gene.

Immunohistochemical analysis
Paraffin embedded lung tissues sections were immunostained with polyclonal anti-rat MIP1α (BioVision, Lyon, France). Antigen retrieval was carried out by microwave for 20 min in 10 mmol/L citrate buffer (pH 6.0). Antibody was diluted 1:100 in blocking solution and applied to the slides, and the slides were incubated at 4°C overnight. Immunohistochemical staining was done by the avidin-biotin complex method (ABC) using the Vectastain Elite ABC system (Vector Laboratories, Burlingame, CA). Biotinylated goat anti-rabbit IgG (Vector Laboratories) was used as a secondary antibody at a dilution of 1:500 and then incubated with the Vectastain Elite ABC system (Vector Laboratories, Burlingame, CA). Sections were lightly counterstained with hematoxylin for microscopic examination.

ELISA for MIP1α in the lung tissues and the supernatants of cell culture
Left lung tissue samples (50-100mg) were thawed, rinsed 3 times with ice cold PBS and homogenized in 1 ml of tissue extraction reagent (PeproTech, London, UK) containing 1% (v/v) Proteinase Inhibitor Cocktail (Sigma-Aldrich, St Louis, MO, USA). The homogenates were clarified by centrifugation at 10,000g, 4°C for 5 min. The protein content in the supernatants was measured with a BCA TM Protein assay kit (Pierce). The levels of MIP1α in the supernatants were measured using rat MIP1α ELISA Development Kit (Cat#: 900-K75, Peprotech, Inc., Rocky Hill, NJ.) according to the manufacturer’s instruction, and expressed as pg/mg lung tissue protein. The levels of MIP1α in cell culture supernatants were measured as above and expressed as pg/ml.

Isolation of PAM and exposure of nTiO2 to PAM cells
Induction and isolation of alveolar macrophages in female SD rats was performed as described previously (Xu et al., 2010). 10^5 primary alveolar macrophages (PAM) were cultured in RPMI1640 containing 2% fetal bovine serum and antibiotics overnight at 37°C, 5% CO2. 500 µg/ml of anTiO2 and rniTiO2 suspensions was then added to the cultures to a final concentration of 10 µg/ml and the cells were incubated for another 24 hours. RNA was isolated from the PAM and the level of MIP1α protein in the conditioned culture media was measured by ELISA.

In vitro cell proliferation assay
A549 cells were seeded into 96-well culture plates at 2x10³ cells per well in 2% fetal bovine serum Dulbecco’s modified Eagle’s medium (Wako Chemicals Co., Ltd). After overnight incubation, the medium was replaced with the conditioned PAM culture media treated with anTiO2 or rniTiO2, and the cells were incubated for another 72 hours, with or without 20 µg/ml of anti-MIP1α neutralizing antibody (R&D Systems, Minneapolis, MN). The relative cell number of A549 cells was determined using a Cell counting Kit-8 (Dojindo Molecular Technologies, Rockville, MD) according to the manufacturer’s instruction.

Cytotoxicity assay in vitro
A549 cells, the primary human lung fibroblast cell line CCD34 (ECACC, Cat. No. 90110514) and PAM were used for cytotoxicity analyses. Cells were seeded in 96 well plates at 5x10³/well and incubated overnight. The cells were then treated with anTiO2 and rniTiO2 suspensions at final concentrations of 0, 2, 10, or 50 µg/ml and then incubated for another 24 hours. The relative cell number was determined as described above.

Cytotoxicity of anTiO2 and rniTiO2 under ultraviolet B irradiation
A549 cells were used for analysis of nTiO2 cytotoxicity under ultraviolet irradiation. First, we determined an irradiation time that did not affect the cell viability as follows: A549 cells were seeded into 96 well plates at 1x10⁴/well in 200 µL Dulbecco’s modified Eagle’s medium (Wako Chemicals Co., Ltd) containing 10% fetal bovine serum and incubated overnight. The cells were then treated with ultraviolet A (UVB) for 0, 30 sec, 1 min, 2 min, 5 min and 10 min with a transilluminator (Vilber Lourmat, France). The light intensity was 1000 mW/cm², and the emission spectrum was from 270 nm to 330 nm with a peak at 312 nm. The non-irradiated control wells were covered with a sterile aluminium sheet to prevent irradiation. The relative cell number was determined after incubation for 48 hours at 37°C, 5% CO2.

Next, we observed the effect of anTiO2 and rniTiO2 on cell viability under UVB. A549 cells were seeded into 96 well plate at 1x10⁴/well in 100 µL culture media and incubated overnight. Then, 100 mL of anTiO2 or rniTiO2 suspensions in DMEM culture medium containing 10% FBS was added into the wells to a final concentration of 0, 2.5 and 10 µg/ml and incubated for 30 min. The cells were irradiated with UVB for 2 min (2 min UVB irradiation did not affect cell viability), and incubated for another 48 hours, before determination of relative cell number.

Statistical analysis
Statistical significance of the in vitro and in vivo findings was analyzed using the two-tailed Student’s t-test. In vitro and in vivo data are presented as means ± standard error of the mean (SEM).
deviations. A value of p<0.05 was considered to be significant.

Results

Characterization of nTiO₂ particles in suspension

TEM images showed that individual anTiO₂ particles were spherical in shape, while individual rnTiO₂ particles had a rod-like shape, and both anTiO₂ and rnTiO₂ formed large aggregates in suspension (Figure 1A and B). Similarly, SEM observation indicated aggregate formation of both types of nTiO₂ particles (Figure 1C and D). Peaks of titanium (green arrows) and oxygen (blue arrows), which are present in the carbon sheets used in the SEM, were observed by elemental scanning (Figure 1E and F). Peaks of other elements were not detected in either the rnTiO₂ or anTiO₂ samples. Analyses of particle size showed that the mean and medium diameters were 5.491±2.727 mm and 5.127 mm for anTiO₂, and 3.799±2.231 mm and 3.491 mm for rnTiO₂ (Figure 1G), confirming aggregate formation of both types of nTiO₂ particles in suspension.

Figure 1. Characterization of nTiO₂ Particles in Suspension. A and B: TEM images of anTiO₂ and rnTiO₂ particles in suspension. C and D: SEM images of anTiO₂ and rnTiO₂ particles. E and F: Element scanning showed peaks of titanium (green arrows), oxygen (blue arrows), carbon (white arrows) and nitrogen (red arrows) in anTiO₂ and rnTiO₂ particles. G: Size distribution of anTiO₂ and rnTiO₂ in suspension.

Histological observation and 8-OHdG level in the lung tissue

Only a few small lung inflammatory lesions were observed in rats treated with anTiO₂ and rnTiO₂ (Figure 2A, B and C). Alveolar macrophage infiltration was found throughout the lung tissue, and most of the alveolar macrophages were seen with phagocytosed anTiO₂ particles or rnTiO₂ particles (Figure 2D, E and F). TEM observation demonstrated that both anTiO₂ and rnTiO₂ were deposited in various sizes in the cytoplasm of the alveolar macrophages (Figure 2G and H). Neither anTiO₂ or rnTiO₂ particles were found in other types of cells in the lung tissue. The number of macrophages per mm² lung tissue section was 67.1±15.8 (saline), 165.0±34.9 (anTiO₂) and 214.2±44.1 (rnTiO₂). The numbers of macrophages in the anTiO₂ and rnTiO₂ treated groups was significantly higher than in the control group (p<0.001), and the anTiO₂ treated group had lower macrophage infiltration than the rnTiO₂ treated group.

The level of 8-OHdG, a parameter of oxidative DNA damage caused by reactive oxygen species (ROS), in the lung tissue in rats treated with anTiO₂ and rnTiO₂ was 1.96±0.77 and 3.07±1.25 (pg per mg DNA), respectively, and was higher than that of the control (1.44±0.63): The increase in 8-OHdG in the lungs of rnTiO₂, but not anTiO₂, treated rats was significantly higher than the control.
MIP1α expression in the lung tissue

RT-PCR suggested an increase in MIP1α mRNA expression in lung tissue treated with anTiO2 or rnTiO2 (Figure. 3A). Real-time PCR analysis indicated that compared with the control group, the increase was 2.79-fold for anTiO2 and 5.35-fold for rnTiO2. MIP1α mRNA expression was also significantly lower in the anTiO2 treated group compared to the rnTiO2 treated group (Figure. 3B). The levels of MIP1α protein in the lung tissue were 32.8±0.31 and 52.7±0.58 pg/mg lung protein in the anTiO2 and rnTiO2 treated groups, both significantly higher than that of the control group (20.8±0.24) (Figure. 3C). Similarly to MIP1α mRNA expression, MIP1α protein expression was significantly lower in the anTiO2 treated group compared to the rnTiO2 treated group.

To find out what cells in the lung accounted for the increased MIP1α protein expression, we examined tissue samples using MIP1α immunohistochemistry. As shown in Figure. 3D, E and F, MIP1α protein was produced by anTiO2 or anTiO2 burdened alveolar macrophages.

Exposure of PAMs to anTiO2 and rnTiO2 and cell proliferation assays in vitro

As in the lung tissue, in vitro exposure of PAM to rnTiO2 induced expression of MIP1α mRNA (Figure. 4A) and protein (Figure. 4B). Treatment with anTiO2 and rnTiO2 caused 11.96-fold and 15.26-fold increases in the expression of MIP1α mRNA, respectively, in cultured PAM. The level of MIP1α protein in the cell culture medium was 32.8±1.1 pg/mL for anTiO2 and 52.7±1.3 pg/mL for rnTiO2, significantly higher than that of the control.

The supernatants of the culture media of PAM treated with rnTiO2, anTiO2, or anTiO2 showed only a tendency to increase A549 proliferation, while those collected from PAM treated with rnTiO2 significantly promoted proliferation of A549 cells (115%) compared to the saline treated group. The promotion effect of the supernatants of PAM treated with rnTiO2 was significantly lower in the anTiO2 treated compared to the rnTiO2 treated cells.

The supernatants of the culture media of PAM treated with anTiO2 showed only a tendency to increase A549 cell proliferation, while those collected from PAM treated with rnTiO2 significantly promoted proliferation of A549 cells (115%) compared to supernatants from the saline treated group (Figure. 4C). The promotion effect of the supernatants of PAM cell cultures treated with anTiO2 or rnTiO2 was significantly lower in the anTiO2 treated compared to the rnTiO2 treated cells.

Figure 3. Expression of MIP1α in the Lung Tissue. A, B and C: Analysis of expression of MIP1α mRNA by RT-PCR (A) and real-time PCR (B) and protein by ELISA (C). D, E, and F: Immunohistochemistry shows MIP1α expressed in alveolar macrophages of lung tissue treated with saline (D), anTiO2 (E) and rnTiO2 (F). *, ** represent p<0.05 and 0.001, respectively, versus saline; ### represent p<0.001, versus rnTiO2.

Figure 4. The Effect of anTiO2 and rnTiO2 on PAM Cells. The expression of MIP1α mRNA in cultured PAM (A) and protein in the culture media (B) indicate that treatment with anTiO2 or rnTiO2 increased MIP1α expression in the PAM. Conditioned cell culture media of PAM treated with rnTiO2, but not anTiO2, had a significant effect on proliferation of A549 cells, and this promotion was attenuated by addition of 20 μg/ml MIP1α neutralizing antibody (C). **, ***represent p<0.01 and 0.001, versus saline; #, ###represent p<0.05 and 0.001, versus rnTiO2.

Figure 5. In vitro Assays. A: The effect of anTiO2 and rnTiO2 on the viability of A549, CCD34 and PAM cells. B: The effect of UVB irradiation on the viability of A549, CCD34, and PAM cells. C: The effect of anTiO2 and rnTiO2 on the viability of A549 under UVB irradiation. *, **, ***represent p<0.05 and 0.001, versus the vehicle (20.8±1.2 pg/mL). Both mRNA and protein expression of MIP1α was significantly lower in the anTiO2 treated compared to the rnTiO2 treated cells.
**In vitro cytotoxicity assays**

In vitro cytotoxicity assays indicated that both anTiO\(_2\) and rnTiO\(_2\) had little effect on the cell viability of A549 and CCD34 cells at a concentration of up to 50 mg/ml. anTiO\(_2\) had a cytotoxic effect on the cell viability of PM at doses of 10 and 50 mg/ml, while rnTiO\(_2\) did not impair the cell viability of PM at any of the examined concentrations (Figure 5A).

To investigate whether UVB irradiation affected the cytotoxic effects of anTiO\(_2\) and rnTiO\(_2\) on cell viability, we first determined the exposure times that ultraviolet B irradiation alone did not impair the viability of A549 cells. As shown in Figure 5B, irradiation for up to 2 min did not have any effect on the viability of A549 cells. With 2 min of UVB irradiation, neither anTiO\(_2\) or rnTiO\(_2\) at doses of 2, 5 or 10 µg/ml resulted in any decrease in the viability of A549 cells (Figure 5C).

**Discussion**

The toxicity of nanoparticles usually includes tiers of biological responses such as induction of ROS and inflammation (Nel et al., 2006). This may contribute to carcinogenic potential (Tsuda et al., 2009). Thus, in the present study, we compared several parameters of inflammation and oxidative stress induced by TIPS of anTiO\(_2\) and rnTiO\(_2\). The results indicated that both anTiO\(_2\) and rnTiO\(_2\) particles were phagocytosed by alveolar macrophages and did not cause strong lung inflammation. Treatment with anTiO\(_2\) and rnTiO\(_2\) increased alveolar macrophage infiltration, MIP1\(\alpha\) expression and 8-OHdG production: anTiO\(_2\) had less effect than rnTiO\(_2\).

Phagocytosis by alveolar macrophages is a major defense mechanism for deposition and clearance of inhaled particles (Heppleston, 1984; Rom et al., 1991; Geiser et al., 2008). However, activation of alveolar macrophages is strongly associated with inflammatory reactions and ROS production (Renwick et al., 2001; Bhatt et al., 2002; Wang et al., 2007). Also, MIP1\(\alpha\), secreted from rnTiO\(_2\) burden alveolar macrophages, is possibly involved in the promotion of lung carcinogenesis (Xu et al., 2010). Similarly, pleural macrophage recruitment and activation are involved in the pathogenesis of asbestosis (Choe et al., 1997). These results indicate two contrasting roles of alveolar macrophages in pathogenesis and host defense.

The toxic effects of nanoparticles are dependent on their size, shape, surface functionality and composition (Albanese et al., 2012). In the present study, we used comparable sizes of anTiO\(_2\) and rnTiO\(_2\) particles. Both types of nTiO\(_2\) had no surface coating and had no obvious difference in elemental composition. Therefore, differences in alveolar macrophage induction, MIP1\(\alpha\) expression and 8-OHdG production between anTiO\(_2\) and rnTiO\(_2\) are likely due to their different crystal structures and shapes. The lower toxicity of anTiO\(_2\) compared to rnTiO\(_2\) in the absence of UVB irradiation in our study is consistent with a previous in vitro study with bulk rutile and anatase TiO\(_2\) (Gurr et al., 2005). In contrast to a previous study (Sayes et al., 2006), in the present study anTiO\(_2\) and rnTiO\(_2\) did not exhibit different toxicities on the cell viability of A549 cells under ultraviolet irradiation.

It should be noted that both types of anTiO\(_2\) and rnTiO\(_2\) particles formed aggregates in suspension, and aggregation may alter their bio-reactivity. Whether anTiO\(_2\) and rnTiO\(_2\) particles have different long-term effects remains to be clarified.

In conclusion, in vivo exposure of the rat lung to anTiO\(_2\) or rnTiO\(_2\) particles increased alveolar macrophage infiltration, MIP1\(\alpha\) expression and 8-OHdG production, with anTiO\(_2\) eliciting lower levels of biological responses than rnTiO\(_2\). Similarly, exposure of primary alveolar macrophages to rnTiO\(_2\) in vitro resulted in the cells producing more MIP1\(\alpha\) mRNA and protein than cells exposed to anTiO\(_2\). Cytotoxicity assays in vitro indicated that both anTiO\(_2\) and rnTiO\(_2\) had very low cellular toxicity even under UVB irradiation.

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