

The Acute Proinflammatory and Prothrombotic Effects of Pulmonary Exposure to Rutile TiO₂ Nanorods in Rats

ABDERRAHIM NEMMAR,^{*,1} KHALED MELGHIT,[†] AND BADRELDIN H. ALI[‡]

^{*}Department of Physiology, Faculty of Medicine and Health Sciences, United Arab Emirates University, Al Ain, UAE; [†]Department of Chemistry, College of Science, Sultan Qaboos University, Muscat 123, Al-Khod, Sultanate of Oman; and [‡]Department of Pharmacology, College of Medicine and Health Sciences, Sultan Qaboos University, Muscat 123, Al-Khod, Sultanate of Oman

Nanotechnology is extensively used in industry and is widely explored for possible applications in medicine. However, its potential respiratory and systemic adverse effects remain unknown. Here pure titanium dioxide (TiO₂) nanorods with rutile structure were prepared at room temperature by using a soft chemistry technique. The structure of the TiO₂ rutile nanorods was confirmed by powder X-ray diffraction, and the size was revealed by transmission electron microscopy. Thereafter, we investigated, in Wistar rats, the acute (24-hr) effects of intratracheal instillation of these rutile TiO₂ nanorods (1 and 5 mg/kg) on lung inflammation (assessed by bronchoalveolar lavage), systemic inflammation, and platelet aggregation in whole blood. Compared with vehicle-exposed rats, rats that underwent intratracheal instillation of TiO₂ nanorods experienced a dose-dependent increase in macrophage numbers at 1 (+50%) and 5 mg/kg (+81%; $P < 0.05$) and an influx of neutrophils at 1 (+294%) and 5 mg/kg (+4117%; $P < 0.01$) in their bronchoalveolar lavage fluid. Both doses of rutile TiO₂ nanorods caused pulmonary and cardiac edema, assessed by analysis of the wet weight-to-dry weight ratios. Similarly, the numbers of monocytes and granulocytes in the blood were increased in a dose-dependent manner after exposure to rutile TiO₂ nanorods. In contrast, the number of platelets was significantly reduced after pulmonary exposure to 5 mg/kg TiO₂ nanorods; this result indicated the occurrence of platelet aggregation *in vivo*. The direct addition of TiO₂ nanorods (0.4–10 µg/ml) to untreated rat blood significantly induced platelet aggregation in a dose-dependent fashion *in vitro*. It is concluded that the intratracheal instillation of rutile TiO₂ nanorods caused upregulation of lung inflammation, pulmonary and cardiac edema, and systemic

inflammation. Rutile TiO₂ nanorods also triggered platelet aggregation *in vivo* and *in vitro*. Exp Biol Med 233:610–619, 2008

Key words: nanotechnology; nanoparticulate; lung; toxicity

Introduction

Nanotechnology is a broad interdisciplinary area of research, grouping physical, chemical, biological, and engineering expertise involved in manufacturing materials at a sub-100-nm scale (1). Whereas benefits of nanotechnology in areas as diverse as diagnosis, imaging, drug delivery, and information and communication technologies are extensively publicized, the discussion of the potential effects of the widespread use of nanotechnology in consumer and industrial products is just beginning to emerge (2, 3).

Accidental or involuntary contact during production or use is most likely to occur via different routes such as skin penetration, ingestion, or inhalation. It is believed that, of all major exposure sites of the body, the lung is the most important site for involuntary exposure to man-made nanoparticulates (4). In addition to affecting the lungs, nanoparticulates that have been inhaled can rapidly translocate to the systemic circulation and reach other organs (5–9). Moreover, Elder *et al.* (10) reported that translocation of inhaled nanosized particles along neurons is a more efficient pathway to the central nervous system than is blood circulation across the blood–brain barrier.

Titanium dioxide (TiO₂) is a poorly soluble particulate that is widely used in the productions of paints, coatings, plastics, foods, and toothpastes. Moreover, nanosized TiO₂ is used in cosmetics, skin care products, and some pharmaceuticals. Therefore, potential widespread exposure may occur during manufacturing and use. The potential pulmonary and cardiovascular toxicity is not well elucidated. Earlier studies indicated that a single intratracheal

¹ To whom correspondence should be addressed at Faculty of Medicine and Health Sciences, Department of Physiology, United Arab Emirates University, PO Box 17666, Al Ain, UAE. E-mail: anemmar@uaeu.ac.ae or anemmar@hotmail.com

Received June 18, 2007.

Accepted December 13, 2007.

DOI: 10.3181/0706-RM-165

1535-3702/08/2335-0610\$15.00

Copyright © 2008 by the Society for Experimental Biology and Medicine

(i.t.) exposure to TiO₂ nanoparticles can cause pulmonary inflammation, fibrosis, DNA damage, and emphysema-like lung injury (11–13). The toxic effects of TiO₂ particles are dose-dependent and size-dependent. Smaller nano-TiO₂ (20 nm) cause a greater pulmonary inflammatory response in rats and mice than do larger TiO₂ particles (250 nm). The toxicity of nano-TiO₂ correlates well with its surface area per unit mass (14, 15).

Along with size and surface, the phase composition of nanoscale TiO₂ seems to play an important role in nanoparticle-induced toxicity. Titania can exist as several different phases (anatase, rutile, brookite, and monoclinic TiO₂). The most common and stable phases are rutile and anatase. Using human dermal fibroblast and lung epithelial cells, Sayes *et al.* (16) recently compared the cytotoxicity of anatase, rutile, and a mixture of anatase and rutile TiO₂ nanoparticles. The most cytotoxic phase is anatase, the next most cytotoxic is the mixture of anatase and rutile, and the least cytotoxic is rutile. Moreover, i.t. instillation of anatase TiO₂ nanorods causes transient pulmonary inflammation (observed at 24 hrs after i.t. exposure but not 1 week to 3 months later; Ref. 17). However, the potential pathophysiological effect of rutile TiO₂ nanorods on pulmonary and cardiovascular endpoints has not been investigated.

In the present study, we synthesized pure rutile TiO₂ nanorods by using a soft chemistry technique and analyzed them by powder X-ray diffraction and transmission electron microscopy. Moreover, we investigated the short-term effect (24 hrs) of exposure to rutile TiO₂ nanorods on lung inflammation and edema in rats and its consequences for peripheral extrapulmonary modification and its effects on the heart, systemic inflammation, and platelet activation.

Material and Methods

Rutile TiO₂ Nanorods. TiO₂ was prepared by a soft chemistry technique similar to the recently published method (18). About 5 ml of TiCl₃ (30% w/v; DBH Chemical Ltd, Poole, UK) was diluted with 45 ml of distilled water and stirred for about 65 hrs at room temperature. Then, 500 ml of distilled water was added, and subsequently about 20 ml of 10% NH₄OH was added. A white gel appeared at a pH of approximately 9. The gel at the bottom of the beaker was easily separated from the solution by decantation. It was washed with distilled water several times until the AgNO₃ test indicated the absence of chloride ion in the residual solution. The wet gel was dried at room temperature. The as-prepared TiO₂ was characterized by powder X-ray diffraction (Philips 1710 diffractometer; Almelo, The Netherlands). The surface area of the sample was determined with a standard BET apparatus (Autosorb-1, Quantachrome, FL).

For electron microscopy, droplets (10 µl) of a suspension of 1 mg of rutile TiO₂ nanorods in 500 µl of saline (0.9% NaCl) containing Tween 80 (0.01%) were placed on matured formvar/carbon film for 30 secs. The

samples were then drained and inverted onto droplets of ultrapure water for 1 hr before being drained, dried, and examined in a JEOL (JEM 1230) electron microscope (Tokyo, Japan).

Animals and I.T. Instillation of Rutile TiO₂ Nanorods. This project was reviewed and approved by our Institutional Review Board, and experiments were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee.

Rutile TiO₂ nanorods were suspended in saline containing Tween 80 (0.01%). To minimize aggregation, particle suspensions were sonicated (Clifton Ultrasonic Bath, Clifton, NJ) for 15 mins and vortexed before their dilution and before i.t. administration. Control animals received saline containing Tween 80 (0.01%).

Eighteen-week-old male Wistar rats (Taconic Farms Inc., Germantown, NY) weighing 359 ± 10 g were used. They were anesthetized by intraperitoneal injection of ketamine (75 mg/kg) and xylazine (10 mg/kg) and placed supine with extended necks on an angled board. A Becton Dickinson 18-gauge cannula (Franklin Lakes, NJ) was inserted via the mouth into the trachea. Suspensions of rutile TiO₂ nanorods (1 or 5 mg/kg) or vehicle only were instilled (150 µl) via a sterile syringe and followed by an air bolus of 100 µl.

Blood Collection, Bronchoalveolar Lavage (BAL) Fluid Analysis, and Pulmonary and Cardiac Edema. Twenty-four hours after i.t. administration of vehicle (control) or rutile TiO₂ nanorods, the rats were anesthetized as described, and blood was drawn from the inferior vena cava and placed in EDTA (4%). A sample was used for platelet and white blood cell (WBC) counts, which were done by using an ABX Micros 60 counter (ABX Diagnostics, Montpellier, France). The remaining blood was centrifuged at 4°C for 15 mins at 900 g, and the plasma samples were stored at –20°C.

The rats were then killed with an overdose of ketamine. BAL was then performed by cannulating the trachea and clamping the left bronchus. The bronchi and right lung were lavaged three times with 5 ml sterile saline. The BAL fluid was pooled in a plastic tube on ice. No difference in the amount of recovered fluid was observed between the different groups. BAL fluid was centrifuged (1,000 g for 10 mins at 4°C). Cell counting was performed in a hemocytometer after resuspension of the pellets and staining with 1% gentian violet. The cell differential counts were performed on cytocentrifuge preparations fixed in methanol and stained with Diff Quick (Dade Behring, Marburg, Germany). The supernatant was stored at –20°C until further analysis.

The presence of pulmonary or cardiac edema was assessed by analysis of the wet weight-to-dry weight ratio. The heart or nonlavaged left lung were removed and placed in a preweighed glass tube for measuring wet left lung or heart weight and dry left lung or heart weight (after 24 hrs at

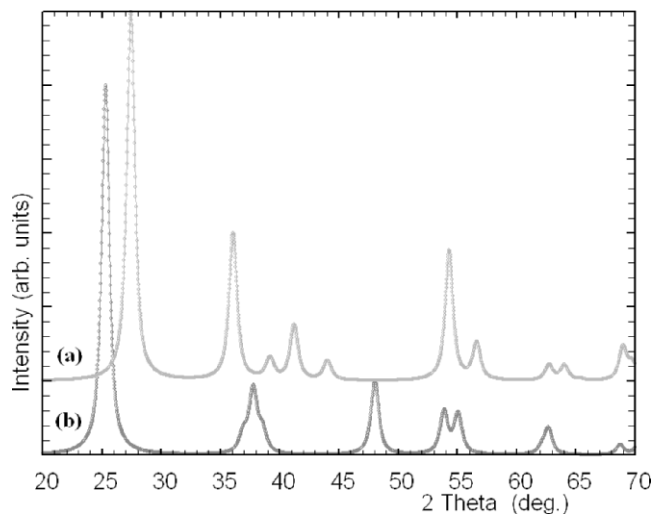


Figure 1. Powder X-ray diffraction of rutile and anatase TiO_2 calculated by using the Mercury computer program (22). (a) Rutile TiO_2 (Joint Committee on Powder Diffraction Standards, file number 21-1276). (b) Anatase TiO_2 (Joint Committee on Powder Diffraction Standards, number 21-1272).

80°C; Ref. 19). The wet weight-to-dry weight ratio was equal to (wet weight – dry weight)/wet weight (20).

Platelet Aggregation in Rat Whole Blood. The platelet aggregation assay in whole blood was the recently described method (21) with slight modifications. After anesthesia, blood from separate animals was withdrawn from the inferior vena cava and placed in citrate (3.2%), and 500- μl aliquots were added to the wells of a 12-well plate, which was then positioned in an MTS 2/4 digital microtiter shaker (IKA WERKE GmbH & CO, Stauffer, Germany) at room temperature. Blood was pretreated with ADP (0.5 μM), vehicle, or rutile TiO_2 nanorods (0.4, 2, or 10 $\mu\text{g}/\text{ml}$). The plate was rotated at 900 rpm. Three minutes later, 25- μl samples were removed and fixed on ice in 225 ml cellFix (Becton Dickinson). After fixation, single platelets were counted in an ABX Micros 60 counter. The degree of aggregation in vehicle and nanorod-treated blood was expressed as the percent of aggregation induced by 0.5 μM ADP.

Statistics. Data were expressed as the means $\pm$ SEM. Comparisons between groups were performed by one-way analysis of variance (ANOVA), which was followed by analysis using the Dunnett test for comparing data from treated animals with those from controls. P values <0.05 were considered significant.

Results

Rutile TiO_2 Nanorods. Figure 1 illustrates a comparison of powder X-ray diffraction patterns calculated by using the Mercury computer program (22) between rutile TiO_2 (Joint Committee on Powder Diffraction Standards, file number 21-1276) and anatase TiO_2 (Joint Committee on Powder Diffraction Standards, number 21-1272). Figure 2A shows a comparison of the powder X-ray diffraction of as-

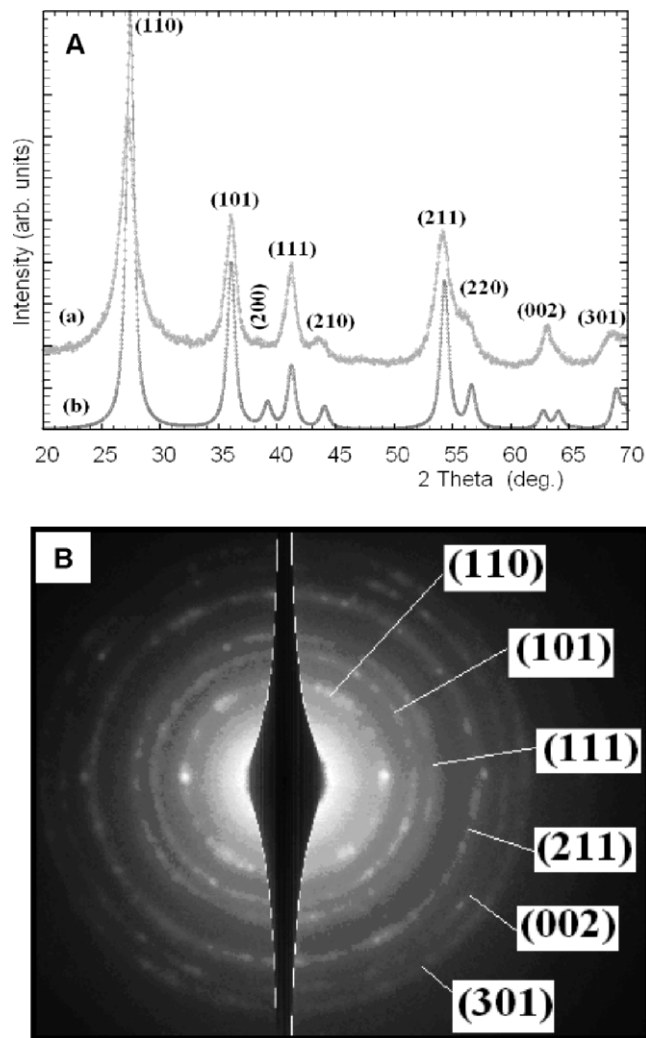


Figure 2. Characterization of as-prepared TiO_2 . (A) Powder X-ray diffraction patterns of rutile TiO_2 . (a) As-prepared TiO_2 nanorods with Miller indices (h k l). Crystal family of planes for each diffraction peak are shown. (b) The pattern of TiO_2 rutile (Joint Committee on Powder Diffraction Standards, file number 21-1276) calculated by using the Mercury computer program (22). (B) A selected area of the electron diffraction pattern with Miller indices (h k l) of as-prepared TiO_2 .

prepared sample with the pattern of rutile TiO_2 (Joint Committee on Powder Diffraction Standards, file number 21-1276) calculated by using Mercury program (22). The two patterns were similar, and all diffraction peaks of as-prepared sample can be indexed by the rutile lattice, where Miller indices (h k l) show a crystal family of planes for each diffraction peak. The prepared sample has broad diffraction peaks that are the consequence of small particle size. Figure 2B illustrates selected areas of the electron diffraction pattern for the as-prepared sample. Clearly shown are the presence of only a rutile TiO_2 spotty ring without additional diffraction spots and rings of other compounds. These analyses showed that the as-prepared sample consisted of a pure crystalline sample with rutile structure.

The instilled surface areas of rutile TiO_2 nanorods,

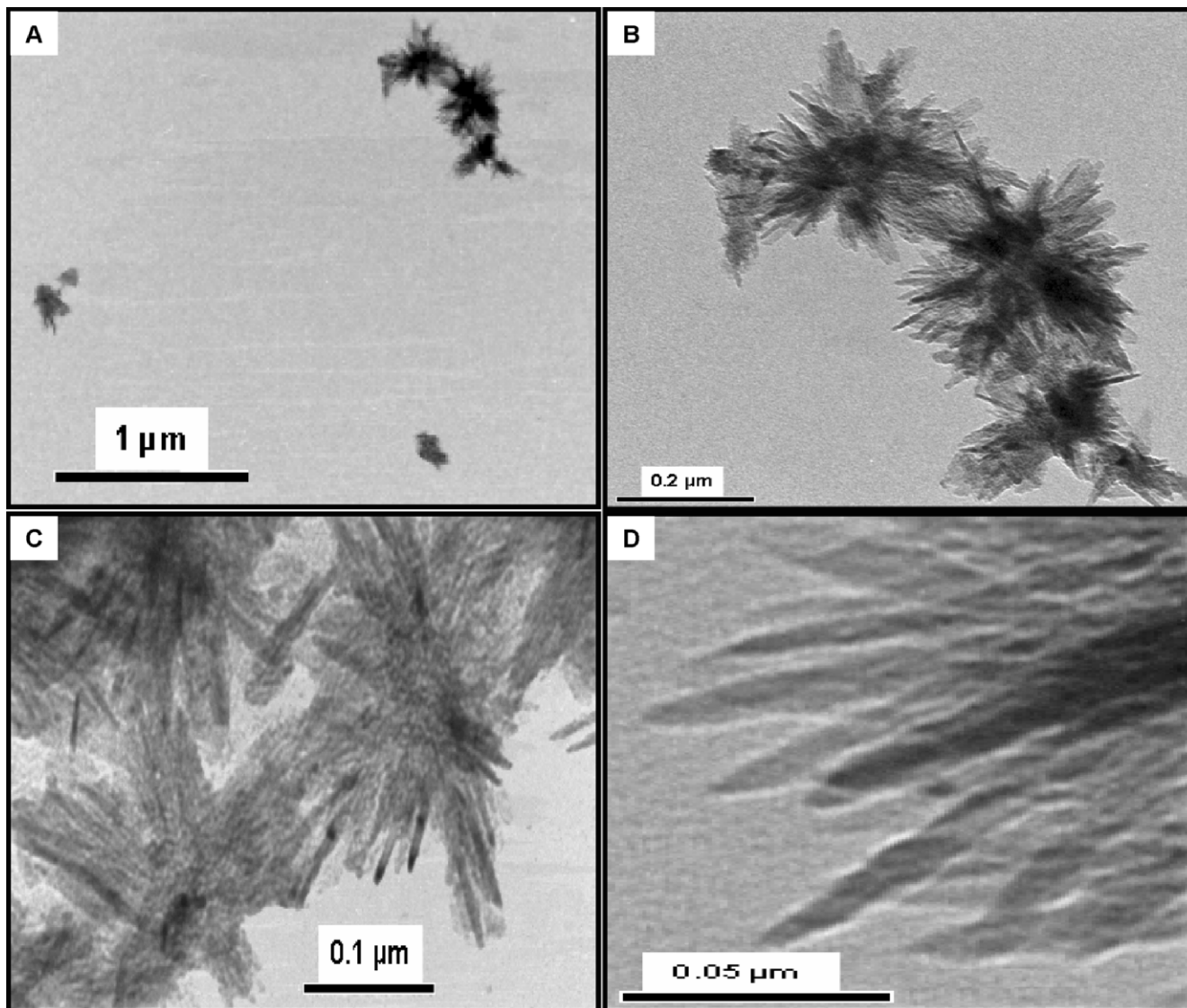


Figure 3. Transmission electron microscopy of a sample of the synthesized rutile TiO₂ nanorods. Some of the range of forms are shown at different magnifications. (A) 1 µm. (B) 0.2 µm (C) 0.1 µm. (D) 0.05 µm.

measured by a standard BET apparatus, were 16.46 cm² and 82.30 cm² for doses of 1 and 5 mg/kg, respectively.

Figure 3 illustrates transmission electron microscopy analysis of rutile TiO₂ nanorods, showing the presence of nanorods with a primary diameter of approximately 4 to 6 nm. Nanosized aggregates were present, and in general the aggregate sizes were less than 1 µm.

Rutile TiO₂ Nanorods Triggered Lung Inflammation. Depending on the i.t. treatment, the cells found in BAL fluid were primarily macrophages and polymorphonuclear neutrophils (PMNs) (Fig. 4). Lymphocytes were not found in control rat BAL fluid. No other cells were observed microscopically. Diff-Quik staining of the cells recovered in BAL fluid revealed that rutile TiO₂ nanorods were mainly taken up by macrophages (Fig. 4B–D). However, cell-free aggregates of nanorods were also seen (Fig. 4B–D).

The i.t. instillation of rutile TiO₂ nanorods at doses of 1

and 5 mg/kg resulted in a marked cellular influx in the lung (Fig. 5). Although the level of significance was reached at 5 mg/kg ($P < 0.05$) but not at 1 mg/kg, there was a clear dose-dependent increase in the number of macrophages (Fig. 5A) and of PMNs (Fig. 5B) in BAL fluid when rats that had undergone pulmonary exposure to rutile TiO₂ nanorods were compared with vehicle-exposed rats.

Rutile TiO₂ Nanorods Caused Pulmonary and Cardiac Edema. Figure 6 shows the results of our analyses of the lung and heart wet weight-to-dry weight ratios. A slight but significant increase of this relation was observed for the lung after i.t. instillation of rutile TiO₂ nanorods at a dose of 1 mg/kg ($P < 0.05$) and of 5 mg/kg ($P < 0.05$) when the treated rats were compared with vehicle-instilled rats (Fig. 6A).

Similar to the lung wet weight-to-dry weight ratios, the heart wet weight-to-dry weight ratio was significantly

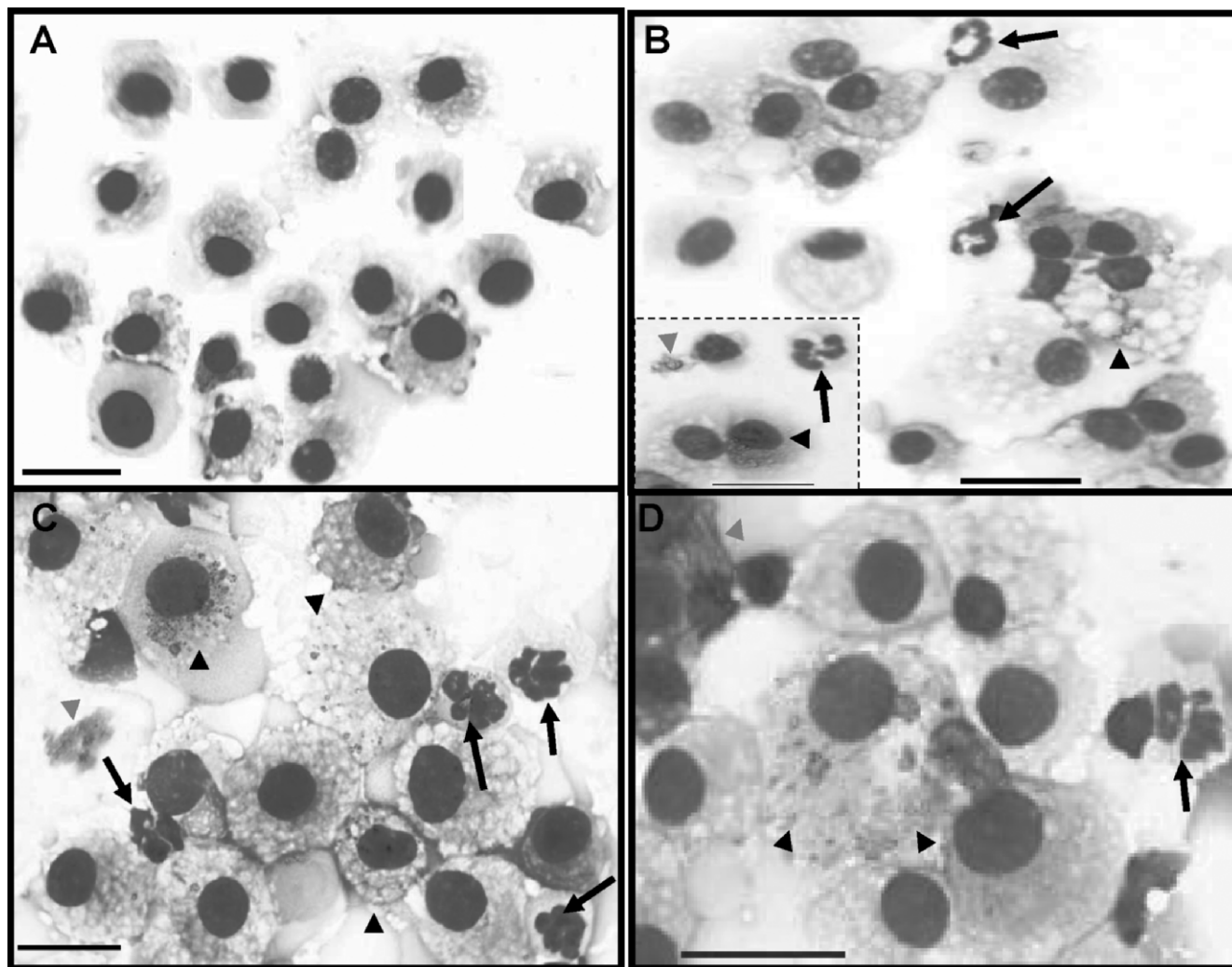


Figure 4. Cytocentrifuge preparation of cells obtained by lavage. The cells were recovered from rats exposed to (A) vehicle, (B) 1 mg/kg rutile TiO₂ nanorods, or (C and D) 5 mg/kg rutile TiO₂ nanorods, 24 hrs after instillation exposure. The micrographs demonstrate the influx of neutrophils (arrows) after exposure by i.t. instillation, the distribution of macrophages containing phagocytized rutile TiO₂ nanorods (arrowheads), and cell-free aggregates of rutile TiO₂ nanorods (red arrowheads). The scale bar represents 20 μ m. A color version of the figure is available in the online article.

greater in rats exposed to rutile TiO₂ nanorods at a dose of (1 mg/kg ($P < 0.05$) and of 5 mg/kg ($P < 0.01$) than in control rats (Fig. 6B).

Rutile TiO₂ Nanorods Affected the Number of Circulatory Monocytes, Granulocytes, and Platelets. Figure 7 illustrates the effect of rutile TiO₂ nanorods on the numbers of WBCs and platelets. Twenty-four hours after their i.t. administration, rutile TiO₂ nanorods, at the dose of 5 mg/kg, caused a significant increase in monocyte and granulocyte numbers than did administration of the vehicle (Fig. 7A and B). Although the differences in the numbers of monocytes and granulocytes did not reach statistical significance at the dose of 1 mg/kg, the monocyte and granulocyte numbers increased in a dose-dependent manner after the administration of rutile TiO₂ nanorods when the nanorods-treated rats were compared with those in the control group (Fig. 7A and B). No effect of rutile TiO₂

nanorods was observed on the number of lymphocytes (Fig. 7C).

Figure 7D shows that, in contrast to the numbers of monocytes and granulocytes, the number of platelets decreased in a dose-dependent manner after the i.t. administration of rutile TiO₂ nanorods; this decrease was seen when the nanorod-treated rats were compared with vehicle-treated rats. However, the level of significance was reached only at the dose of 5 mg/kg ($P < 0.05$).

Rutile TiO₂ Nanorods Caused Platelet Aggregation *In Vitro* in Whole Blood. Because the number of platelets decreased *in vivo*, a result indicating the presence of platelet aggregation, we investigated platelet aggregation in the whole blood of rats. Figure 8 shows that low concentrations of rutile TiO₂ nanorods (0.4, 2, and 10 μ g/ml blood) caused platelet aggregation in a dose-dependent manner. Direct addition of as little as 0.4 μ g/ml resulted in a

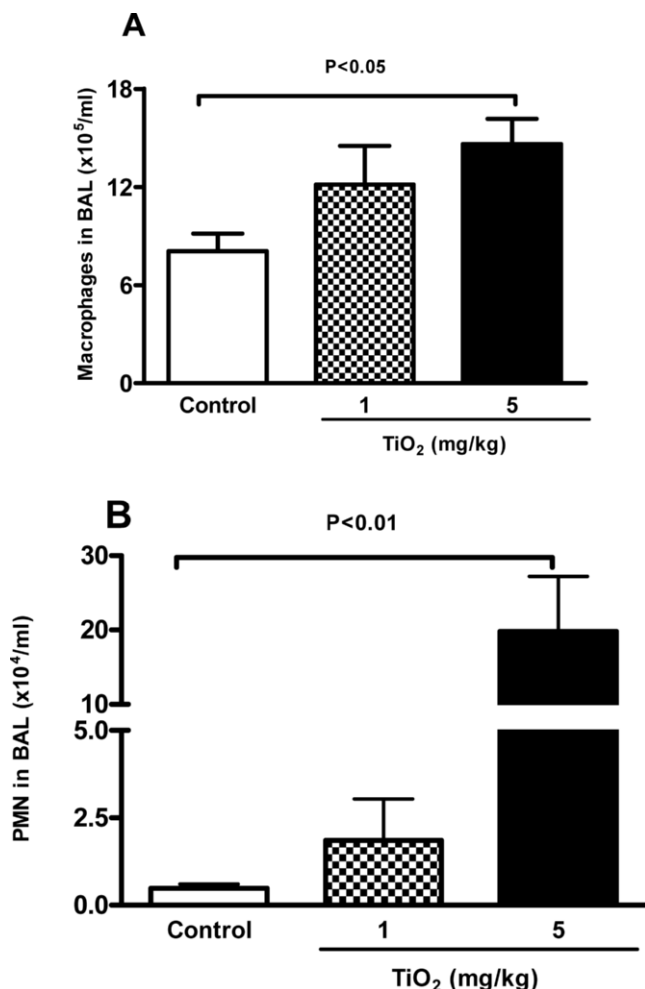


Figure 5. Rutile TiO₂ nanorods induced lung inflammation. (A) The number of macrophages and (B) the number of PMNs in BAL fluid were assessed 24 hrs after i.t. instillation of vehicle or rutile TiO₂ nanorods. Data are the mean $\pm$ SEM for each group (6 to 7 in each group). Statistical analysis by one-way ANOVA followed by the Dunnett multiple-comparison test was performed.

clear trend in platelet aggregation, and the effect was significant at doses of 2 μ g/ml ($P < 0.05$) and 10 μ g/ml ($P < 0.001$). The maximum platelet aggregation appeared to be reached at a dose of 2 μ g/ml, as no further increase in platelet aggregation was observed at the higher dose (10 μ g/ml).

Discussion

The present work provides, for the first time, evidence that rutile TiO₂ nanorods deposited in the lung can upregulate lung inflammation, pulmonary and cardiac edema, and systemic inflammation. Rutile TiO₂ nanorods were also shown to trigger platelet aggregation *in vivo* and *in vitro*.

The future of nanotechnology will largely depend on public acceptance of the risks of nanomaterials in relation to their benefits. Previous experience with materials such as asbestos advises caution in using novel substances without

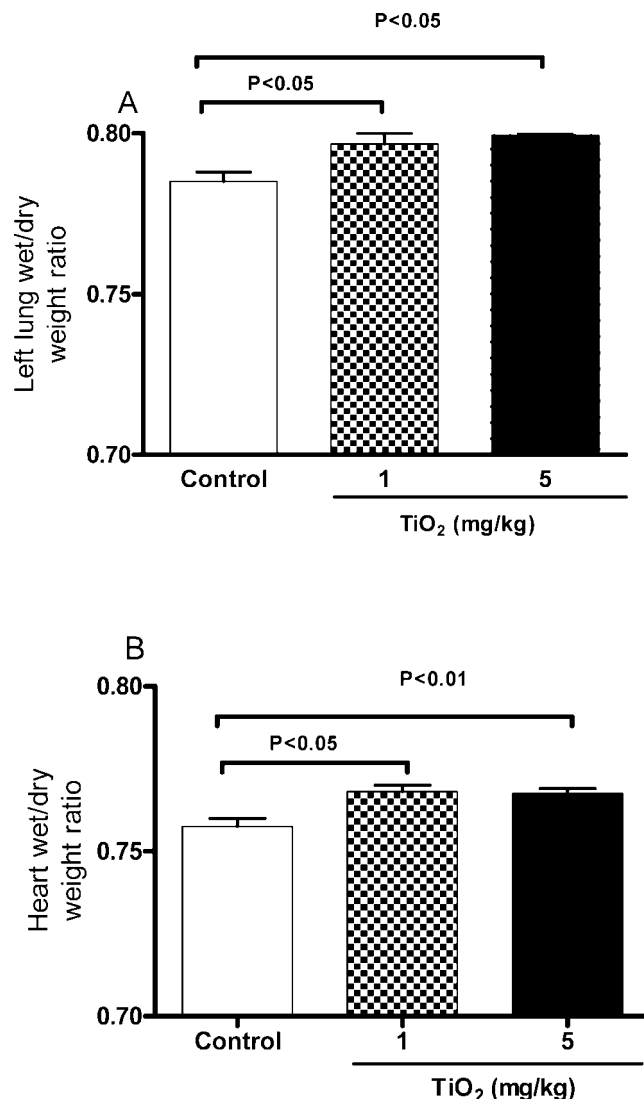


Figure 6. Rutile TiO₂ nanorods induced pulmonary and cardiac edema. (A) The wet weight-to-dry weight ratio of the left lung and (B) the wet weight-to-dry weight ratio of the cardiac tissue 24 hrs after i.t. instillation of vehicle or rutile TiO₂ nanorods are shown. Data are the mean $\pm$ SEM for each group (6 to 7 in each group). Statistical analysis by one-way ANOVA followed by the Dunnett multiple-comparison test was performed.

full evaluation of their potential health risks (23). Engineered nanomaterials typically possess nanostructure-dependent properties (e.g., chemical, mechanical, electrical, optical, magnetic, biological properties), which make them desirable for commercial or medical applications. However, these same properties may potentially lead to nanostructure-dependent biological activity that differs from and is not directly predicted by the bulk properties of the constituent chemicals and compounds (24, 25).

Pulmonary toxicity studies in rats have demonstrated that low-solubility ultrafine particles or nanoparticles (size < 100 nm) produce more potent inflammatory responses than do larger particles of similar chemistry at the same

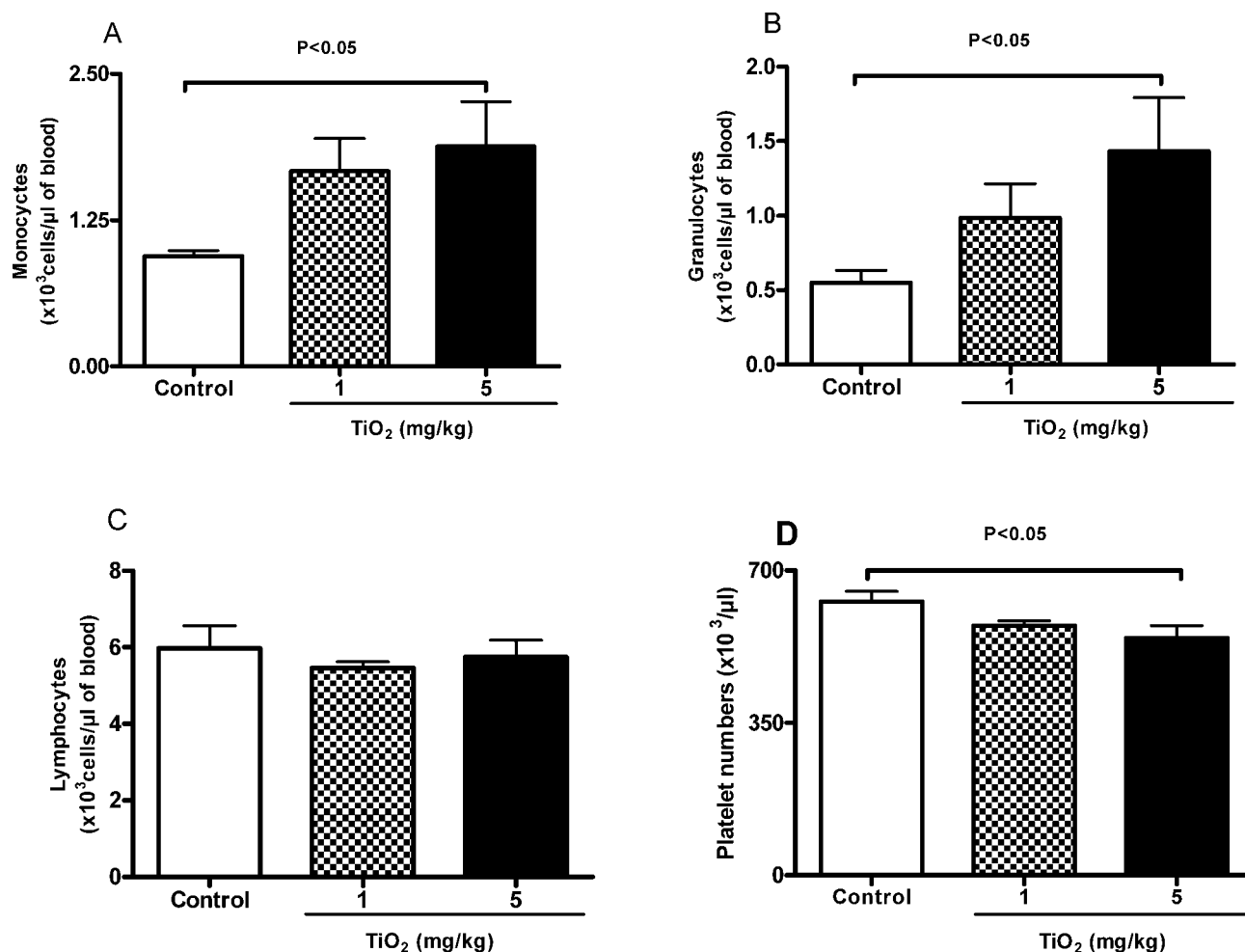


Figure 7. Rutile TiO₂ nanorods induced changes in the numbers of circulatory cells. The number of (A) monocytes, (B) granulocytes, (C) lymphocytes, and (D) platelets were evaluated 24 hrs after i.t. instillation of vehicle or rutile TiO₂ nanorods. Data are the mean $\pm$ SEM for each group (6 to 7 in each group). Statistical analysis by one-way ANOVA followed by the Dunnett multiple-comparison test was performed.

mass concentrations or doses (4). Some evidence suggests that inhaled nanoparticles, after deposition in the lung, largely escape alveolar macrophage surveillance and gain greater access to the pulmonary interstitium through translocation from alveolar spaces through epithelium (15, 26).

The potential pathophysiologic effects of rutile TiO₂ nanorods are not known. To clarify this point, we applied a soft chemistry technique to produce rutile TiO₂ nanorods. The purity of these nanorods was verified by powder X-ray diffraction and transmission electron microscopy electron-diffraction analysis. We have previously shown that the rutile phase of nanorods possesses an excellent photodegradation of Congo red under sunlight. This effect has been related to their shape and size (18). The transmission electron microscopy analysis of rutile TiO₂ nanorods showed nanorods with a primary diameter of approximately 4 to 6 nm (Fig. 3C and D). We also found nanosized aggregates, and in general the aggregate sizes were less than 1 μ m.

The i.t. instillation of a bolus of particles has been shown to be a convenient and valid mode of administration

of foreign compounds into the airways (27). By this method of delivery, the actual dose delivered to the lungs of each animal can be established accurately, and this method is simpler than inhalation; thus, the introduction of a range of doses to the lung in a short time is permitted. However, this technique has drawbacks that include the essentially non-physiologic nature and the administration of the particles as a bolus rather than as an infusion that lasts several hours. The consequence of the latter on our findings remains to be established in studies using exposure by inhalation.

The exposure doses chosen for this study (1 and 5 mg/kg) and the time point reflected i.t. instillation doses used in previous studies of diesel exhaust, carbon nanotubes, and TiO₂ as a form of nanoparticles or anatase nanorods (17, 21, 28, 29). Our data showed that 24 hrs after their i.t. instillation, rutile TiO₂ nanorods cause macrophage and neutrophil influx in the alveoli, reflecting increased epithelial permeability. This finding is consistent with those from the study of Warheit *et al.* (17), who showed that similar doses of TiO₂ nanodots or anatase nanorods caused a short-lived influx of neutrophils in BAL fluid that was

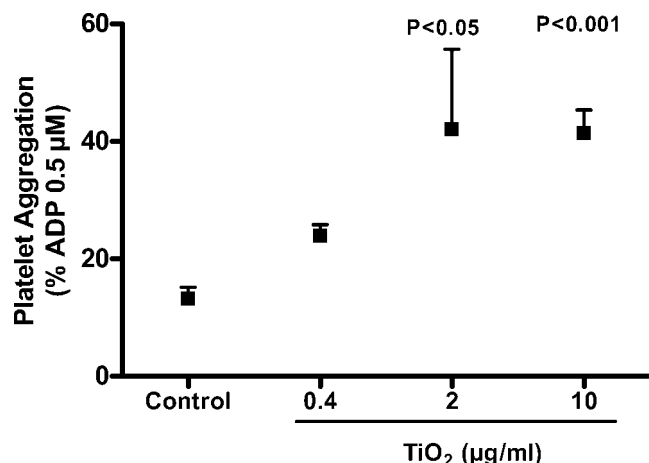


Figure 8. Rutile TiO₂ nanorods induced platelet aggregation in whole blood *in vitro*. Platelet aggregation in untreated rat whole blood 3 mins after the addition of vehicle or rutile TiO₂ nanorods (0.4, 2, or 10 μg/ml) was assessed. The degree of aggregation is expressed as the percent of aggregation induced by ADP (0.5 μM). Data are the mean ± SEM for each group (6 in each group). Statistical analysis by one-way ANOVA followed by the Dunnett multiple-comparison test was performed.

observed at 24 hrs. However, this effect was not sustained 1 week or 1 or 3 months after exposure. It was not clear from that study whether the number of macrophages was affected by TiO₂ nanodots or anatase nanorods (17). Recently, Chen *et al.* (13) showed that TiO₂ nanoparticles can induce severe pulmonary emphysema. They found that PIGF, chemokines, and the complement cascade may cause inflammatory cell chemotaxis, cell proliferation, and apoptosis, resulting in serious lung injury. In addition, the present study showed an increase in lung wet weight-to-dry weight ratio, indicating pulmonary edema formation. The increase in lung weight ratio has been observed in rats after i.t. instillation of quartz or positively charged polystyrene nanoparticles but not after instillation of carbon black or diesel particles (30, 31).

There are three primary hypotheses being investigated to explain the extrapulmonary effect of nanoparticles (4, 32). Inhaled particles have been suggested to affect the autonomic nervous system, and these effects may lead to changes in the pattern of breathing, heart rate, and heart rate variability. Inhaled particles may affect the cardiovascular system through inflammatory mediators (e.g., cytokines and histamine) produced in the lungs and released into the circulation (4, 32). Several studies have shown that nanoparticles, owing to their small size, could avoid normal phagocytic defenses in the respiratory system and gain access to the systemic circulation and thus to different extrapulmonary sites or even to the central nervous system (5–8, 10, 33). Our data showed that i.t. instillation of rutile TiO₂ nanorods causes cardiac edema. This observation has not been previously reported. Cardiac edema was reportedly observed in rats exposed to environmental particles that were particulate matter with a size <2.5 μm (PM_{2.5}; Ref. 20). The mechanism whereby rutile TiO₂ causes cardiac

edema remains to be established. However, this effect could be related to the translocation of rutile TiO₂ nanorods, which directly affected the heart, and/or through the release of an inflammatory mediator by the lungs.

In addition to cardiac edema, the number of circulatory inflammatory cells such as monocytes and granulocytes were increased after pulmonary exposure to rutile TiO₂ nanorods. An increase in leukocytes in association with nanoparticle exposure is usually interpreted as an indication of an increased inflammatory response. Our findings are in agreement with those of previous studies that reported an increase in the number of blood leukocytes after pulmonary exposure of rats to nanoparticles (34) or in association with increasing particulate air pollution in humans (35, 36). Other studies performed in humans and animals exposed to particulate matter with a size <10 μm (PM₁₀) showed the development of leukocytosis, which was accompanied by stimulation of the bone marrow that included the accelerated release of PMNs and monocytes from the marrow (36–38).

We recently demonstrated a prothrombotic effect of diesel exhaust particles in hamsters and an activation of platelets (28, 39, 40). Studies of rats additionally showed evidence of thrombus formation after the deposition of nanoparticles in the respiratory tract (41). A procoagulant effect has also been shown in mice and rats, after intravascular infusion of nanoparticles or diesel particles (42, 43). We have also reported that polystyrene nanoparticles in hamster models can modulate thrombus formation, and Suwa *et al.* (44) observed a systemic inflammatory response and a progression of the atherosclerotic process in hyperlipidemic rabbits in association with PM₁₀.

Our findings showed that i.t. instillation of rutile TiO₂ nanorods leads to a dose-dependent decrease in platelet number, indicating that platelet aggregation occurred *in vivo*. Our data are in agreement with the results of Rückerl *et al.* (45), who reported a decrease in platelet number along with an increase in soluble CD40 ligand, a marker of platelet activation, after human exposure to environmental nanoparticles. In contrast, in our previous studies, we did not find any changes in platelet number after pulmonary exposure to diesel particles or carbon nanotubes (21, 28, 39, 40). This effect could be related to the shape and/or surface chemistry of the rutile TiO₂ nanorods (4). Because this decrease in platelets could be the result of platelet aggregation due to a direct effect of rutile TiO₂ nanorods that have presumably translocated to the systemic circulation, we performed additional *in vitro* studies whereby we assessed the effect of rutile TiO₂ nanorods on platelet aggregation in whole blood. The addition of TiO₂ nanorods into rat blood *in vitro* caused platelet activation in a dose-dependent manner. This effect was seen with as little as 0.4, 2, and 10 μg of rutile TiO₂ nanorods per milliliter, which are concentrations that can presumably be achieved in the circulation after i.t. instillation of 0.3, 1.5, and 7.5 mg/kg or 0.1, 0.5, and 1.5 mg per rat (5, 7).

We conclude that pulmonary exposure to rutile TiO₂ nanorods leads to lung inflammation and to systemic inflammation and activation of circulating blood platelets. Further experimental studies are needed to determine the mechanisms behind these observations and to study the effect of size and surface modification on respiratory and cardiovascular endpoints. Our findings suggest that the development of novel nanoparticulates for pharmacology, therapeutics, diagnostics, or industrial purposes must proceed in tandem with assessment of any toxicologic and environmental side effects.

We thank Mr. Issa Al-Amri (Electron Microscopy Unit, College of Medicine and Health Sciences, Sultan Qaboos University) for performing the electron microscopy analysis.

1. Seaton A, Donaldson K. Nanoscience, nanotoxicology, and the need to think small. *Lancet* 365:923–924, 2005.
2. Ferrari M. Cancer nanotechnology: opportunities and challenges. *Nat Rev Cancer* 5:161–171, 2005.
3. Hoet PH, Bruske-Hohlfeld I, Salata OV. Nanoparticles—known and unknown health risks. *J Nanobiotechnology* 2:12, 2004.
4. Oberdorster G, Oberdorster E, Oberdorster J. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. *Environ Health Perspect* 113:823–839, 2005.
5. Oberdorster G, Sharp Z, Atudorei V, Elder A, Gelein R, Lunts A, Kreyling W, Cox C. Extrapulmonary translocation of ultrafine carbon particle following whole-body inhalation exposure of rats. *J Toxicol Environ Health A* 65:1531–1543, 2002.
6. Nemmar A, Hoet PH, Vanquickenborne B, Dinsdale D, Thomeer M, Hoylaerts MF, Vanbilloen H, Mortelmans L, Nemery B. Passage of inhaled particles into the blood circulation in humans. *Circulation* 105:411–414, 2002.
7. Nemmar A, Vanbilloen H, Hoylaerts MF, Hoet PH, Verbruggen A, Nemery B. Passage of intratracheally instilled ultrafine particles from the lung into the systemic circulation in hamster. *Am J Respir Crit Care Med* 164:1665–1668, 2001.
8. Kreyling W, Semmler M, Erbe F, Mayer P, Schulz H, Oberdorster G, Ziesenis A. Translocation of ultrafine insoluble iridium particles from lung epithelium to extrapulmonary organs is size dependent but very low. *J Toxicol Environ Health A* 65:1513–1530, 2002.
9. Geiser M, Rothen-Rutishauser B, Kapp N, Schurch S, Kreyling W, Schulz H, Semmler M, Hof VI, Heyder J, Gehr P. Ultrafine particles cross cellular membranes by nonphagocytic mechanisms in lungs and in cultured cells. *Environ Health Perspect* 113:1555–1560, 2005.
10. Elder A, Gelein R, Silva V, Feikert T, Opanashuk L, Carter J, Potter R, Maynard A, Ito Y, Finkelstein J, Oberdorster G. Translocation of inhaled ultrafine manganese oxide particles to the central nervous system. *Environ Health Perspect* 114:1172–1178, 2006.
11. Afaq F, Abidi P, Matin R, Rahman Q. Cytotoxicity, pro-oxidant effects and antioxidant depletion in rat lung alveolar macrophages exposed to ultrafine titanium dioxide. *J Appl Toxicol* 18:307–312, 1998.
12. Rahman Q, Lohani M, Dopp E, Pemsel H, Jonas L, Weiss DG, Schiffmann D. Evidence that ultrafine titanium dioxide induces micronuclei and apoptosis in Syrian hamster embryo fibroblasts. *Environ Health Perspect* 110:797–800, 2002.
13. Chen HW, Su SF, Chien CT, Lin WH, Yu SL, Chou CC, Chen JJ, Yang PC. Titanium dioxide nanoparticles induce emphysema-like lung injury in mice. *FASEB J* 20:2393–2395, 2006.
14. Oberdorster G. Pulmonary effects of inhaled ultrafine particles. *Int Arch Occup Environ Health* 74:1–8, 2001.
15. Ferin J, Oberdorster G, Penney DP. Pulmonary retention of ultrafine and fine particles in rats. *Am J Respir Cell Mol Biol* 6:535–542, 1992.
16. Sayes CM, Wahi R, Kurian PA, Liu Y, West JL, Ausman KD, Warheit DB, Colvin VL. Correlating nanoscale titania structure with toxicity: a cytotoxicity and inflammatory response study with human dermal fibroblasts and human lung epithelial cells. *Toxicol Sci* 92:174–185, 2006.
17. Warheit DB, Webb TR, Sayes CM, Colvin VL, Reed KL. Pulmonary instillation studies with nanoscale TiO₂ rods and dots in rats: toxicity is not dependent upon particle size and surface area. *Toxicol Sci* 91:227–236, 2006.
18. Melghit K, Al-Rabaniah SS. Photodegradation of Congo red under sunlight catalysed by nanorod rutile TiO₂. *J Photochem Photobiol A* 184:331–334, 2006.
19. Rhoden CR, Wellenius GA, Ghelfi E, Lawrence J, Gonzalez-Flecha B. PM-induced cardiac oxidative stress and dysfunction are mediated by autonomic stimulation. *Biochim Biophys Acta* 1725:305–313, 2005.
20. Rivero DH, Soares SR, Lorenzi-Filho G, Saiki M, Godleski JJ, Antonangelo L, Dolhnikoff M, Saldiva PH. Acute cardiopulmonary alterations induced by fine particulate matter of Sao Paulo, Brazil. *Toxicol Sci* 85:898–905, 2005.
21. Nemmar A, Hoet PH, Vandervoort P, Dinsdale D, Nemery B, Hoylaerts MF. Enhanced peripheral thrombogenicity after lung inflammation is mediated by platelet-leukocyte activation: role of P-selectin. *J Thromb Haemost* 5:1217–1226, 2007.
22. Macrae CF, Edgington PR, McCabe P, Pidcock E, Shields GP, Taylor R, Towler M, De Streek J. Mercury: visualization and analysis of crystal structures. *J Appl Crystallogr* 39:453–457, 2006.
23. Hoet PHM, Nemmar A, Nemery B. Health impact of nanomaterials? *Nat Biotechnol* 22:19, 2004.
24. Oberdorster G, Maynard A, Donaldson K, Castranova V, Fitzpatrick J, Ausman K, Carter J, Karn B, Kreyling W, Lai D, Olin S, Monteiro-Riviere N, Warheit D, Yang H. Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy. Part I. *Fibre Toxicol* 2:8, 2005.
25. Maynard AD, Aitken RJ, Butz T, Colvin V, Donaldson K, Oberdorster G, Philbert MA, Ryan J, Seaton A, Stone V, Tinkle SS, Tran L, Walker NJ, Warheit DB. Safe handling of nanotechnology. *Nature* 444:267–269, 2006.
26. Oberdorster G, Ferin J, Lehnert BE. Correlation between particle size, in vivo particle persistence, and lung injury. *Environ Health Perspect* 102(Suppl 5):173–179, 2004.
27. Driscoll KE, Costa DL, Hatch G, Henderson R, Oberdorster G, Salem H, Schlesinger RB. Intratracheal instillation as an exposure technique for the evaluation of respiratory tract toxicity: uses and limitations. *Toxicol Sci* 55:24–35, 2000.
28. Nemmar A, Hoet PH, Dinsdale D, Vermeylen J, Hoylaerts MF, Nemery B. Diesel exhaust particles in lung acutely enhance experimental peripheral thrombosis. *Circulation* 107:1202–1208, 2003.
29. Warheit DB, Webb TR, Reed KL, Frerichs S, Sayes CM. Pulmonary toxicity study in rats with three forms of ultrafine-TiO₂ particles: differential responses related to surface properties. *Toxicology* 230:90–104, 2007.
30. Murphy SA, Berube KA, Pooley FD, Richards RJ. The response of lung epithelium to well characterised fine particles. *Life Sci* 62:1789–1799, 1998.
31. Nemmar A, Delaunois A, Nemery B, Dessy-Doize C, Beckers JF, Sulon J, Gustin P. Inflammatory effect of intratracheal instillation of ultrafine particles in the rabbit: role of C-fiber and mast cells. *Toxicol Appl Pharmacol* 160:250–261, 1999.
32. Vermeylen J, Nemmar A, Nemery B, Hoylaerts MF. Ambient air pollution and acute myocardial infarction. *J Thromb Haemost* 3:1955–1961, 2005.
33. Wallenborn JG, McGee JK, Schladweiler MC, Ledbetter AD, Kodavanti UP. Systemic translocation of particulate matter-associated

- metals following a single intratracheal instillation in rats. *Toxicol Sci* 98:231–239, 2007.
34. Gilmour PS, Ziesenis A, Morrison ER, Vickers MA, Drost EM, Ford I, Karg E, Mossa C, Schroepel A, Ferron GA, Heyder J, Greaves M, MacNee W, Donaldson K. Pulmonary and systemic effects of short-term inhalation exposure to ultrafine carbon black particles. *Toxicol Appl Pharmacol* 195:35–44, 2004.
 35. Ghio AJ, Hall A, Bassett MA, Cascio WE, Devlin RB. Exposure to concentrated ambient air particles alters hematologic indices in humans. *Inhal Toxicol* 15:1465–1478, 2003.
 36. Tan WC, Qiu D, Liam BL, Ng TP, Lee SH, van Eeden SF, D'Yachkova Y, Hogg JC. The human bone marrow response to acute air pollution caused by forest fires. *Am J Respir Crit Care Med* 161: 1213–1217, 2000.
 37. Goto Y, Hogg JC, Shih CH, Ishii H, Vincent R, van Eeden SF. Exposure to ambient particles accelerates monocyte release from bone marrow in atherosclerotic rabbits. *Am J Physiol Lung Cell Mol Physiol* 287:L79–L85, 2004.
 38. Goto Y, Ishii H, Hogg JC, Shih CH, Yatera K, Vincent R, van Eeden SF. Particulate matter air pollution stimulates monocyte release from the bone marrow. *Am J Respir Crit Care Med* 170:891–897, 2004.
 39. Nemmar A, Hoet PHM, Vermeylen J, Nemery B, Hoylaerts MF. Pharmacological stabilization of mast cells abrogates late thrombotic events induced by diesel exhaust particles in hamsters. *Circulation* 110: 1670–1677, 2004.
 40. Nemmar A, Nemery B, Hoet PHM, Vermeylen J, Hoylaerts MF. Pulmonary inflammation and thrombogenicity caused by diesel particles in hamsters—role of histamine. *Am J Respir Crit Care Med* 168:1366–1372, 2003.
 41. Silva VM, Corson N, Elder A, Oberdorster G. The rat ear vein model for investigating in vivo thrombogenicity of ultrafine particles (UFP). *Toxicol Sci* 85:983–989, 2005.
 42. Khandoga A, Stampfl A, Takenaka S, Schulz H, Radykewicz R, Kreyling W, Krombach F. Ultrafine particles exert prothrombotic but not inflammatory effects on the hepatic microcirculation in healthy mice in vivo. *Circulation* 109:1320–1325, 2004.
 43. Nemmar A, Al Maskari S, Ali BH, Al Amri IS. Cardiovascular and lung inflammatory effects induced by systemically administered diesel exhaust particles in rats. *Am J Physiol Lung Cell Mol Physiol* 292: L664–L670, 2007.
 44. Suwa T, Hogg JC, Quinlan KB, Ohgami A, Vincent R, van Eeden SF. Particulate air pollution induces progression of atherosclerosis. *J Am Coll Cardiol* 39:935–942, 2002.
 45. Ruckerl R, Phipps RP, Schneider A, Frampton M, Cyrus J, Oberdorster G, Wichmann HE, Peters A. Ultrafine particles and platelet activation in patients with coronary heart disease—results from a prospective panel study. *Part Fibre Toxicol* 4:1, 2007.