

# Titanium Dioxide Exposure Induces Acute Eosinophilic Lung Inflammation in Rabbits

Gil Soon CHOI<sup>1†</sup>, Chulho OAK<sup>1†</sup>, Bong-Kwon CHUN<sup>2</sup>, Donald WILSON<sup>3</sup>, Tae Won JANG<sup>1</sup>, Hee-Kyoo KIM<sup>1</sup>, Mannhong JUNG<sup>1</sup>, Engin TUTKUN<sup>4</sup> and Eun-Kee PARK<sup>5\*</sup>

<sup>1</sup>Department of Internal Medicine, Kosin University College of Medicine, Republic of Korea

<sup>2</sup>Department of Pathology, Kosin University College of Medicine, Republic of Korea

<sup>3</sup>Department of Occupational Toxicology, Institute of Industrial Ecological Sciences, University of Occupational and Environmental Health, Japan

<sup>4</sup>Ankara Occupational Diseases Hospital, Ministry of Health, Turkey

<sup>5</sup>Department of Medical Humanities and Social Medicine, Kosin University College of Medicine, Republic of Korea

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**Abstract:** Titanium dioxide (TiO<sub>2</sub>) is increasingly widely used in industrial, commercial and home products. TiO<sub>2</sub> aggravates respiratory symptoms by induction of pulmonary inflammation although the mechanisms have not been well investigated. We aimed to investigate lung inflammation in rabbits after intratracheal instillation of P25 TiO<sub>2</sub>. One ml of 10, 50 and 250 μg of P25 TiO<sub>2</sub> was instilled into one of the lungs of rabbits, chest computed-tomography was performed, and bronchoalveolar lavage (BAL) fluid was collected before, at 1 and 24 h after P25 TiO<sub>2</sub> exposure. Changes in inflammatory cells in the BAL fluids were measured. Lung pathological assay was also carried out at 24 h after P25 TiO<sub>2</sub> exposure. Ground glass opacities were noted in both lungs 1 h after P25 TiO<sub>2</sub> and saline (control) instillation. Although the control lung showed complete resolution at 24 h, the lung exposed to P25 TiO<sub>2</sub> showed persistent ground glass opacities at 24 h. The eosinophil counts in BAL fluid were significantly increased after P25 TiO<sub>2</sub> exposure. P25 TiO<sub>2</sub> induced a dose dependent increase of eosinophils in BAL fluid but no significant differences in neutrophil and lymphocyte cell counts were detected. The present findings suggest that P25 TiO<sub>2</sub> induces lung inflammation in rabbits which is associated with eosinophilic inflammation.

**Key words:** Allergy, Eosinophil, Inflammation, P25 TiO<sub>2</sub> nanoparticle

## Introduction

Over the past decades, advances in nanotechnology have led to their rapid applications in the fields of medi-

cine, pharmaceuticals, biotechnology, energy production and environmental sciences<sup>1</sup>). The increasing use of nanomaterials in various products at workplaces and in the home setting, including many consumer items such as clothing and plastic wares<sup>2</sup>) therefore pose an obvious risk to humans.

Titanium dioxide (TiO<sub>2</sub>) nanoparticles (NPs) are one of the most abundantly utilized nanomaterials because of their chemical stability, low toxicity and relatively cheap price<sup>3</sup>). It is used as a white pigment in paint, food color-

\*To whom correspondence should be addressed.

E-mail: ekpark@kosin.ac.kr

†These authors contributed equally to this work.

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ing, as an ultraviolet blocker in cosmetics, disinfectant in environment and wastewater, and as a photosensitizer for photodynamic therapy. Oral ingestion and entry through the dermal route are mainly mediated by therapeutic or cosmetic application<sup>4, 5</sup>). The respiratory route is most important because the intake of NPs into the body is from atmospheric air via the upper respiratory tract<sup>6</sup>). TiO<sub>2</sub> NPs are increasingly being manufactured, leading to increased occupational exposure and release into the atmospheric environment. Nano-sized particles are generally more toxic to the lung than their larger-sized counterparts<sup>7</sup>) which are why there has recently been increasing concern about the impact of TiO<sub>2</sub> NPs in the lung.

Several epidemiological studies have reported that TiO<sub>2</sub> NPs exposure at the work place aggravate respiratory symptoms<sup>8, 9</sup>). Besides, earlier studies indicated that inhalation TiO<sub>2</sub> NPs can induce pulmonary response such as inflammation, fibrosis, emphysema-like lung injury, and lung cancer<sup>6, 10, 11</sup>). However, the pulmonary effects of TiO<sub>2</sub> NPs are not fully understood. Previous animal studies have shown that exposure to TiO<sub>2</sub> NPs causes oxidative stress, induce lung inflammation in the airways and alveolar spaces<sup>12, 13</sup>). Moreover, it has been reported that TiO<sub>2</sub> NPs are able to induce neutrophilic pulmonary inflammation<sup>14, 15</sup>). Recent studies found that TiO<sub>2</sub> NPs cause lung inflammation by activation of T-helper 2 cells and that the exposure of high concentration of TiO<sub>2</sub> NPs in the lung induced an innate immune activation<sup>16, 17</sup>). Although *in vitro* and *in vivo* studies suggest that TiO<sub>2</sub> NPs cause various forms of pulmonary inflammation<sup>11, 18</sup>), to our knowledge, relatively few studies have investigated the pulmonary effect in a rabbit model, and its pathogenic mechanism. The present study investigated the effect of TiO<sub>2</sub> NPs on rabbit lungs, evaluated by lung image analysis, bronchoalveolar lavage (BAL) fluids examination, and histopathologic analysis.

## Materials and Methods

### *Titanium dioxide (TiO<sub>2</sub>)*

P25 TiO<sub>2</sub> nanoparticles (Brunauer-Emmett-Teller (BET) specific surface area of 53.8 m<sup>2</sup>/g) were obtained from Degussa. The particles were suspended as follows: 1.5 g of P25 TiO<sub>2</sub> powder was suspended in 100 ml of distilled water in a pyrex glass beaker, and sonicated for 15 min by a Branson Digital Cell Disruptor Sonifier 250 (Branson, USA) with a double-stepped microtip (3 mm diameter); this process was repeated 3 times. To stabilize temperature during sonication, the beaker was placed in a bucket of ice

throughout the process. The particle suspension was then centrifuged at 3,000 × g for 20 min at 20 °C. The supernatant was carefully collected and filtered through a 1 μm filter to remove the large agglomerates (>1 μm). A given volume of particle suspension was evaporated, after which the weight of the remaining evaporate was measured and P25 TiO<sub>2</sub> concentration determined (w/v; in mg/ml). The hydrodynamic size distribution by number of P25 TiO<sub>2</sub> particles suspended in water was analyzed using a Dynamic Light Scattering Zetasizer Nano (Malvern Instruments, UK), and the average particle size was calculated to be 61.9 ± 5.1 nm.

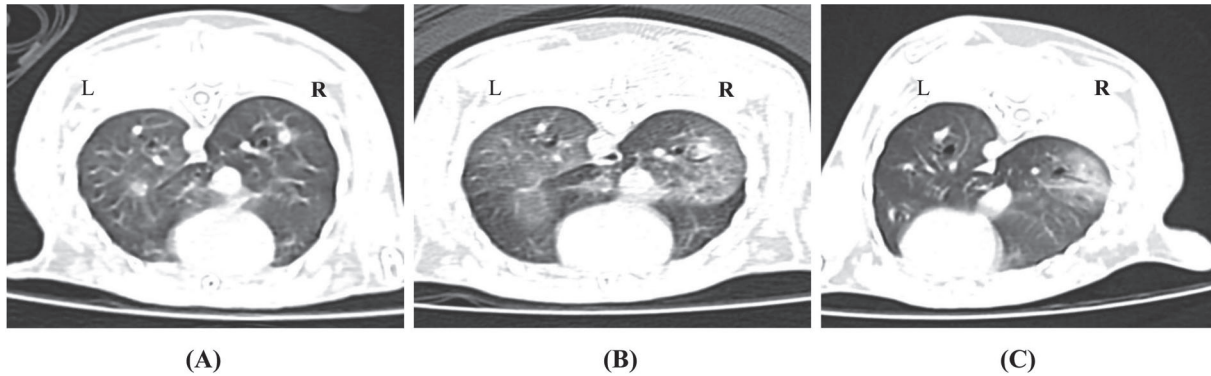
### *Animals and study protocol*

Nine Male New Zealand white rabbits (Taesung Laboratory Animal Science, Busan, Republic of Korea) weighing 3.0 to 3.5 kg were used for this experiment. The rabbits were housed at 20–25 °C and 50–70% relative humidity with a 12 h light/dark cycle. They had free access to water and diet and were acclimatized for at least 1 wk before starting the experiments. Radiologic image analysis (computer-tomography (CT)) was performed to ascertain lung inflammation at 1 and 24 h after P25 TiO<sub>2</sub> exposure, and also to investigate the pathogenic mechanism, bronchoalveolar lavage (BAL) was performed at before P25 TiO<sub>2</sub> exposure, 1 and 24 h after P25 TiO<sub>2</sub> exposure. For further histological analysis, all rabbits were euthanized using CO<sub>2</sub> gas at 24 h after P25 TiO<sub>2</sub> exposure.

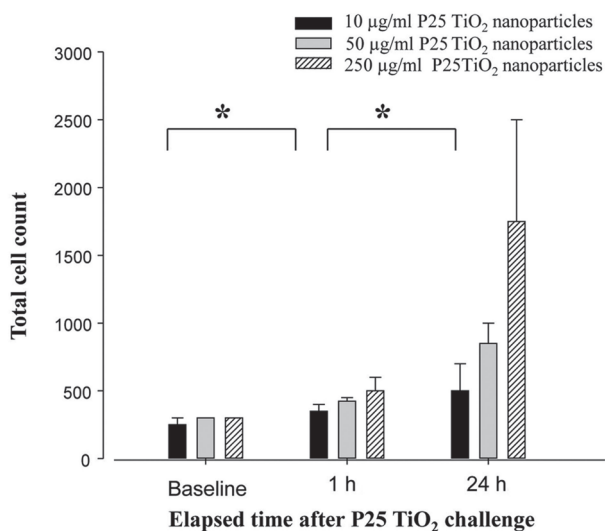
Animal experimental procedure was approved by the Animal Research Ethical Committee in Kosin Gospel Hospital, Busan, Republic of Korea.

### *P25 TiO<sub>2</sub> nanoparticles exposure*

Rabbits were anesthetized by intramuscular injection of ketamine 5 mg/kg (Huons Co., Korea) and xylazine 0.8 mg/kg (Bayer, Republic of Korea). Oxygen saturation was monitored by pulse oxymeter in the ear. Transbronchial P25 TiO<sub>2</sub> instillation was performed using an ultrathin bronchoscope (BF-XP260F, Olympus; Tokyo, Japan). The ultrathin bronchoscope was inserted into the target bronchus as deep as possible under direct vision. The instillation catheter was inserted beyond the visible bronchus through working channel. One ml of 10 μg P25 TiO<sub>2</sub> was once instilled into the right lung through the catheter and 1 ml of normal saline (as control) was instilled into the left lung (N=3). One ml of 50 and 250 μg P25 TiO<sub>2</sub> were instilled in the same way (N=3 in each group).



**Fig. 1.** Lung inflammatory change by chest CT image analysis. Both lungs were instilled with 10  $\mu\text{g}/\text{ml}$  of P25 TiO<sub>2</sub> in the right lung and normal saline in the left lung. (A) normal lung at baseline; (B) lung inflammation with ground glass opacity in both lung fields 1 h after instillation of P25 TiO<sub>2</sub> NP; (C) after 24 h of P25 TiO<sub>2</sub> NP exposure, ground glass opacities in the left lung (control) disappeared but they remained in the right lower lung field.



**Fig. 2.** Total cell count change in bronchoalveolar lavage fluid (BALF). Total cell count in BALF was significantly increased at 1 and 24 h after P25 TiO<sub>2</sub> NP challenge. The bars represent mean  $\pm$  SE; \* $p < 0.05$  significantly different from baseline; Mann-Whitney U test.

### Bronchoalveolar Lavage and Cell Counting

Bronchoalveolar lavage (BAL) was performed before P25 TiO<sub>2</sub> exposure, at 1 and 24 h after P25 TiO<sub>2</sub> exposure through an ultrathin bronchoscope, which was wedged into the 1st branch bronchus of the right lung. Sterile saline solution (2 ml) was instilled through the bronchoscope. The fluid was immediately recovered by gentle suction after each instillation. The measurement of recovered fluids showed an approximately 90% recovery. To maximize cell viability, the harvested BALF was immediately placed

on ice and centrifuged at 1,000  $\times$  g for 10 min. The supernatants were immediately stored at  $-80^{\circ}\text{C}$  for further analysis. The cell pellet was used to prepare slides, which were stained according to the May-Grunwald and Giemsa procedures to morphologically assess the cells in the fluid. The differential cell counts were then counted by hemocytometer.

### Lung Pathologic Examination Assay

The lung was harvested for pathologic examination at 24 h after P25 TiO<sub>2</sub> exposure. Tissue pretreatments and preparation of hematoxylin and eosin (H&E) stained slices were carried out as previously described<sup>16</sup>. They were evaluated by light microscopy.

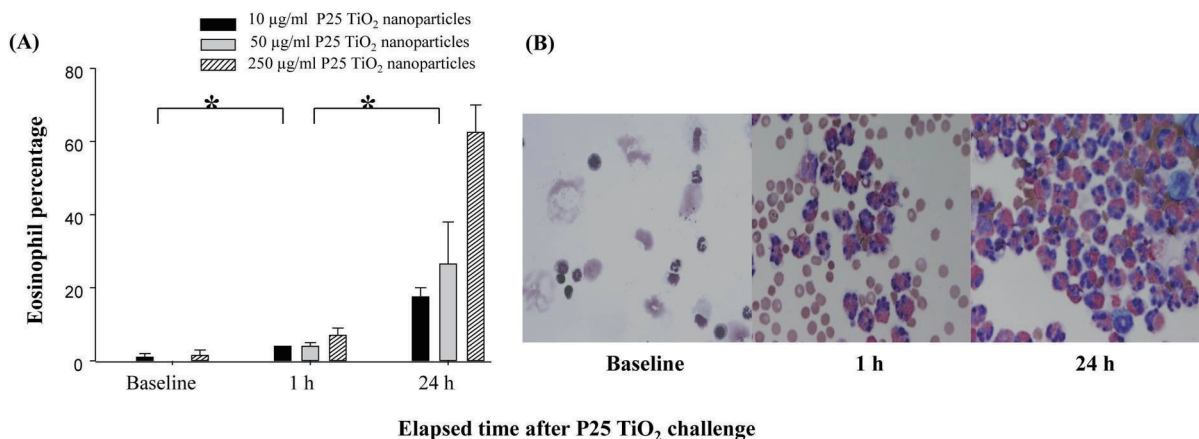
### Statistical Analysis

Results were expressed as mean  $\pm$  standard error (SE). Mann-Whitney U test was used in the case of two independent samples. All analyses were carried out using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). A  $p < 0.05$  was considered statistically significant.

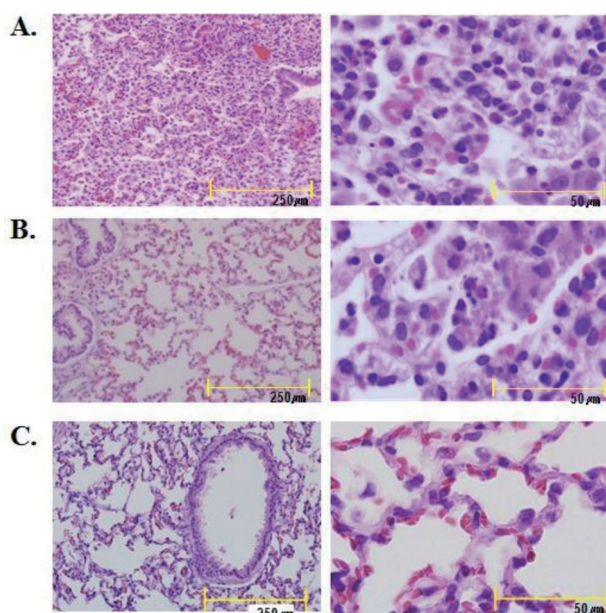
### Results

#### Lung image analysis

To ascertain lung inflammation by P25 TiO<sub>2</sub> exposure in the rabbit, chest CT was performed after P25 TiO<sub>2</sub> instillation. Both lungs were clear before the experiment (Fig. 1A), but at 1 h ground glass opacities (GGO) were noted in each lung instilled with P25 TiO<sub>2</sub> (10  $\mu\text{g}/\text{ml}$ ) and normal saline in Fig. 1B. At 24 h after exposure, persistent lung inflam-



**Fig. 3.** Eosinophil percentage change in bronchoalveolar lavage fluid (BALF). The increase in eosinophil percentage was noted at 1 and 24 h after P25 TiO<sub>2</sub> NP challenge with dose-dependent responses. (A) the eosinophil count in BALF increased at 1 and 24 h after P25 TiO<sub>2</sub> NP challenges; (B) the photos of eosinophil count in BALF at the dose of 50 µg/ml compared to baseline. The bars represent mean  $\pm$  SE; \* $p$ <0.05 significantly different from baseline; Mann-Whitney U test.



**Fig. 4.** Pathological examination of the lungs following P25 TiO<sub>2</sub> NP challenge. After 24 h of P25 TiO<sub>2</sub> instillation, (A) severe eosinophilic inflammation was noted in the lung that was instilled with 50 µg/ml; (B) mild eosinophilic inflammation was noted in the lung exposed to the dose of 10 µg/ml; (C) normal baseline. Left pane at magnification of 100 $\times$  and right pane at a magnification of 1,000 $\times$ .

mation with GGO was noted in the right lung instilled with P25 TiO<sub>2</sub>, while lung inflammation disappeared in the control lung, instilled with normal saline in Fig. 1C. Similar results were obtained from the experiments using 50 and 250 µg/ml of P25 TiO<sub>2</sub> (data not shown).

#### *Inflammatory cell changes in BAL fluids*

After one single instillation of 10 µg/ml P25 TiO<sub>2</sub> in the rabbit lung, the total cell count in BAL fluids was increased at 1 h ( $p=0.24$ ) and 24 h ( $p=0.42$ ) (Fig. 2). At 50 and 250 µg/ml of P25 TiO<sub>2</sub> the total cell count in BAL fluids was high at 1 h and 24 h compared to the baseline.

There was an especially marked increase in eosinophil percentage at 1 h ( $4.0 \pm 0.4\%$ ) and 24 h ( $17.5 \pm 5.5\%$ ) after 10 µg/ml of P25 TiO<sub>2</sub> ( $p<0.05$ , Fig. 3A). When exposed to higher concentrations of P25 TiO<sub>2</sub> (50 and 250 µg/ml), a dose-dependent increase in eosinophil percentage was detected at 1 h and 24 h (Fig. 3A). The eosinophil count on BAL fluids increased at 1 and 24 h after 50 µg/ml P25 TiO<sub>2</sub> exposure when compared to baseline (Fig. 3B).

#### *Pathogenic changes after P25 TiO<sub>2</sub> exposure*

Analysis of BAL fluids confirmed that P25 TiO<sub>2</sub> induced inflammation in lung tissues. After 24 h of P25 TiO<sub>2</sub> instillation (50 µg/ml), severe eosinophilic inflammation was noted in the alveolar, peribronchial and perivascular regions, with moderate hemorrhage (Fig. 4A). Mild eosinophilic inflammation was noted in lung tissues exposed to 10 µg/ml P25 TiO<sub>2</sub> (Fig. 4B). On the other hand, in the control lung (not exposed to P25 TiO<sub>2</sub>), there was no eosinophilic inflammation (Fig. 4C).

## **Discussion**

Exposure to TiO<sub>2</sub> NPs during production or use is most likely to occur via different routes such as skin penetration, ingestion, or inhalation, but, it is believed that the lung is

the most important target organ<sup>19</sup>). Although there have been a few studies on the pulmonary effects of TiO<sub>2</sub> exposure, to the best of our knowledge, the current study is the first investigation in a rabbit model evaluating acute lung changes after P25 TiO<sub>2</sub> exposure. Generally, mice or rat models are widely used in research due to ease of handling and their being relatively cost-effective compared to other models. Despite these advantages, mice and humans have considerable differences in lung structure and function which limits their suitability to lung disease studies<sup>20, 21</sup>). The rabbit is known to have a very similarity to human in terms of airway anatomy and responses to inflammatory mediators, although it has not been widely used probably due to limitation of cost and reagent availability. Differences in the pulmonary effects of TiO<sub>2</sub> NPs in mouse and rat species have previously been reported<sup>22, 26</sup>). Considering rabbit is phylogenetically closer to human than rodents, our study may further provide important knowledge to understanding the acute lung impacts of TiO<sub>2</sub> exposure in human.

TiO<sub>2</sub> NPs was previously reported to induce pulmonary response<sup>11</sup>), which has been mainly evaluated subacute and chronic change by histopathological analysis. There were limitations in that the sequential acute changes following TiO<sub>2</sub> exposure were not investigated. This is why image analysis was utilized in our study to evaluate acute lung inflammation following TiO<sub>2</sub> NPs intratracheal instillation. We observed ground glass opacities of acute pneumonitis at 1 h after single P25 TiO<sub>2</sub> NPs exposure. Furthermore we observed persistent pneumonitis in the P25 TiO<sub>2</sub> exposed lung, as well as newly developed pneumonitis in the P25 TiO<sub>2</sub> unexposed opposite lung at 24 h. These results indicate that single instillation of P25 TiO<sub>2</sub> can induce severe acute pulmonary inflammation. Moreover, previous studies reported that high dose TiO<sub>2</sub> NPs cause more severe lung inflammation compared with that of low dose of TiO<sub>2</sub>, as well as induce persistent pulmonary inflammation<sup>23–25</sup>). This information may have clinical implications regarding safety in handling of TiO<sub>2</sub> NPs.

To understand the pathogenic mechanism of this acute lung inflammation by P25 TiO<sub>2</sub> exposure, in the present study, BAL fluids and histopathology of lung sections were examined. We found that eosinophils were significantly increased during acute response (1 h) after P25 TiO<sub>2</sub> NPs exposure, which persisted at 24 h in BAL fluids. Furthermore, eosinophil increases showed a dose-dependent pattern. This finding is consistent with those from the study in rat<sup>16</sup>). Although we did not observe any significant changes of other inflammatory cells, some stud-

ies in mouse models have shown increase of neutrophils and macrophages in the lung, as well as epithelial change after challenge with TiO<sub>2</sub><sup>10, 22, 26–28</sup>). However, these inflammatory changes were associated with different properties of TiO<sub>2</sub> NPs, like crystal structure, surface chemistry, and surface area<sup>12, 29–31</sup>). Therefore, it may be difficult to compare results between NP studies. TiO<sub>2</sub> NP exposure in rats induced innate immune activation of eosinophils in the acute and long-lasting lymphocyte responses<sup>12</sup>). Recently, it has been reported that lung challenge with TiO<sub>2</sub> NP in mice cause inflammation by activation of T-helper 2 cells<sup>32</sup>). It has also been shown that lung exposure to TiO<sub>2</sub> NP aggravates an asthmatic response and also promotes allergic sensitization and lung inflammation in a mouse model<sup>17, 33</sup>). In addition, our histopathologic analysis showed severe eosinophil inflammation in the lung after P25 TiO<sub>2</sub> challenge, compared with those of the control lung that was not exposed to P25 TiO<sub>2</sub>. Considering that eosinophils are the main effector cells in an allergic inflammation such as asthma<sup>34, 35</sup>), we speculate that P25 TiO<sub>2</sub> induce allergic lung inflammation by eosinophil activation. Additional investigations are needed to elucidate how eosinophil activation happens following TiO<sub>2</sub> exposure.

In conclusion, this is the first study that investigated lung inflammation after P25 TiO<sub>2</sub> exposure in a rabbit model and found the particles to induce eosinophilic lung inflammation. Further research is necessary to investigate the mechanism and implications of this eosinophil activation induced by TiO<sub>2</sub> NP.

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