Pathological Study of the Lung of Rabbits Exposed to Suspension of Different Crystalline Nano-titanium Dioxide

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Abstract: Objective. *To compare the pathology changes of rabbit lung tissue contaminated by nano and micro TiO_2 suspension liquid in different crystal forms.*

Methods. *Each rabbit according to 2.5 $ml \cdot kg^{-1} \cdot bw^{-1}$ dose, one-time non-exposed pipe drip dye, and put them to death after 21 days. Lung was taken with paraffin embedding, tissue section and hematoxylin and eosin (HE) staining for observation.*

Results. *In anatase TiO_2 nanoparticles group, white single lung nodules could be seen on the surface of the lung. Microscopically, in the center of the nodules, the high density inflammatory lesion is given priority to lymphocytes aggregation. In macrophages, anatase TiO_2 nanoparticles were shown brown and granule, which were deposited in pulmonary interstitial. Alveolar septum was widening with edema of alveolar epithelial cells. In rutile TiO_2 nanoparticles group, alveolar epithelial cell was swelling and fell off. Alveolar space was narrow, where a large number of nano rutile TiO_2 macrophages and neutrophils infiltrations could be seen. Rutile TiO_2 nanoparticles could also be seen deposited on the pulmonary interstitial in abundance. Alveolar septum was widening, with fibrosis of chronic inflammation cells and fibroblasts. No significant difference was between the micron rutile and anatase TiO_2 groups, while the alveolar structures were clear, with narrow alveolar space, widen alveolar septum, visible fibrosis, angiotelectasis hyperemia, and inflammatory lesion with lymphocytic infiltration predominantly in small bronchial submucosa. Micro rutile and anatase TiO_2 were deposited in pulmonary interstitial and devoured by macrophages. The macrophages with foreign body were in black. But no obvious pathological changes were seen in the control group. Conclusion: Both of the TiO_2 could lead to the damage to alveolar structure at the experimental doses. For two kinds of crystal types, contamination with nano TiO_2 was more serious than micron TiO_2, while anatase TiO_2 could result in pulmonary nodules.*

Keywords: *Pathology, Nanometer rutile TiO_2, Nanometer anatase TiO_2, Nodules.*

Introduction

Micro titanium dioxide is often considered as low toxic dust. In various toxicology studies on dust, titanium dioxide (TiO_2) is often used as the control of inert dust. However, nano TiO_2 maybe has a potential hazard. The biological effect of nano TiO_2 has a close relationship with its ultra features, UV absorbency and efficient photocatalytic activity. In vivo and in vitro studies have found that nano TiO_2 has a greater damage to the lungs than the micro TiO_2 dose,

which may be associated with the large specific surface area of nano materials. Different crystal types and sizes are both likely to affect its toxicity [12, 14, 15, 22].

Nano titanium dioxide has a wide contact with human body. Nano titanium dioxide of different particle sizes may enter into the human body in a variety of ways, so it is extremely urgent to study its toxicity. Once the nano titanium dioxide enters into the body, it can lead to organ injury at different levels. Generally, contact inhalation is the major way for particles to enter into the body. A number of studies have shown that for rats exposed to nano TiO_2 particles, with the extension of time, the scavenging capacity of pulmonary macrophage is decreased, while more TiO_2 particles are detained in the lungs, and gradually invade the interstitial tissue and peripheral lymph node, thus causing the alveolar epithelial damage and inflammations such as and hyperplasia, etc. [7]. Bermudez et al. [3] gave intratracheal instillation of nano TiO_2 particles to rats. They observed inflammations including hyperplasia and increased permeability of rat alveolar epithelial cells, fibrosis of the alveolar septum, etc. And macrophages and neutrophils are also increased significantly in the bronchoalveolar lavage fluid (BALF). Nano TiO_2 particles can also cause hyperplasia of alveolar type II cells and epithelial cell apoptosis, and finally leads to pulmonary fibrosis, pulmonary emphysema-like damage, even acute inflammation and cell damage effect within 24 h [20]. Chen et al. [5] gave intratracheal instillation of nano TiO_2 (0.1 mg, 0.5 mg, 19~21 nm). They found apparent emphysema, macrophage accumulation and widespread destruction of the alveolar membrane, etc. Chronic exposure in high dose of TiO_2 can lead to lung cancer, while subchronic exposure may cause lung overload and other inflammatory change [6].

Some researches have shown that nano TiO_2 in different crystal forms have different effects on the organisms. Sayes et al. [19] infected human lung epithelial cells. The results showed that toxicity of anatase type (SSA = 153 m^2/g) is larger than that of rutile type (SSA = 123 m^2/g) by two magnitudes. Warheit et al. [21] gave intratracheal instillation of nano TiO_2 with different crystal forms to SD rats. Results showed that for lung inflammation of SD rats, cell toxicity, alveolar epithelial cell proliferation and histopathologic response, anatase/rutile type has greater influence than the rutile type. They think that photocatalytic activity and the nature of different crystal type lead to different toxicities. Jiang et al. [11] used fluorochrome to determine the reactive oxygen species generated from nano TiO_2 in amorphous, anatase, rutile and anatase type/rutile type, so as to determine the oxidative damage. Results showed that nano TiO_2 particle (3 nm, amorphous type) caused more serious damage than nano TiO_2 particle (4 nm, anatase type). The conclusion of nano TiO_2 particle larger than 30 nm was consistent with the nano TiO_2 particle of the smaller size, and amorphous type led to greater oxidative damage than the anatase type. In the same way, TiO_2 particle of 40 nm and 50 nm were used. And the results showed that the abilities to cause oxidative damage from strong to weak are as the following order of the anatase type, anatase/rutile, and rutile type. They thought the reason of higher oxide toxicity of anatase type than the rutile type might be due to the photosensitivity and surface chemical properties. Anatase type has stronger photosensitivity than the rutile type [17]. In addition, the surface chemical properties of anatase type makes it easy to absorb O_2^-, O^- and free water molecules [8], while rutile type is easy to absorb free water molecules [2], which makes the anatase type more likely to cause oxidative damage [17].

Sarcoidosis is a systemic granulomatous disease of unknown etiology, with lymph nodes around the hilum of the lung and pulmonary interstitial as the most common sites, which can involve multiple extrapulmonary systems and organs, such as skin, eye, spleen and heart, etc. It is characterized as non-caseous epithelioid cell granulomas under microscopy.

Many hypotheses have been put forward for the causes of sarcoidosis, such as bacteria, viruses and treponemal infection, allergy, genetic factors, inhalation of dust components such as pollen and metal dust, etc. [4, 9, 13, 16, 18], but no recognized evidence has been found yet. No report has yet been seen over the rabbit pulmonary sarcoid granulomas caused by nano TiO_2, neither comparison on lung injury by nano TiO_2 with two kinds of crystal types. Therefore, this research intends to adopt micro and nano TiO_2 with two kinds of crystal types. Non-exposure intratracheal instillation is intended for infection, and the pathological changes in morphology are observed. By comparing lung injuries caused by nano TiO_2 in two kinds of crystal types and by micro TiO_2 in two kinds of crystal types, so as to lay an experiment basis for studying the potential effects on the human body.

Materials and methods

Material sources and preparation of powder suspension

Finely milled micro titanium dioxide powder (45 micrometer, anatase type and rutile type) with untreated surface, and nano titanium dioxide powder (rutile type) with untreated surface were purchased from Anhui Kena New Materials Co., Ltd. Primary particle size was ≤ 30 nm, specific surface area was ≥ 80 (m^2/g), mass fraction of nano TiO_2 was ≥ 99.9%. Nano titanium dioxide powder (anatase type) with untreated surface was purchased from Hangzhou Wanjing New Materials Co., Ltd. Primary particle size was ≤ 30 nm, specific surface area was ≥ 80 (m^2/g), mass fraction of nano TiO_2 was ≥ 99.9%. All kinds of TiO_2 are sterilized with high pressure, and made into suspension of 20 mg/ml with physiological saline, and then were sterilized under high temperature.

Experimental animals

Thirty rabbits (body weight 1.3÷2.5 kg) were provided by Experimental Animal Center of Xinjiang Medical University. After weighted, the rabbits were divided into 5 groups subsequently according to the digital method, namely saline control group (SLDZ group), micro titanium dioxide in rutile type (wj-TiO_2 group), micro titanium dioxide in anatase type (wr-TiO_2 group), nano titanium dioxide in rutile type (nj-TiO_2 group), and nano titanium dioxide in anatase type (nr-TiO_2 group), six in each group. Feeding environment: T = 10°C, illumination time 9:00-16:00 h. Free-access to forage and drinking water was allowed. The rabbits were fed with complete particle feed.

Infection

Four kinds of TiO_2 powder were made into suspension of 20 mg/ml with physiological saline respectively. Each rabbit was infected by non-exposure intratracheal instillation at 2.5 $ml \cdot kg^{-1} \cdot bw^{-1}$ (treatment group: perfusion suspension through respiratory tract once; the control group: physiological saline). Under the same conditions, rabbits in each group were fed for 20 days. The rabbits were given normal diet and drinking during the period [1].

Pathological morphological observation: the rats were executed and the lung tissues were taken. After routine paraffin section, the samples were observed under microscopy after hematoxylin and eosin (HE) staining.

Results

Gross specimen observation

21 days after the infection, rat lungs in saline control group were light pink with smooth surface, and no abnormal changes were seen. In micro TiO_2 group and nano TiO_2 group,

white lesions were distributed on the surface of the rabbit lungs. Among them, one nodule was observed in the right lung in nr-TiO_2 group (Fig. 2E).

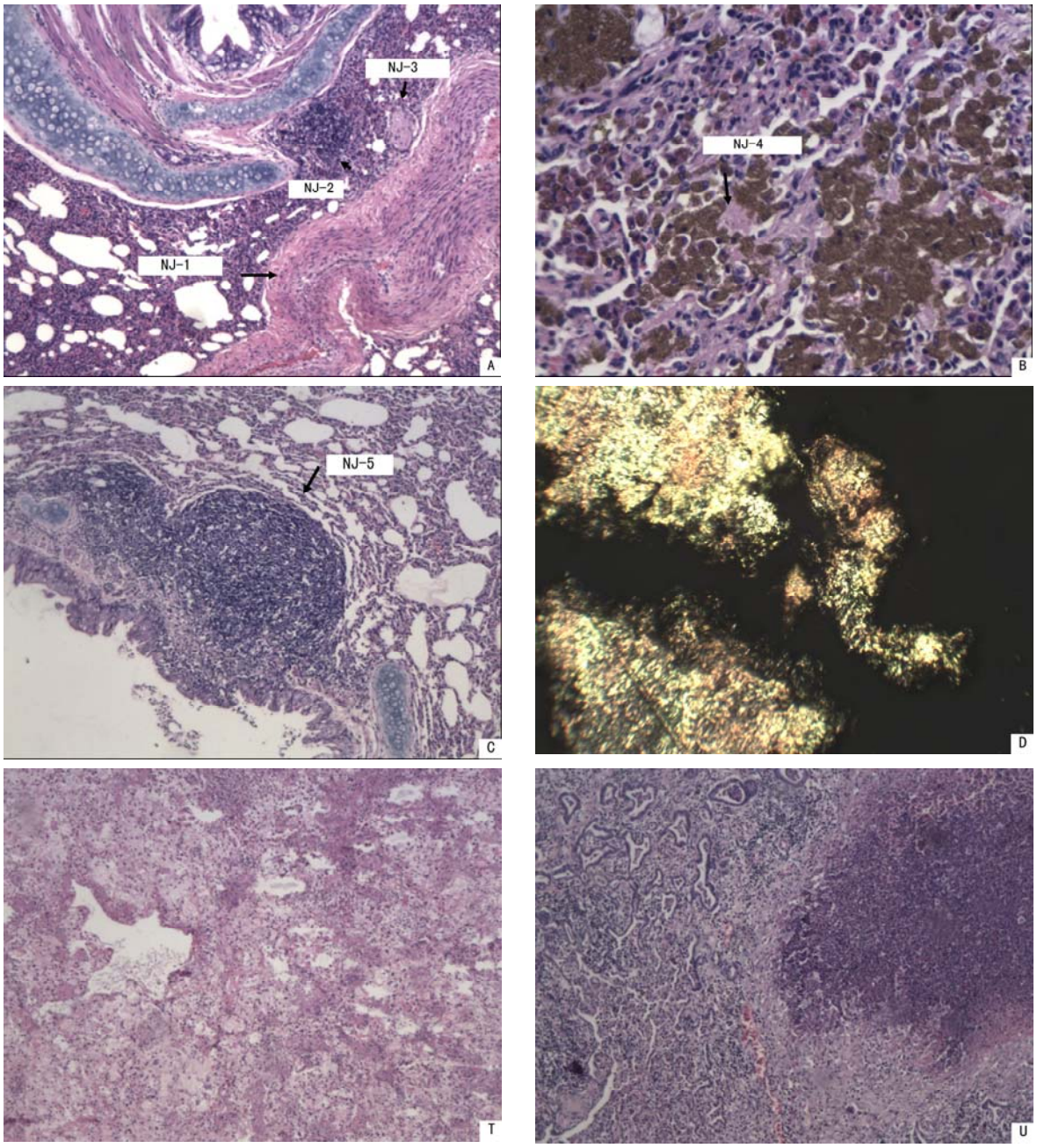

Fig. 1 A, B, and C sections – nano TiO_2 rutile type group (HE);
D section – nano TiO_2 rutile type group. Under orthogonal polarized microscope, nano TiO_2 rutile in the rabbit pulmonary tissue was reddish with white light (×100)
A(NJ-1) – pulmonary arterial stenosis; A(NJ-2) – inflammatory lesion; A(NJ-3) – alveolar edema; B(NJ-4) – collagen fiber cohesion; C(NJ-5) – inflammatory lesion;
T section – necrotic tissue; U section – there is necrotic tissue.

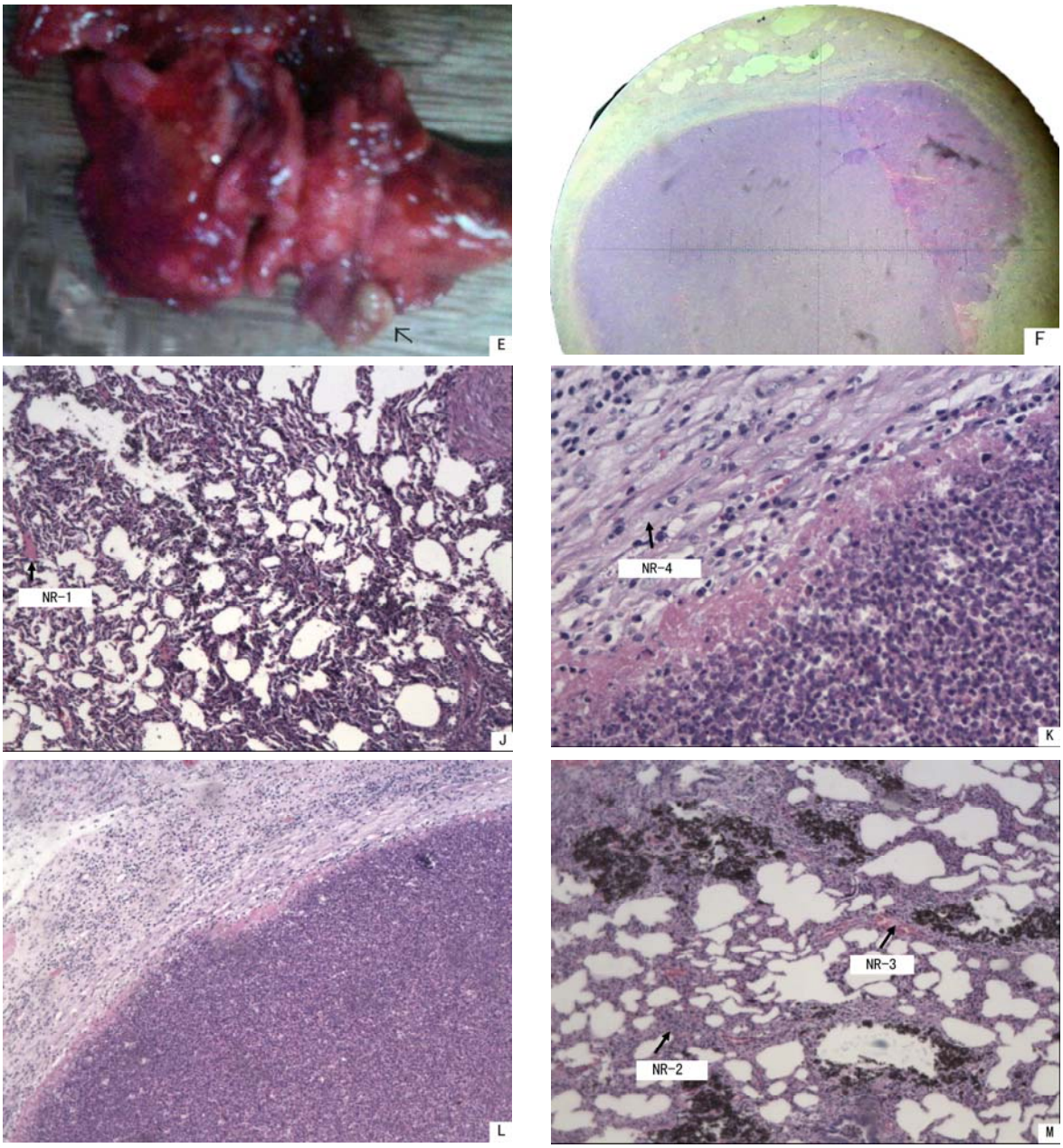

Fig. 2 E, F, J, K, L, and M sections – nano TiO_2 anatase type group (HE).
E – view, a gray nodule was in the lower right corner with medium quality and a size of 9mm×7mm×7mm figure arrow; F – pulmonary nodules (×100); J(NR-1) – alveolar edema; K(NR-4) – collagen fibers (×400); L – pulmonary nodules (×200); M(NR-2) – widened alveolar septa (×200); M(NR-3) – blood vessels expansion (×200).

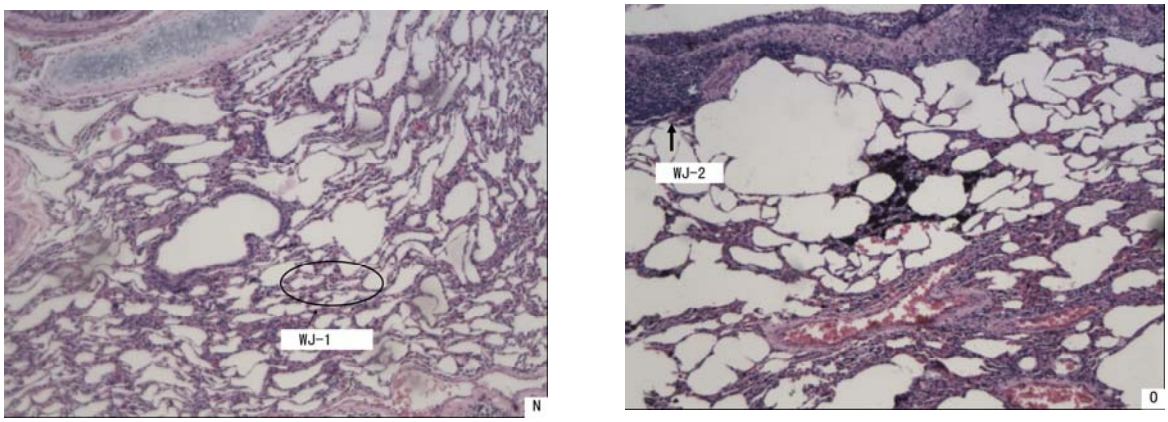

Fig. 3 N and O sections – micro TiO_2 rutile type group (HE)
N(WJ-1) – narrowed alveolar cavity (×200); O(WJ-2) – inflammatory lesion (×400).

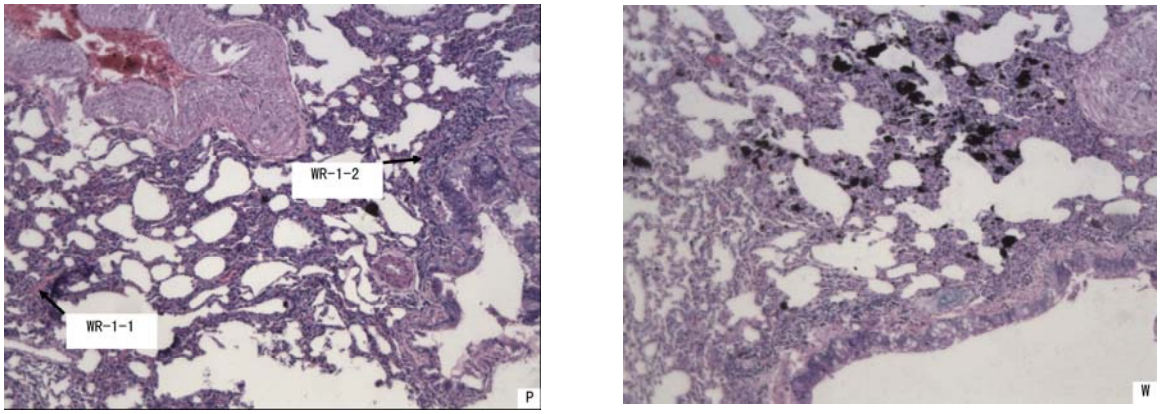

Fig. 4 P and W sections – micro TiO_2 anatase type group (HE)
P(WR-1-1) – widened septa, vasodilation;
P(WR-1-2) – fibrosis associated with lymphocytic infiltrates (×200);
W – dark and brown granules for micro TiO_2 anatase type (×200).

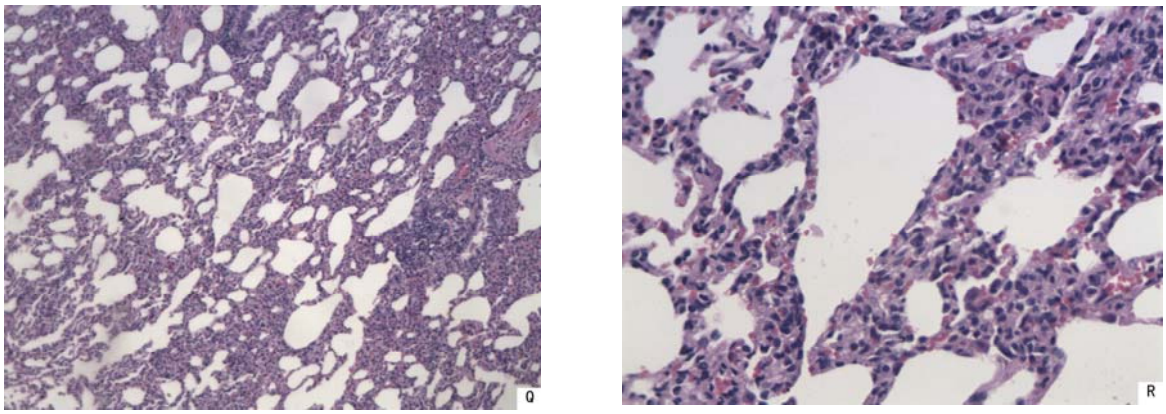

Fig. 5 Q and R sections – physiological saline control group (HE)
Q (×200); R (×400).

Observation under microscopy after HE staining

nj-TiO_2 group

Alveolar epithelial cell were swelled and fell off. The alveolar cavity was decreased. A large number of nano TiO_2 rutile macrophages and neutrophils infiltrations can be seen in the alveolar cavities. Nano TiO_2 rutile macrophages are also deposited on the pulmonary interstitial (Fig. 1B). The alveolar cavity was widened, chronic inflammation cells and fibroblasts are visible. Fibrosis was appeared. The capillaries were expanded and congested. Increases of the intimal-medial thickness of the pulmonary artery caused pulmonary artery stenosis. It was difficult to identify the alveolar structures in severe cases. The alveolar epithelial cells were swelled and fell off. Eosinophils, neutrophils, lymphocytes, plasma cells, and brownish nano TiO_2 rutile macrophages were inside (Fig. 1B). Inflammatory lesions given priory to lymphocytic infiltration could also be seen in bronchial submucosa. Alveolar edema could be seen occasionally. Consolidation fibrosis and necrotic lung tissue existed (Fig. 1U).

nr-TiO_2 group

The contents of the Nano TiO_2 anatase macrophages were brown granular, which deposited in pulmonary interstitial. The alveolar septa were widened, accompanied with a large number of lymphocyte infiltration. There were capillary expansion and hyperemia, and edema of alveolar epithelial cells. Alveolar edema can be seen occasionally.

Also there was a white single nodule with slightly protruding on the surface of the left lung in the rabbit. The nodule was circular nodule with clear boundary under the microscopy. Obvious leaflets could be seen within the nodule. In the center of the nodule, there was a high density inflammatory lesion given priory to the lymphocytes. The edge was coated with collagen fiber, and fibroblast cells, lymphocytes, and small blood vessels could be seen. Around the nodules, diffuse inflammatory cell infiltration which is mainly composed of lymphocytes was also seen (Fig. 2L).

wj-TiO_2 group

The alveolar structure was clearly visible. Alveolar cavity was decreased, while alveolar septa were widened. Fibrosis was visible. There were capillary expansion and hyperemia, and inflammatory lesion mainly composed of lymphocyte infiltrations was seen bronchial submucosa. Micro TiO_2 rutile was scattered and deposited in the pulmonary interstitum, and then swallowed by the macrophages, which was black.

wr-TiO_2 group

The alveolar structure was clearly visible. Alveolar septa was widened, fibrosis was seen associated with lymphocytic infiltration, and capillary expansion and hyperemia. The foreign bodies were scattered and deposited in the pulmonary interstitum, and then swallowed by the macrophages, which was black with density in cytoplast.

SLDZ group

YS21: No obvious lesions. Bronchial morphology at all levels was clear, and the structures of alveoli and alveolar septum were normal.

Discussion

Histopathological results in this study showed that, the rabbits were infected with two crystal types of micro and nano TiO_2 of 2.5 $ml \cdot kg^{-1} \cdot bw^{-1}$ once by non-exposure intratracheal instillation. After 21 days, inflammations were observed, including capillary expansion in

rabbit lungs of micro and nano TiO_2 model group, and large amount of TiO_2 particles deposition. It is speculated that these performances may be caused by the stimulation of particles on the lungs. Nano TiO_2 group appeared obvious damage: alveolar septa were widened, accompanied by a large number of lymphocyte infiltration. Capillary expansion hyperemia and edema of alveolar epithelial cells were seen, and occasionally there was alveolar edema. There was also consolidation fibrosis. In nano TiO_2 rutile group, there were necrotic lung and pulmonary artery stenosis. The alveolar structure was difficult to recognize. Pulmonary nodules were also found in nano TiO_2 anatase group. Sarcoidosis is a systemic granulomatous disease of unknown etiology, with lymph nodes around the hilum of the lung and pulmonary interstitial as the most common sites, which can involve multiple extrapulmonary systems and organs, such as skin, eye, spleen and heart, etc. It is characterized as non-caseous epithelioid cell granulomas under microscopy. Many hypotheses have been put forward for the causes of sarcoidosis, such as bacteria, viruses and treponemal infection, allergy, genetic factors, inhalation of dust components such as pollen and metal dust, etc. [4, 9, 13, 16, 18], but no recognized evidence has been found yet. This experiment suggested inhalation with nano TiO_2 anatase particles may be associated with sarcoidosis.

Such changes were not seen in the control group and micro TiO_2 model group. Nano TiO_2 with two kinds of crystal types has obviously greater injury to rabbit lungs than the micro TiO_2 of two crystal types. In makes us recognize and understand the absorption process of particulate matter in human body and its biological effects. When the particle size is decreased to nanometer range, although the chemical composition has not changed, the surface binding force and chemical activity of the particles will be increased significantly, and its nature and strength of the biological effect on the body is likely to be changed. In addition, due to the small size, nanoparticles can enter into the cells and organelles through the pores on biological membrane or by cell endocytosis. Thus it can interact with biological macromolecules in the cell, and damage the normal space structure of biofilm and biological macromolecules, etc. [5, 20]. Under the nanoscale, with the reduction of the particle size, the specific surface was increased rapidly, when the atoms distributed within the lattice were reduced, while gathered at the particle surface were increased rapidly, which led to the production of discontinuous crystal plane with a large number of structure defects, thus greatly improving the overall reactivity particle surface [2]. Researchers believe that toxic effects showed in most of the nanometer materials were because that the electronic active site (electron donating or receiving group) on the surface of the material can react with oxygen to form a super oxygen anion (O_2^-) and further produce reactive oxygen free radicals (ROS) by disproportionation reaction. In addition, high reactivity produced by huge specific surface area of nano materials also brings a certain degree of challenge to the traditional toxicological safety evaluation. On the one hand, unit dose selection of the nano materials for in vivo and in vitro experiments has become a focus. More and more studies have demonstrated that compared with mass concentration, the trend of the toxic effects of nanoparticles is more related to its surface area. With the same chemical composition under the unit quality, nano particles have significantly larger toxic effects than the micro particles. If converting the unit dose into surface area, however, it will be found that the surface area of nano particles is much larger than the micro particles. Therefore, when evaluating toxicity of nano-materials, the selection of unit dose still needs to be further cleared. This experiment also shows that the chemicals with the same chemical composition but different crystalline structures show different biological effects. The toxicity mechanism research of nanometer titanium dioxide is still at the initial stage at home and abroad. Because the toxicology research time is shorter, many problems have not yet been determined, such as the influences of nanometer titanium dioxide with different crystal shape, particle size or different infection means on the body.

In the future, various standards of nanometer material should be developed to establish the evaluation system [10].

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