

## 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 (anTiO<sub>2</sub>) and rutile (rnTiO<sub>2</sub>), are widely used in industry, commercial products and biosystems. TiO<sub>2</sub> has been evaluated as a Group 2B carcinogen. Previous reports indicated that anTiO<sub>2</sub> is less toxic than rnTiO<sub>2</sub>, however, under ultraviolet irradiation anTiO<sub>2</sub> is more toxic than rnTiO<sub>2</sub> *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 anTiO<sub>2</sub> and rnTiO<sub>2</sub>. Female SD rats were treated with 500 µg/ml of anTiO<sub>2</sub> or rnTiO<sub>2</sub> suspensions by intra-pulmonary spraying 8 times over a two week period. In the lung, treatment with anTiO<sub>2</sub> or rnTiO<sub>2</sub> increased alveolar macrophage numbers and levels of 8-hydroxydeoxyguanosine (8-OHdG); these increases tended to be lower in the anTiO<sub>2</sub> treated group compared to the rnTiO<sub>2</sub> treated group. Expression of MIP1α mRNA and protein in lung tissues treated with anTiO<sub>2</sub> and rnTiO<sub>2</sub> was also significantly up-regulated, with MIP1α mRNA and protein expression significantly lower in the anTiO<sub>2</sub> group than in the rnTiO<sub>2</sub> group. In cell culture of primary alveolar macrophages (PAM) treated with anTiO<sub>2</sub> and rnTiO<sub>2</sub>, expression of MIP1α mRNA in the PAM 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 anTiO<sub>2</sub> treated cultures compared to the rnTiO<sub>2</sub> treated cultures. Furthermore, conditioned cell culture media from PAM cultures treated with anTiO<sub>2</sub> had less effect on A549 cell proliferation compared to conditioned media from cultures treated with rnTiO<sub>2</sub>. However, no significant difference was found in the toxicological effects on cell viability of ultra violet irradiated anTiO<sub>2</sub> and rnTiO<sub>2</sub>. In conclusion, our results indicate that anTiO<sub>2</sub> 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 rnTiO<sub>2</sub>.

**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 (anTiO<sub>2</sub> and rnTiO<sub>2</sub>) 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, anTiO<sub>2</sub> has better photocatalytic activity than rnTiO<sub>2</sub> (Kakinoki et al., 2004). Accordingly, anTiO<sub>2</sub> is mainly used in paints, such as surface painting of the walls and windows of buildings and vehicles, and photocatalytic systems, while rnTiO<sub>2</sub> is preferentially used in cosmetics, sunscreen and food additives.

Large quantity production and widespread application of nTiO<sub>2</sub> 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 rnTiO<sub>2</sub> promotes DHPN-induced lung carcinogenesis in rats, and the promotion effect is possibly associated with rnTiO<sub>2</sub> 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 anTiO<sub>2</sub> 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 anTiO<sub>2</sub> and rnTiO<sub>2</sub>. anTiO<sub>2</sub> (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<sub>2</sub> 200 nm particles do not (Gurr *et al.*, 2005). In contrast, both nano-sized and 200nm rnTiO<sub>2</sub> are toxic *in vitro* (Gurr *et al.*, 2005; Sayes *et al.*, 2006). On the other hand, under ultraviolet irradiation, anTiO<sub>2</sub> is 100 times more toxic to human dermal fibroblasts and A549 cells than rnTiO<sub>2</sub>, and is more potent than rnTiO<sub>2</sub> 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<sub>2</sub> and rnTiO<sub>2</sub> *in vivo*, however, are still lacking.

Respiratory exposure to nTiO<sub>2</sub> 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<sub>2</sub> and rnTiO<sub>2</sub> to the rat lung by trans-tracheal intra-pulmonary spraying (TIPS) and compared lung inflammation and several toxicological parameters induced by anTiO<sub>2</sub> and rnTiO<sub>2</sub>. The results indicated that obvious lung inflammatory lesions were not observed in the rats, and anTiO<sub>2</sub> or rnTiO<sub>2</sub> particles were phagocytosed by alveolar macrophages. Analysis of alveolar macrophage induction, 8-OHdG level in the lung, and MIP1 $\alpha$  expression both *in vivo* in the lung and *in vitro* in PAM indicated that anTiO<sub>2</sub> elicited lower levels of biological responses than rnTiO<sub>2</sub>. Long-term toxic effects of anTiO<sub>2</sub> and rnTiO<sub>2</sub> still need to be clarified.

## Materials and Methods

### *Preparation and characterization of nTiO<sub>2</sub> suspension*

Nanosized TiO<sub>2</sub> 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<sub>2</sub> and rnTiO<sub>2</sub> particles were suspended in saline at 500  $\mu$ g/ml and then autoclaved. The suspensions were sonicated for 20 min shortly before use to prevent aggregate formation.

Characterization of nTiO<sub>2</sub> was conducted as follows: The shapes of nTiO<sub>2</sub> in suspension were imaged by transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Element analysis was performed by a an X-ray microanalyzer (EDAX, Tokyo, Japan), after aliquots of nTiO<sub>2</sub> were loaded onto a carbon sheet. For size distribution analysis, aliquots of the 500  $\mu$ g/ml nTiO<sub>2</sub> suspension were loaded onto clean glass slides and photographed under a polarized light microscope (Olympus BX51N-31P-O polarized light microscope, Tokyo, Japan), and the photos were then analyzed by an image analyzer system (IPAP, Sumika Technos Corporation, Osaka, Japan). Over 1000 particles of anTiO<sub>2</sub> and rnTiO<sub>2</sub> 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<sub>2</sub>; and Group 3, rnTiO<sub>2</sub>) aged 9 weeks were acclimated for 7 days prior to the start of the study. Saline and nTiO<sub>2</sub> suspensions were administered by TIPS to the animals under isoflurane anesthesia: The nozzle of a Microsprayer (series IA-1B Intratracheal Aerosolizer, Penn-century, Philadelphia, PA) connected to a 1 ml syringe was inserted into the trachea through the larynx and a total volume of 0.5 ml suspension was sprayed into the lungs synchronizing with spontaneous inspiration by the animal (Xu *et al.*, 2010). Rats were treated once every the other day over a 2 week period, a total of eight treatments. The total amount of anTiO<sub>2</sub> and rnTiO<sub>2</sub> 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<sup>2</sup>.

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-hydroxydeoxy guanosine 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 mg 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 MIP1 $\alpha$  were 5'-TTTGTGAGACCAGCAGCCTTT -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'-AGCCATGTACGTAGCCATCC-3' (forward) and 5'-CTCTCAGCTGTGGTGGTGAA-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 MIP1 $\alpha$ ; and 95°C 20 sec, 60°C 20 sec, 72°C 30sec, 15 cycles for b-actin. Real-time PCR analysis of MIP1 $\alpha$  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 MIP1 $\alpha$  (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 for 1 hour and visualized using avidin-conjugated alkaline phosphatase complex (ABC kit, Vector laboratories) and Alkaline Phosphatase Substrate Kit (Vector Laboratories). Sections were lightly counterstained with hematoxylin for microscopic examination.

#### ELISA for MIP1 $\alpha$ 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 BCATM Protein assay kit (Pierce). The levels of MIP1 $\alpha$  in the supernatants were measured using rat MIP1 $\alpha$  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 MIP1 $\alpha$  in cell culture supernatants were measured as described above and expressed as pg/ml.

#### Isolation of PAM and exposure of nTiO<sub>2</sub> to PAM cells

Induction and isolation of alveolar macrophages in female SD rats was performed as described previously (Xu et al., 2010). 10<sup>6</sup> primary alveolar macrophages (PAM) were cultured in RPMI1640 containing 2% fetal bovine serum and antibiotics overnight at 37°C, 5% CO<sub>2</sub>. 500  $\mu$ g/ml of anTiO<sub>2</sub> and rnTiO<sub>2</sub> suspensions was then added

to the cultures to a final concentration of 10  $\mu$ g/ml and the cells were incubated for another 24 hours. RNA was isolated from the PAM and the level of MIP1 $\alpha$  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 2 $\times$ 10<sup>3</sup> 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 anTiO<sub>2</sub> or rnTiO<sub>2</sub>, and the cells were incubated for another 72 hours, with or without 20  $\mu$ g/ml of anti-MIP1 $\alpha$  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 5 $\times$ 10<sup>3</sup>/well and incubated overnight. The cells were then treated with anTiO<sub>2</sub> and rnTiO<sub>2</sub> suspensions at final concentrations of 0, 2, 10, or 50  $\mu$ g/ml and then incubated for another 24 hours. The relative cell number was determined as described above.

#### Cytotoxicity of anTiO<sub>2</sub> and rnTiO<sub>2</sub> under ultraviolet B irradiation

A549 cells were used for analysis of nTiO<sub>2</sub> 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 1 $\times$ 10<sup>3</sup>/well in 200  $\mu$ L Dulbecco's modified Eagle's medium (Wako Chemicals Co., Ltd) containing 10% fetal bovine serum and incubated overnight. The cells were irradiated with ultraviolet B (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/cm<sup>2</sup>, 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% CO<sub>2</sub>.

Next, we observed the effect of anTiO<sub>2</sub> and rnTiO<sub>2</sub> on cell viability under UVB. A549 cells were seeded into 96 well plate at 1 $\times$ 10<sup>3</sup>/well in 100  $\mu$ L culture media and incubated overnight. Then, 100  $\mu$ L of anTiO<sub>2</sub> or rnTiO<sub>2</sub> suspensions in DMEM culture medium containing 10% FBS was added into the wells to final concentration of 0, 2, 5 and 10  $\mu$ g/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 and 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 $\pm$ standard

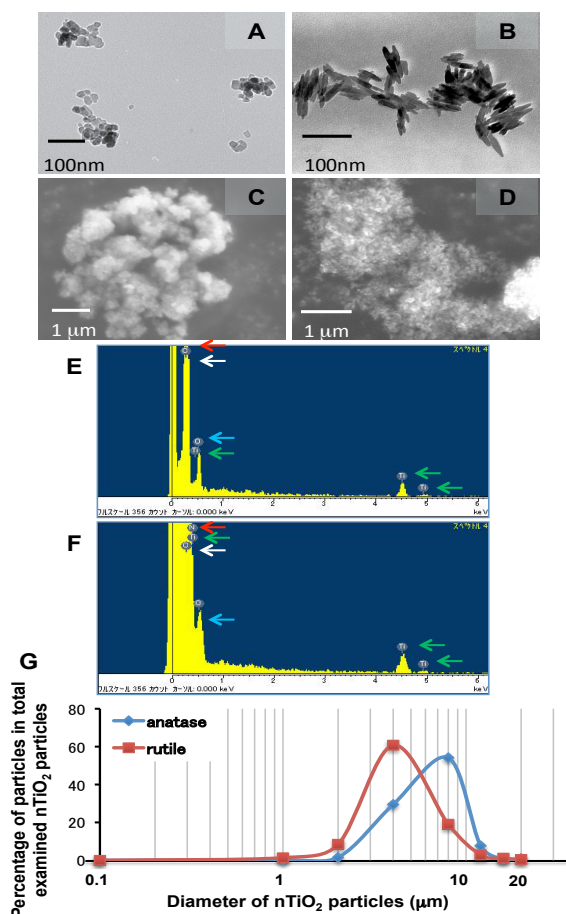


deviations. A value of  $p < 0.05$  was considered to be significant.

## Results

### Characterization of nTiO<sub>2</sub> particles in suspension

TEM images showed that individual anTiO<sub>2</sub> particles were spherical in shape, while individual rnTiO<sub>2</sub> particles had a rod-like shape, and both anTiO<sub>2</sub> and rnTiO<sub>2</sub> formed large aggregates in suspension (Figure. 1A and B). Similarly, SEM observation indicated aggregate formation of both types of nTiO<sub>2</sub> particles (Figure. 1C and D). Peaks of titanium (green arrows) and oxygen (blue arrows), which are present in both types of nTiO<sub>2</sub> particles, and carbon (white arrows) and nitrogen (red arrow), 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 rnTiO<sub>2</sub> or anTiO<sub>2</sub> samples. Analyses of particle size showed that the mean and medium diameters were  $5.491 \pm 2.727$  nm and  $5.127$  nm for anTiO<sub>2</sub>, and  $3.799 \pm 2.231$  nm and  $3.491$  nm for rnTiO<sub>2</sub> (Figure. 1G), confirming aggregate formation of both types of nTiO<sub>2</sub> particles in suspension.

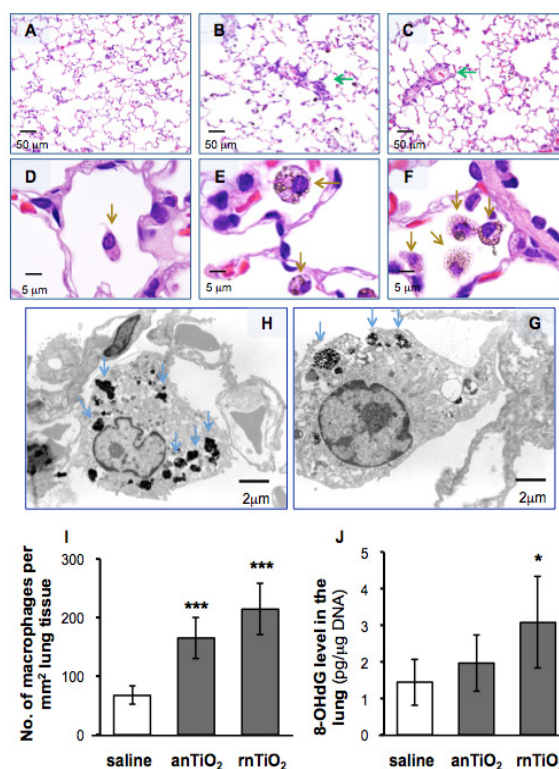


**Figure 1. Characterization of nTiO<sub>2</sub> Particles in Suspension.** A and B: TEM images of anTiO<sub>2</sub> and rnTiO<sub>2</sub> particles in suspension. C and D: SEM images of anTiO<sub>2</sub> and rnTiO<sub>2</sub> particles. E and F: Elemental scanning showed peaks of titanium (green arrows), oxygen (blue arrows), carbon (white arrows) and nitrogen (red arrows) in anTiO<sub>2</sub> and rnTiO<sub>2</sub> particles. G: Size distribution of anTiO<sub>2</sub> and rnTiO<sub>2</sub> 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 anTiO<sub>2</sub> and rnTiO<sub>2</sub> (Figure. 2A, B and C). Alveolar macrophage infiltration was found throughout the lung tissue, and most of the alveolar macrophages were seen with phagocytosed anTiO<sub>2</sub> particles or rnTiO<sub>2</sub> particles (Figure. 2D, E and F). TEM observation demonstrated that both anTiO<sub>2</sub> and rnTiO<sub>2</sub> were deposited in various sizes in the cytoplasm of the alveolar macrophages (Figure. 2G and H). Neither anTiO<sub>2</sub> or rnTiO<sub>2</sub> particles were found in other types of cells in the lung tissue. The number of macrophages per mm<sup>2</sup> lung tissue section was  $67.1 \pm 15.8$  (saline),  $165.0 \pm 34.9$  (anTiO<sub>2</sub>) and  $214.2 \pm 44.1$  (rnTiO<sub>2</sub>). The numbers of macrophages in the anTiO<sub>2</sub> and rnTiO<sub>2</sub> treated groups was significantly higher than in the control group ( $p < 0.001$ ), and the anTiO<sub>2</sub> treated group had lower macrophage infiltration than the rnTiO<sub>2</sub> 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 anTiO<sub>2</sub> and rnTiO<sub>2</sub> was  $1.96 \pm 0.77$  and  $3.07 \pm 1.25$  (pg per mg DNA), respectively, and was higher than that of the control ( $1.44 \pm 0.63$ ). The increase in 8-OHdG in the lungs of rnTiO<sub>2</sub>, but not anTiO<sub>2</sub>, treated rats was significantly higher than the control



**Figure 2. Histological Observation and 8-OHdG Level in the Lung Tissue.** A, B and C: Histological images of lung tissue treated with saline, anTiO<sub>2</sub> and rnTiO<sub>2</sub>, respectively. Green arrows indicate small inflammatory lesions. D (saline), E (anTiO<sub>2</sub>) and F (rnTiO<sub>2</sub>): Higher magnification images of alveolar macrophages (brown arrows). nTiO<sub>2</sub> particles are clearly observed. G and H: TEM images of alveolar macrophages with anTiO<sub>2</sub> and rnTiO<sub>2</sub> particles in their cytoplasm (blue arrows). I and J: The numbers of alveolar macrophages and 8-OHdG levels in the lung tissue. \*, \*\*\* represent  $p < 0.05$  and  $0.001$ , respectively, versus saline

( $p < 0.05$ ) (Figure. 2J).

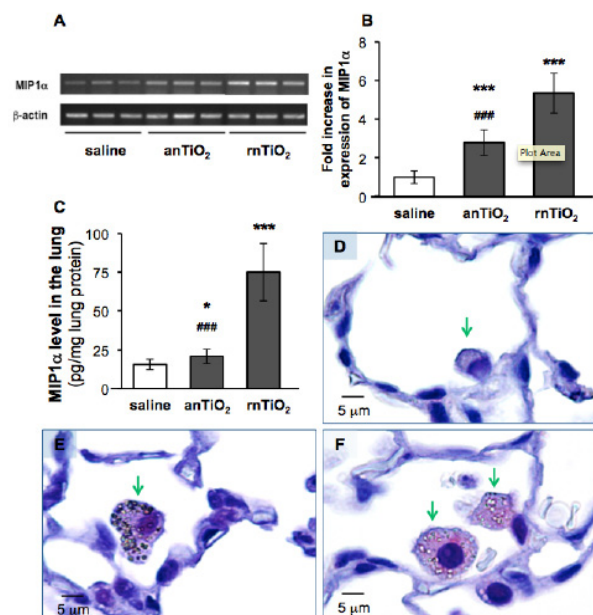
#### MIP1 $\alpha$ expression in the lung tissue

RT-PCR suggested an increase in MIP1 $\alpha$  mRNA expression in lung tissue treated with anTiO<sub>2</sub> or rnTiO<sub>2</sub> (Figure. 3A). Real-time PCR analysis indicated that compared with the control group, the increase was 2.79-fold for anTiO<sub>2</sub> and 5.35-fold for rnTiO<sub>2</sub>. MIP1 $\alpha$  mRNA expression was also significantly lower in the anTiO<sub>2</sub> treated group compared to the rnTiO<sub>2</sub> treated group (Figure. 3B). The levels of MIP1 $\alpha$  protein in the lung tissue were  $32.8 \pm 0.31$  and  $52.7 \pm 0.58$  pg/mg lung protein in the anTiO<sub>2</sub> and rnTiO<sub>2</sub> treated groups, both significantly higher than that of the control group ( $20.8 \pm 0.24$ ) (Figure. 3C). Similarly to MIP1 $\alpha$  mRNA expression, MIP1 $\alpha$  protein expression was significantly lower in the anTiO<sub>2</sub> treated group compared to the rnTiO<sub>2</sub> treated group.

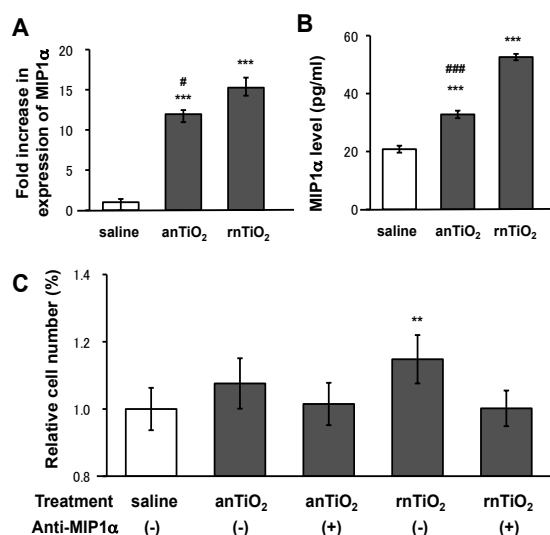
To find out what cells in the lung accounted for the increased MIP1 $\alpha$  protein expression, we examined tissue samples using MIP1 $\alpha$  immunohistochemistry. As shown in Figure. 3D, E and F, MIP1 $\alpha$  protein was produced by anTiO<sub>2</sub> or rnTiO<sub>2</sub> burdened alveolar macrophages.

#### Exposure of PAMs to anTiO<sub>2</sub> and rnTiO<sub>2</sub> and cell proliferation assays *in vitro*

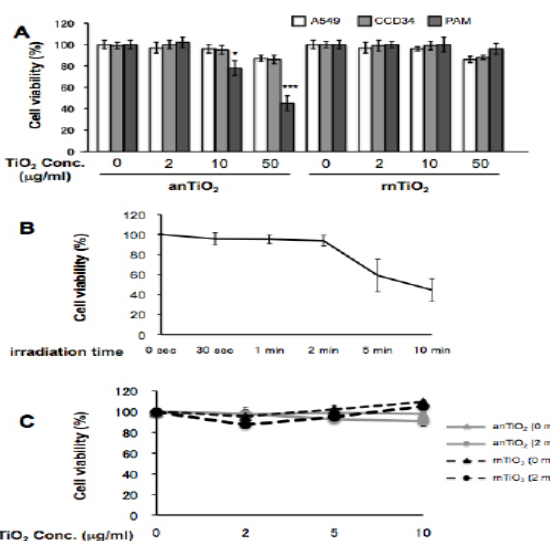
As in the lung tissue, *in vitro* exposure of PAM to rnTiO<sub>2</sub> induced expression of MIP1 $\alpha$  mRNA (Figure. 4A) and protein (Figure. 4B). Treatment with anTiO<sub>2</sub> and rnTiO<sub>2</sub> caused 11.96-fold and 15.26-fold increases in the expression of MIP1 $\alpha$  mRNA, respectively, in cultured PAM. The level of MIP1 $\alpha$  protein in the cell culture medium was  $32.8 \pm 1.1$  pg/mL for anTiO<sub>2</sub> and  $52.7 \pm 1.3$  pg/mL for rnTiO<sub>2</sub>, significantly higher than that of the control



**Figure 3. Expression of MIP1 $\alpha$  in the Lung Tissue.** A, B and C: Analysis of expression of MIP1 $\alpha$  mRNA by RT-PCR (A) and real-time PCR (B) and protein by ELISA (C). D, E, and F: Immunohistochemistry shows MIP1 $\alpha$  expressed in alveolar macrophages of lung tissue treated with saline (D), anTiO<sub>2</sub> (E) and rnTiO<sub>2</sub> (F). \*, \*\*\* represent  $p < 0.05$  and  $0.001$ , respectively, versus saline; ### represent  $p < 0.001$ , versus rnTiO<sub>2</sub>.



**Figure 4. The Effect of anTiO<sub>2</sub> and rnTiO<sub>2</sub> on PAM Cells.** The expression of MIP1 $\alpha$  mRNA in cultured PAM (A) and protein in the culture media (B) indicate that treatment with anTiO<sub>2</sub> or rnTiO<sub>2</sub> increased MIP1 $\alpha$  expression in the PAM. Conditioned cell culture media of PAM treated with rnTiO<sub>2</sub>, but not anTiO<sub>2</sub>, had a significant effect on proliferation of A549 cells, and this promotion was attenuated by addition of 20  $\mu$ g/ml MIP1 $\alpha$  neutralizing antibody (C). \*\*, \*\*\* represent  $p < 0.01$  and  $0.001$ , versus saline; #, ### represent  $p < 0.05$  and  $0.001$ , versus rnTiO<sub>2</sub>.



**Figure 5. In vitro Assays.** A: The effect of anTiO<sub>2</sub> and rnTiO<sub>2</sub> on the viability of A549, CCD34 and PAM cells. B: The effect of UVB irradiation on the viability of A549 cells. C: The effect of anTiO<sub>2</sub> and rnTiO<sub>2</sub> on the viability of A549 under UVB irradiation. \*, \*\*\* represent  $p < 0.05$  and  $0.001$ , versus the vehicle.

( $20.8 \pm 1.2$  pg/mL). Both mRNA and protein expression of MIP1 $\alpha$  was significantly lower in the anTiO<sub>2</sub> treated PAM compared to the rnTiO<sub>2</sub> treated cells.

The supernatants of the culture media of PAM treated with anTiO<sub>2</sub> showed only a tendency to increase A549 cell proliferation, while those collected from PAM treated with rnTiO<sub>2</sub> 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 anTiO<sub>2</sub> or

rnTiO<sub>2</sub> was attenuated by anti-MIP1 $\alpha$  neutralizing antibodies, indicating MIP1 $\alpha$  is probably a mediator of the increase in A549 cell proliferation.

#### *In vitro* cytotoxicity assays

*In vitro* cytotoxicity assays indicated that both anTiO<sub>2</sub> and rnTiO<sub>2</sub> had little effect on the cell viability of A549 and CCD34 cells at a concentration of up to 50 mg/ml. anTiO<sub>2</sub> had a cytotoxic effect on the cell viability of PAM at doses of 10 and 50 mg/ml, while rnTiO<sub>2</sub> did not impair the cell viability of PAM at any of the examined concentrations (Figure. 5A).

To investigate whether UVB irradiation affected the cytotoxic effects of anTiO<sub>2</sub> and rnTiO<sub>2</sub> on cell viability, we first determined the exposure times that ultraviolet B irradiation itself 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<sub>2</sub> or rnTiO<sub>2</sub> at doses of 2, 5 or 10  $\mu$ g/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<sub>2</sub> and rnTiO<sub>2</sub>. The results indicated that both anTiO<sub>2</sub> and rnTiO<sub>2</sub> particles were phagocytosed by alveolar macrophages and did not cause strong lung inflammation. Treatment with anTiO<sub>2</sub> and rnTiO<sub>2</sub> increased alveolar macrophage infiltration, MIP1 $\alpha$  expression and 8-OHdG production: anTiO<sub>2</sub> had less effect than rnTiO<sub>2</sub>.

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<sub>2</sub> burdened 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 asbestos (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<sub>2</sub> and rnTiO<sub>2</sub> particles. Both types of nTiO<sub>2</sub> 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<sub>2</sub> and rnTiO<sub>2</sub> are likely due to their different crystal structures and shapes. The lower toxicity of anTiO<sub>2</sub> compared to rnTiO<sub>2</sub> in the absence of UVB irradiation in our study

is consistent with a previous *in vitro* study with bulk rutile and anatase TiO<sub>2</sub> (Gurr et al., 2005). In contrast to a previous study (Sayes et al., 2006), in the present study anTiO<sub>2</sub> and rnTiO<sub>2</sub> did not exhibit different toxicities on the cell viability of A549 cells under ultraviolet irradiation.

It should be noted that both types of anTiO<sub>2</sub> and rnTiO<sub>2</sub> particles formed aggregates in suspension, and aggregation may alter their bio-reactivity. Whether anTiO<sub>2</sub> and rnTiO<sub>2</sub> particles have different long-term effects remains to be clarified.

In conclusion, *in vivo* exposure of the rat lung to anTiO<sub>2</sub> or rnTiO<sub>2</sub> particles increased alveolar macrophage infiltration, MIP1 $\alpha$  expression and 8-OHdG production, with anTiO<sub>2</sub> eliciting lower levels of biological responses than rnTiO<sub>2</sub>. Similarly, exposure of primary alveolar macrophages to rnTiO<sub>2</sub> *in vitro* resulted in the cells producing more MIP1 $\alpha$  mRNA and protein than cells exposed to anTiO<sub>2</sub>. Cytotoxicity assays *in vitro* indicated that both anTiO<sub>2</sub> and rnTiO<sub>2</sub> had very low cellular toxicity even under UVB irradiation.

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## References

- Albanese A, Tang PS, Chan WC (2012). The effect of nanoparticle size, shape, and surface chemistry on biological systems. *Annu Rev Biomed Eng*, **14**, 1-16.
- Bhatt NY, Kelley TW, Khramtsov VV, et al (2002). Macrophage-colony-stimulating factor-induced activation of extracellular-regulated kinase involves phosphatidylinositol 3-kinase and reactive oxygen species in human monocytes. *J Immunol*, **169**, 6427-34.
- Choe N, Tanaka S, Xia W, et al (1997). Pleural macrophage recruitment and activation in asbestos-induced pleural injury. *Environ Health Perspect*, **105**, 1257-60.
- Geiser M, Casaulta M, Kupferschmid B, et al (2008). The role of macrophages in the clearance of inhaled ultrafine titanium dioxide particles. *Am J Respir Cell Mol Biol*, **38**, 371-6.
- Gurr JR, Wang AS, Chen CH, et al (2005). Ultrafine titanium dioxide particles in the absence of photoactivation can induce oxidative damage to human bronchial epithelial cells. *Toxicology*, **213**, 66-73.
- Heppleston AG (1984). Pulmonary toxicology of silica, coal and asbestos. *Environ Health Perspect*, **55**, 111-27.
- IARC (2010). Carbon black, titanium dioxide, and talc. *IARC Monogr Eval Carcinog Risks Hum*, **93**, 1-413.
- Kakinoki K, Yamane K, Teraoka R, et al (2004). Effect of relative humidity on the photocatalytic activity of titanium dioxide and photostability of famotidine. *J Pharm Sci*, **93**, 582-9.
- Maynard AD, Aitken RJ, Butz T, et al (2006). Safe handling of nanotechnology. *Nature*, **444**, 267-9.
- Mohr U, Ernst H, Roller M, et al (2006). Pulmonary tumor types induced in Wistar rats of the so-called "19-dust study". *Exp Toxicol Pathol*, **58**, 13-20.
- Nel A, Xia T, Madler L, et al (2006). Toxic potential of materials at the nanolevel. *Science*, **311**, 622-7.
- Renwick LC, Donaldson K, Clouter A (2001). Impairment of alveolar macrophage phagocytosis by ultrafine particles.

*Toxicol Appl Pharmacol*, **172**, 119-27.

Rom WN, Travis WD, Brody AR (1991). Cellular and molecular basis of the asbestos-related diseases. *Am Rev Respir Dis*, **143**, 408-22.

Sayes CM, Wahi R, Kurian PA, et al (2006). Correlating nanoscale titania structure with toxicity: a cytotoxicity and inflammatory response study with human dermal fibroblasts and human lung epithelial cells. *Toxicol Sci*, **92**, 174-85.

Schulte P, Geraci C, Zumwalde R, et al (2008). Occupational risk management of engineered nanoparticles. *J Occup Environ Hyg*, **5**, 239-49.

Tsuda H, Xu J, Sakai Y, Futakuchi M, Fukamachi K (2009). Toxicology of engineered nanomaterials - A review of carcinogenic potential. *Asian Pac J Cancer Prev*, **10**, 975-980.

Wang H, Li J, Quan X, et al (2007). Formation of hydrogen peroxide and degradation of phenol in synergistic system of pulsed corona discharge combined with TiO<sub>2</sub> photocatalysis. *J Hazard Mater*, **141**, 336-43.

Wu J, Liu W, Xue C, et al (2009). Toxicity and penetration of TiO<sub>2</sub> nanoparticles in hairless mice and porcine skin after subchronic dermal exposure. *Toxicol Lett*, **191**, 1-8.

Xu J, Futakuchi M, Iigo M, et al (2010). Involvement of macrophage inflammatory protein 1alpha (MIP1alpha) in promotion of rat lung and mammary carcinogenic activity of nanoscale titanium dioxide particles administered by intra-pulmonary spraying. *Carcinogenesis*, **31**, 927-35.