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Pulmonary toxicity induced by three forms of titanium dioxide nanoparticles via intra-tracheal instillation in rats

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Abstract

Titanium dioxide (TiO_2) nanoparticles are in wide commercial use worldwide. To evaluate if acute pulmonary toxicity can be induced by nano-TiO_2 particles, rats were intra-tracheally instilled with 0.5, 5, or 50 mg/kg of 5, 21, and 50 nm TiO_2 primary particles. Toxic effects were determined with the coefficients of lung tissues to body weight, histopathology, biochemical parameters of blood, activity of lactate dehydrogenase (LDH), alkaline phosphatase (ALP) and acid phosphatase (ACP) in tissues, and the phagocytotic ability of alveolar macrophages (AMs). All the indicators were observed in sacrificed rats one week post-exposure.

There was a significant difference of coefficients of pulmonary tissues between the high-dose group and the low- or moderate-dose groups with an exposure of 5 nm TiO_2. At the same time, 5 nm TiO_2 primary particles increased the activity of LDH and ALP when exposure dose was >5 mg/kg. A significant difference in LDH and ALP activity was observed between the 50 mg/kg group and 0.5 or 5 mg/kg group with exposure of 5 nm TiO_2. Lung tissues showed increased ALP activity only if treated with 5 and 50 mg/kg of 21 nm TiO_2 particles. There was no significant difference in LDH and ALP activity in the 50 nm TiO_2 group and control group. Histopathologic examination of lung tissues indicated that the pulmonary response to exposure to TiO_2 particles in rats manifested as dose-dependent inflammatory lesions, which mainly consisted of infiltration of inflammatory cells and interstitial thickening. Analysis of uptake of neutral red dye showed that 50 nm TiO_2 particles significantly increased phagocytotic ability of AMs compared with controls ($P < 0.05$), whereas exposure with 5 nm TiO_2 reduced the phagocytotic ability of AMs when the exposure dose was 50 mg/kg. These results suggest that particle size and exposure dose may have important roles in pulmonary toxicity. The toxic effect of TiO_2 nanoparticles in lung tissue exhibited a dose–response relationship. After exposure with TiO_2 particles of >5.0 mg/kg, 5 and 21 nm TiO_2 particles induced standing pulmonary lesions; and 5 nm TiO_2 particles may suppress the phagocytotic ability of AMs if exposure dose was $\geqslant$50 mg/kg. Pulmonary toxicity caused by 5 nm TiO_2 particles was more severe than that caused by 21 and 50 nm TiO_2 particles.
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Keywords: Titanium dioxide particles; Nanoparticles; Pulmonary toxicity; Intra-tracheal instillation

1. Introduction

As new types of photo-catalyst, anti-ultraviolet light agents, and photoelectric effect agents, titanium dioxide (TiO_2) nanoparticles are widely used commercially in white pigments preparation, anti-aging research, water purification techniques, and cosmetic applications [1]. According to the National Nanotechnology Initiative of America, nano-TiO_2 is one of the most highly manufactured nanomaterials worldwide.

Conventional TiO_2 particles are commonly used as negative controls in toxicology studies of particles due to their low solubility and low toxicity [2–4]. TiO_2 nanoparticles have a bigger surface area compared with the materials of conventional size. Surface atoms, surface energy and

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surface tension of TiO_2 nanoparticles increase rapidly with the decreasing diameter of particles. Their thermomagnetism, photosensitivity, surface stabilities, and other physicochemical characteristics are different from those of conventional size TiO_2 because of the small-size effects, surface effect, quantum-size effect, and macroscopic quantum tunneling effect of TiO_2 nanoparticles. Studies showed that three major characteristics of TiO_2 nanoparticles (ultramicro size, ultraviolet absorbency, high-effect photocatalysis activity) were closely associated with their biological effects [5,6]. The ultrafine size of TiO_2 nanoparticles enables them to pass through cell membranes and nuclear membranes, and they can affect cell ultrastructure and damage the cell membrane [7–10].

Humans are increasingly exposed to TiO_2 nanoparticle material because of its widespread use. The main exposure areas are the respiratory tract and skin. Studies on the pulmonary toxicity of TiO_2 nanoparticles in mammals indicate that intra-tracheal instillation of TiO_2 particles to the animals evoked a pulmonary inflammatory response in a size-dependent way [4,11–16]. It was also demonstrated that TiO_2 nanoparticles changed the integrity of the cell membrane and phagocytic activity in cultured macrophages [17].

The surface of TiO_2 particles interacts with the cell membrane and subcellular structures. Toxicological effects are therefore closely related to the specific surface area of nanoparticles [16,18–20]. With the rapid development of nanotechnology, small-sized TiO_2 nanoparticles (<10 nm) are commercially available. Their physicochemical properties change greatly with decreasing size [21,22]. Previous studies have focused on comparing the effect of several different-sized TiO_2 particles [23].

In this study, we evaluated acute pulmonary toxicity induced by different dosages of TiO_2 primary particles of sizes 5, 21, and 50 nm via intra-tracheal instillation in rats. Changes in the coefficients of tissues to body weight, histopathology, biochemical parameters of blood, enzyme activity in pulmonary tissues, and phagocytotic ability of alveolar macrophages (AMs) were investigated.

2. Materials and methods

2.1. Preparation and characterization of TiO_2 nanoparticles

TiO_2 nanoparticles of size 21 nm were purchased from Degussa Corporation (Hanau, Germany). TiO_2 nanoparticles of size 5 and 50 nm were generous gifts from Hongsheng Materials Technology Company Limited (Zhejiang, China). According to the product reports offered by the companies, crystal phases were determined by a powder X-ray diffractometer (XRD, PW1700, Philips, Netherlands); particle size and distribution were analyzed by transmission electron microscopy (JEM200-CX, JEOL, Tokyo, Japan) and scanning electron microscopy (SEM, S-570, Hitachi, Japan); specific surface area and composition were determined by BET surface area analyzer (OMNISORP-100CX, Coulter, USA) and inductively coupled plasma spectrometry (ICP, JA1100, Jarrell-Ash, MA, USA).

Particles were heated to 123 ± 2 °C for 15 min to reduce the risk of endotoxin contamination. Each TiO_2 powder was dispersed into an aqueous solution buffered with 0.15% NaCl solution to sufficiently disperse particles for sizing analyses. Solutions containing TiO_2 particles were treated by ultrasound for 15–20 min and mechanically vibrated for 2–3 min. TiO_2 nanoparticles, in a stable suspending state, were diluted to 0.2, 2, and 20 mg/ml.

2.2. Animals and treatment

Sprague–Dawley rats (60 males, 60 females; 187 ± 12 g) were purchased from the Shanghai Slack Experimental Animal Center (Shanghai, China). Rats were acclimatized for five days before experimentation. Procedures complied with the national regulations related to animal welfare.

Rats were randomly divided into ten groups. One control group was instilled with 0.15% NaCl solution via the intra-tracheal route. Nine TiO_2 experimental groups were instilled (intra-tracheal) with three sizes (5, 21, and 50 nm TiO_2 suspension) of TiO_2 particles at 0.5, 5, and 50 mg/kg. Animals were fasted overnight before treatment. After rats were anesthetized via inhalation with ether, TiO_2 suspension was given via intra-tracheal instillation. Food and water were provided 2 h later.

One week later, half of the rats were sacrificed by exsanguination via the abdominal aorta. Whole blood and serum samples were collected for blood biochemical assay. Lung tissues were excised and weighed. A piece of lung tissue was immediately fixed in 10% formalin solution for histopathologic diagnosis. Remaining samples were stored at −60 °C for enzyme activity analysis, including lactate dehydrogenase (LDH), alkaline phosphatase (ALP) and acid phosphatase (ACP).

2.3. Coefficients of tissues and organs

After weighing the body and tissues, the coefficient of lung tissue to body weight was calculated as the ratio of tissues (wet weight, mg) to body weight (g).

2.4. Blood biochemical assay and enzyme activity

Total protein and albumin in plasma were measured using a Biochemical Autoanalyzer (Beckman LX-20, CA, USA). Activity of ALP, ACP, and LDH in lung tissues was measured with commercial reagent kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) to determine the degree of damage to lung tissue.

2.5. Histopathologic examination

Tissues fixed in a 10% formalin solution were embedded in paraffin, sectioned 5 μm in thickness, and mounted on

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glass slides. After hematoxylin–eosin (HE) staining, slides were examined using an optical microscope (Nikon E600, Tokyo, Japan) by a pathologist.

2.6. Uptake of neutral red dye in AMs

Remaining animals were sacrificed one week after exposure. Lungs of sham and TiO_2-exposed rats were lavaged thrice with 5 ml of warmed phosphate-buffered saline (PBS) solution *in situ* using a closed chest technique. Obtained lung lavage fluid was centrifuged at 1000 rpm for 10 min. Cells were separated from lung lavage fluid and incubated in Dulbecco's modified eagle's medium (DMEM) with 10% fetal bovine serum for 2 h at 37 °C in an atmosphere of 5% CO_2 and 95% air. Each dish was washed twice with the DMEM to remove non-adherent cells. Viability of adherent cells was determined by the trypan blue exclusion test (>90% viable cells). The final cell suspension was adjusted to 1×10^6 cells/well in a 24-well culture dish. Uptake of neutral red into cells was determined by a previously described method [24].

2.7. Statistical analyses

Statistical analyses were done using SPSS11.5 software. Data were expressed as means ± SD. One-way analysis of variance (ANOVA) was carried out to compare the differences of means among multi-group data. Dunnett's test was carried out when each group of experimental data was compared with solvent-control data. Statistical significance for all tests was judged at a probability level of 0.05.

3. Results

3.1. Physicochemical characteristics of TiO_2 nanoparticles

Particle sizes of the three groups of TiO_2 were approximately 5, 21, and 50 nm. BET specific surface area for 5 nm TiO_2 particles was 210 m^2/g; 50 m^2/g for 21 nm TiO_2 particles; and 30 m^2/g for 50 nm TiO_2 particles. All particles had an almost identical energy spectrum. Purity of different-sized particles was >98% (Table 1).

An aqueous solution buffered with 0.15% NaCl solution to TiO_2 was used to disperse TiO_2 nanoparticles. All types of TiO_2 particle were agglomerated to some extent after dispersion. Aqueous solution with 0.15% NaCl provided an optimum size distribution for dispersed particle agglomerates because nanoparticles form agglomerates near their isoelectric point to the near-neutral pH of the buffer solution.

Table 1
Physical properties of TiO_2 nanoparticles.

Particle size (nm)	Crystalline phase	Median size (nm)	Specific surface area (m^2/g)	Purity (%)
5	Anatase	5	210	>99
21	80% anatase/ 20% rutile	21	50	>99.5
50	Rutile	50	30	⩾98.0

3.2. Coefficients of pulmonary tissues

The coefficients of pulmonary tissues to body weight are presented in Fig. 1. They are defined as milligrams (wet weight of tissues)/g (body weight). A significant difference was observed between the high-dose group and the low- or moderate-dose groups with exposure of 5 nm TiO_2. The increased coefficients suggested that the inflammation may be induced and maintained in the pulmonary tissues for one week after the exposure of 5 nm TiO_2. Exposure to TiO_2 particles of size 21 or 50 nm showed no difference compared with the control group.

3.3. Biochemical parameters of plasma and enzyme activities in pulmonary tissues

There was no significant difference in total protein and albumin from the plasma of all experimental groups compared with that of the control group (Fig. 2). Enzyme activity of ALP, ACP, and LDH in pulmonary tissues varied with TiO_2 particle size (Fig. 3). A significant difference in LDH activity was observed between the high-dose group and control group with the exposure of 5 nm TiO_2; but LDH activity of low-dose or moderate-dose exposure with 5 nm TiO_2 showed no difference with that of the control group. There was a significant difference in ALP activity between the high-dose group and low- or moderate-dose groups with exposure of 5-nm TiO_2. This result also indicated that 5 nm TiO_2 primary particles could induce increased activity of LDH and ALP if the exposure dose was >5 mg/kg (though these changes were not statistically significant). Lung tissues showed an increased activity of ALP only when treated with 5 and 50 mg/kg of 21 nm TiO_2 particles. There were no significant difference in LDH and ALP activity among exposure groups of 50 nm TiO_2 and the control group. No significant difference was

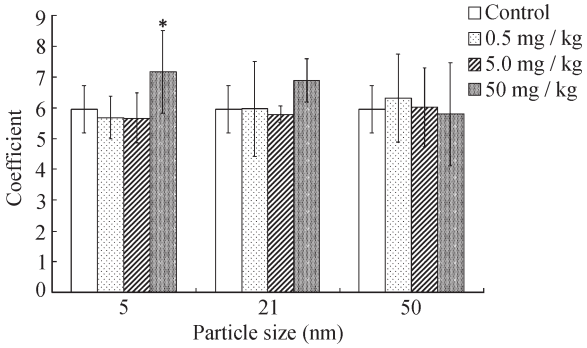

Fig. 1. Coefficients of pulmonary tissues from rats exposed to TiO_2 nanoparticles and the corresponding controls at one week post-exposure. Sprague–Dawley rats (6 males, 6 females) were in each group. Asterisk (*) means that the coefficients of pulmonary tissues from high-dose (50 mg/kg)-exposed groups were significantly higher than other groups after exposure with 5 nm TiO_2 particles ($P < 0.05$).

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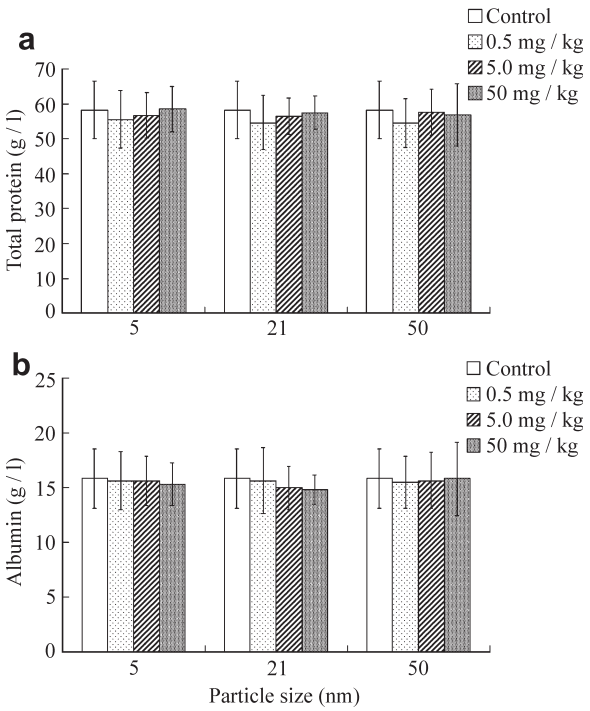

Fig. 2. Total protein (a) and albumin level (b) in plasma from rats exposed to TiO_2 nanoparticles and the corresponding controls at one week post-exposure. Sprague–Dawley rats (6 males, 6 females) were in each group. There was no significant difference in the two parameters among all treatment groups and control rats.

found in pulmonary ACP activity in all the experimental groups and control group.

The influence of particle size on the target organ showed that when the exposure dose was 50 mg/kg, 5 and 21 nm TiO_2 primary particles could induce increased activity of LDH and ALP, whereas 50 nm TiO_2 primary particles induced the same level of activity of LDH and ALP as control rats. When the exposure dose was 0.5 mg/kg, the activity of LDH and ALP enzymes from lung tissues after treatment with 50 nm TiO_2 primary particles were significantly higher than that after treatment with 5 and 21 nm TiO_2 primary particles.

3.4. Histopathologic evaluation

Histopathologic evaluation of lung tissues showed no difference in pathologic lesions between TiO_2 treatment groups. In low-dose and moderate-dose TiO_2 groups, slight inflammation was present and aggregations of lymphocytes and macrophages were revealed. Thickening of the alveolar wall, collapse of terminal bronchioles, and interstitial thickening were observed in some rats exposed to high-dose TiO_2 nanoparticles. Fig. 4a shows the representative normal architecture of lung tissue sections of controls, including the terminal bronchiole, and the corresponding alveolar duct and alveolus. Fig. 4b shows the representative infiltration of inflammatory cells and alveolar wall thickening from lung tissues exposed to low-dose TiO_2 particles.

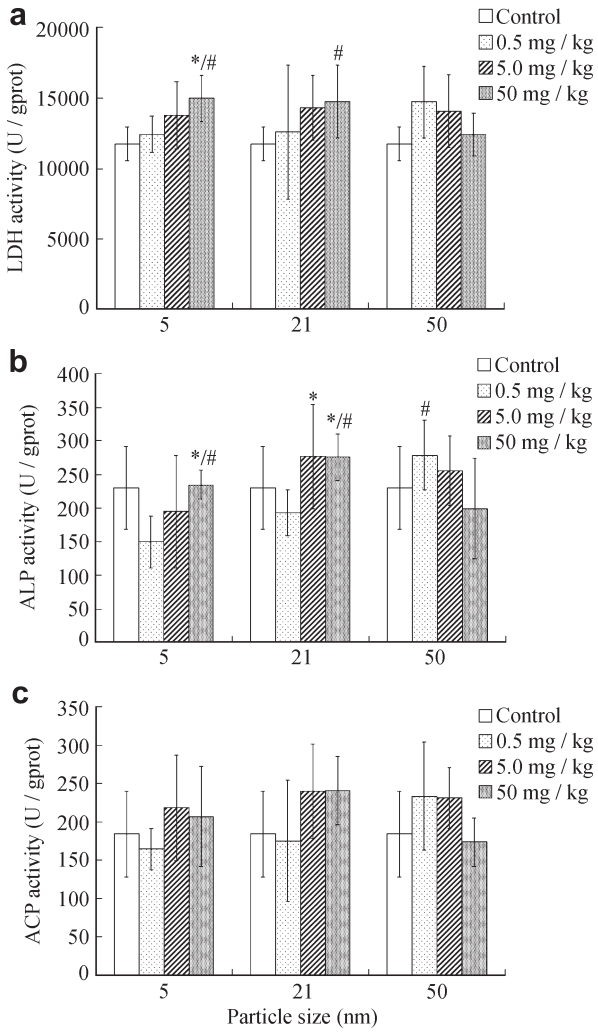

Fig. 3. Activity of LDH (a), ALP (b), and ACP (c) in pulmonary tissues of rats exposed to TiO_2 nanoparticles and the corresponding controls at one week post-exposure. Sprague–Dawley rats (6 males, 6 females) were in each group. Asterisk (*) represents the significant difference of relevant parameters among TiO_2 treatment groups with the different dose exposures of same-size particles and control rats ($P < 0.05$); pound (#) represents the significant difference of relevant parameters among TiO_2 treatment groups with the same dose exposure of the different size particles ($P < 0.05$).

Fig. 4c shows the representative interstitial thickening and terminal bronchiole collapse from lung tissues exposed to high-dose TiO_2 particles.

These results suggested that the pulmonary response to exposure to TiO_2 particles in rats manifests as inflammatory lesions in a dose-dependent fashion. This consisted mainly of cell infiltration and interstitial thickening. The latter implicated persistent and progressive lung inflammatory responses, and the possibility of chronic lung injury.

3.5. Uptake of neutral red dye and phagocytotic ability of AMs

Analysis of uptake of neutral red dye showed different effects on the phagocytotic ability of AMs after *in vivo*

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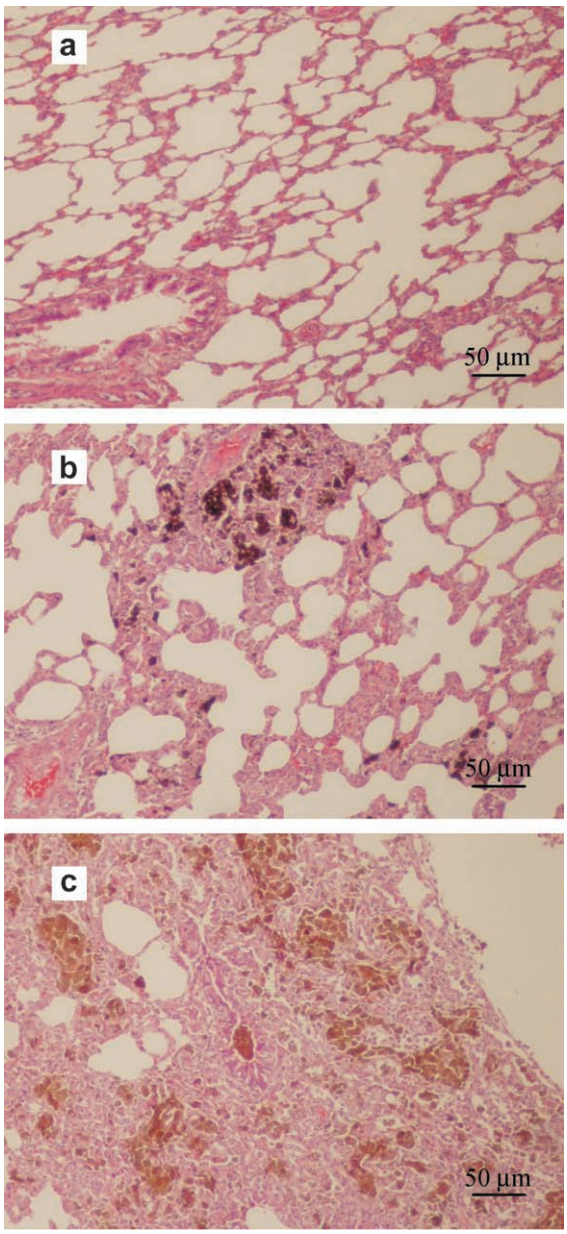

Fig. 4. Light micrographs of lung tissue from a solvent-control rat one week after instillation (a), a rat exposed to 5 nm TiO_2 particles (0.5 mg/kg) one week post-instillation (b), and a rat exposed to 5 nm TiO_2 particles (50 mg/kg) one week post-instillation (c).

exposure to 5, 21, and 50 nm TiO_2 (Fig. 5). A significant difference in AM phagocytotic ability was observed between all dose groups with an exposure of 50 nm TiO_2 and control group ($P < 0.05$). Phagocytotic ability of AM increased according to the exposure dose of TiO_2 particles. Moderate-dose exposure of 5 and 21 nm TiO_2 induced the highest phagocytotic ability of AMs ($P < 0.05$) and phagocytotic ability of AMs decreased after high-dose exposure of 5 and 21 nm TiO_2 particles.

Analysis of the size of particles influencing AM phagocytosis showed that, with low-dose and moderate-dose exposure, 21 nm TiO_2 primary particles could induce higher phagocytotic ability of AMs than 5 and 50 nm

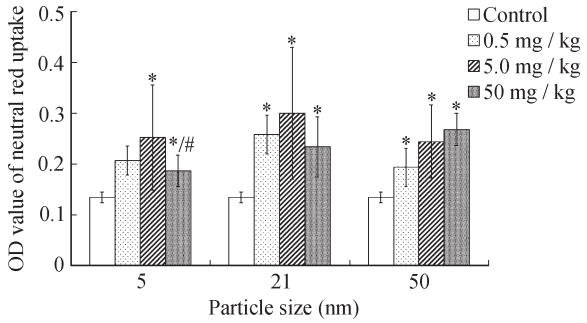

Fig. 5. Uptake of neutral red dye in mouse AMs from rats exposed to TiO_2 nanoparticles and the corresponding controls at one week post-exposure. Sprague–Dawley rats (6 males, 6 females) were in each group. Asterisk (*) represents the significant difference of relevant parameter among TiO_2 treatment groups with the different dose exposure of the same-size particles and control rats ($P < 0.05$); pound (#) represents the significant difference of relevant parameter between 5 nm and 50 nm TiO_2 treatment groups with an exposure dose of 50 mg/kg ($P < 0.05$).

TiO_2. When the exposure dose was 50 mg/kg, phagocytotic ability of AMs induced by 50 nm TiO_2 primary particles was the strongest, followed by 21 nm TiO_2, whereas phagocytotic ability of AMs with the exposure of 5 nm TiO_2 was rapidly decreased. Phagocytotic ability of AMs showed a size-dependent reduction with exposure to 50 mg/kg TiO_2, suggesting that small-size TiO_2 primary particles may suppress the phagocytotic ability of AMs.

4. Discussion

TiO_2 is considered a non-toxic compound, and its LD_{50} is >10,000 g/kg. TiO_2 nanoparticles are different from conventional TiO_2 particles with respect to their toxicological mechanism, which may cause a difference in toxicity. Some studies demonstrated that ultrafine particles exposed to the respiratory tract could induce enhanced pulmonary inflammation and particle translocation effects compared with fine particles [23,25,26]. Warheit et al. [14,15] studied the responses of inflammatory cells or injured cells in fine-TiO_2 particles, nano-TiO_2 dots and nano-TiO_2 rods, and demonstrated no significant differences between ultrafine- or nano-TiO_2 particles and pigment-sized particles of similar chemical composition. This contradiction may be attributed to inconsistent specific surface area, chemical composition, agglomeration of particles, and species specificity in these studies. The present study was done to assess pulmonary toxicity in response to intra-tracheally instilled TiO_2 primary nanoparticles of varying sizes dispersed with 0.15% NaCl.

We used rats to construct the TiO_2 exposure model because rats were considered likely to develop lung inflammatory responses after TiO_2 exposure. Bermudez et al. [11] found that there were significant inter-species differences in pulmonary responses after exposure to TiO_2 particles. The analysis of pulmonary parameters, including particle clearance from lungs, inflammation, proliferation of lung epithelial cells, and histopathologic alterations, showed that

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rats developed a more severe pulmonary inflammatory response than mice or hamsters.

In an aqueous suspension, fine particles are likely to form agglomerates driven by particle surface force. Ultrasonic vibration could disrupt Coulomb forces and van der Waals forces among these agglomerates so that the latter are dispersed *de novo*, but agglomerates may form again once ultrasonic stops. The usual technique involves a dispersant to promote a stable and uniform dispersed system. The main effect of a dispersant is to enhance the hydrophilicity of particles. Ionized dispersants are absorbed onto the surface of particles and generate a double-layer structure with surface charges of particles. With an increasing concentration of dispersant, the repulsion among particles becomes great, which disperses particles. Whereas, if the dispersant concentration is more than a certain threshold, the potential of double-layer structure with surface charges of particles begin to descend, which could cause particle agglomeration. PBS and saline are the common diluent solutions *in vivo*, so the dispersal effects of TiO_2 nanoparticles were analyzed with the two solutions and ultrapure water. Severe agglomeration of TiO_2 nanoparticles in these solvents was observed. An aqueous solution buffered with 0.15% NaCl solution to TiO_2 maintained better dispersion of particles. In conjunction with ultrasonic vibration for 15 min, the system of TiO_2 was homogeneous colloidal, and no significant deposition was observed for 2 h at room temperature. The agglomeration and de-agglomeration of TiO_2 nanoparticles in the present dispersal system led to the complex constitution of TiO_2 particles *in vivo*. The toxicological effects of TiO_2 nanoparticles should therefore include the combined influence of all sizes of particles in a dispersal system on target tissues.

Recent studies on TiO_2 nanoparticles showed that they usually produced only transient inflammatory effects and recovered after a short-term exposure [27]. Even with subacute inhalation of TiO_2 nanoparticles, the inflammatory response was sustained for only 2–3 weeks [25]. Our results showed that different doses and sizes of TiO_2 nanoparticles induced various severities of lung toxicity. The activity of LDH and ALP began to increase after exposure of 5 nm TiO_2 particles at 5 mg/kg. The changes became significant, accompanied with an increased coefficient of lung tissues and fibrosis, after exposure with 5 nm TiO_2 particles at 50 mg/kg. This indicated that when the exposure dose with 5 nm TiO_2 particles was >5 mg/kg, treated rats began to develop lung lesions, represented as type-II cell membrane injury, leakage of cytoplasmic contents, and compensatory proliferation and fibrosis of lung tissues. The results suggested that 5 nm TiO_2 particles instilled intra-tracheally across alveolar epithelial cells through interspaces on the membrane and the interaction with membrane proteins during this process may break the integrity of type-II cell membrane, leading to an increased ALP activity in lung tissues. Breakage of the cytomembrane may cause extravasation of cytoplasmic contents and the release of LDH into extracellular fluid. Lung tissues of rats showed increased ALP activity only with an exposure dose of >5 mg/kg with 21 nm TiO_2 particles; 21 nm TiO_2 particles could cause type-II cell membrane lesions, but membrane integrity was not damaged. Among groups treated with 50 nm TiO_2 particles, indicators reflecting lung injury showed a decreasing trend with increasing dosage. Compared with controls, activities of ALP, ACP, and LDH from group at 50 mg/kg were consistent, whereas those of 5.0 and 0.5 mg/kg gradually increased.

Lung injury induced by relatively small-size particles (5 and 21 nm) and large-size particles (50 nm) was different to that caused by exposure to high-dose (50 mg/kg) and low-dose (0.5 mg/kg) TiO_2. When exposure dose was 50 mg/kg, 5 and 21 nm TiO_2 primary particles could induce lung lesions, but 50 nm TiO_2 primary particles could not. When exposure dose was 0.5 mg/kg, lung lesions treated with 50 nm TiO_2 primary particles were significantly more severe than those treated with 5 and 21 nm TiO_2 primary particles. We conclude that low doses of small-size nanoparticles could be transported to other organs through the circulation which, to a certain extent, may reduce the burden to lung tissue. Due to their large size, 50 nm TiO_2 particles cannot enter the circulation through pulmonary alveoli, but they can deposit in the alveolar wall, which promotes damage to lung tissue. ALP activity of low-dose exposure with 5 and 21 nm TiO_2 was lower than that of the control group. ALP activity is used as an indicator of the secretory activity of type-II alveolar epithelial cells. Our results suggested that (1) low-dose exposure with 5 and 21 nm TiO_2 may stabilize the cell membrane, decreasing the release of ALP in the type-II cell membrane; and (2) 5 and 21 nm TiO_2 particles may inhibit the activity of type-II alveolar epithelial cells and reduce ALP secretion.

A non-specific immune response results when xenobiotics enter the body. Macrophages are activated and clear the threat through phagocytosis. Renwick et al. found that ultrafine particles impair AM phagocytotic ability to a greater extent than fine particles [13]. The effects on the phagocytotic ability of AMs after exposure to different sizes of TiO_2 nanoparticles were investigated in our study. TiO_2 exposure activated the phagocytotic ability of AMs, and the effects on the phagocytotic ability of AMs were different with exposure to different sizes of TiO_2. The phagocytotic ability of AM after exposure with 5 nm TiO_2 was significantly reduced compared with that with 50 nm TiO_2. This suggested that exposure to small-size TiO_2 primary particles suppressed the phagocytotic ability of AMs.

5. Conclusions

This study suggested that particle size and exposure dose of TiO_2 nanoparticles have an important impact on pulmonary toxicity. The toxic effect of TiO_2 nanoparticles to lung tissue exhibited a threshold dose–response relationship. Exposure with >5.0 mg/kg TiO_2 particles, 5 and 21 nm TiO_2 can induce pulmonary lesions; 5 nm TiO_2 primary particles may suppress the phagocytotic ability of

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AMs if the exposure dose is $\geqslant 50$ mg/kg. Pulmonary toxicity caused by 5 nm TiO_2 particles was more severe than that caused by 21 and 50 nm TiO_2 particles. The characteristics of pulmonary toxicity may be related to the physicochemical properties of TiO_2 nanoparticles. The smaller the size of the nanoparticle, the greater the specific surface area, and the stronger the reaction activity. These characteristics can reduce the stability of the cell membrane, and cause cell injury. It can also influence the activity and function of cells through interaction with macromolecules. It is consistent with the hypothesis that different sizes of nanoparticles have the same specific surface area–response (effect) curve [4,11].

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