

Systematic Influence Induced by 3 nm Titanium Dioxide Following Intratracheal Instillation of Mice

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This work reported the systematic influence of titanium dioxide nanoparticles (TiO_2 NPs) with a diameter of 3 nm on mice. Mice were repeated intratracheally instilled with TiO_2 NPs, once per-week for 4 consecutive weeks, at total dose of 13.2 mg/kg. At 28 days post-instillation, the biochemical parameters in bronchoalveolar lavage fluid (BALF) and brain homogenate as well as histopathologic changes of tissues were examined to describe the subacute toxicity of instilled TiO_2 NPs. The results showed that instilled TiO_2 NPs could induce lung damage, and change the permeability of alveolar-capillary barrier. The TiO_2 NPs were able to get access to blood circulation and reach extrapulmonary tissues, then lead to injury at the different level, such as liver and kidney. Our results also indicated that TiO_2 NPs might pass through the blood–brain barrier (BBB), and induce the brain injury through oxidative stress response.

Keywords: Titanium Dioxide, Intratracheal Instillation, Systematic Influence.

1. INTRODUCTION

Due to its outstanding photocatalysis and high stability, TiO_2 is widely used in many fields, including coatings, catalysis, lotions, environmental applications, and so on. These wide usages made people have more opportunity to exposure to this material, and made it necessary to study its health implications. Generally, it is believed that TiO_2 fine particles (>100 nm in diameter) wouldn't cause significant adverse effects on human and animals.^{1–3} However, when its size decreased to a few or a few tens of nanometers, things were changed.⁴ The small size and large surface area not only endow TiO_2 NPs with unique physical and chemical properties, but also result in potential impact on human health and the environment. Oberdörster et al. reported that the level of lung inflammation in rats was associated with the size of TiO_2 NPs, and the ultrafine TiO_2 (20 nm) elicited a significantly greater inflammatory than larger size TiO_2 (250 nm).⁵ To date, most studies on biological effects of TiO_2 NPs focused on the toxicity of respiratory tract, and the results showed that inhaled or instilled TiO_2 NPs could lead to

lung toxicity and epithelium inflammation^{6–11} as well as tumorigenesis.¹²

On the other side, the inhaled NPs might enter the blood circulation, then reach extrapulmonary tissues.¹³ Several studies supported this hypothesis. Nemmar et al. reported that $^{99\text{m}}\text{TcO}_4^-$ labeled albumin particles (80 nm) and carbon particles (100 nm) could penetrate alveolus-capillary barrier after inhaled and instilled respectively.^{14–15} Xu et al. also found that $^{99\text{m}}\text{Tc}$ labeled $\text{C}_{60}(\text{OH})_x$ was able to pass through alveolus-capillary barrier and entered blood circulation quickly.¹⁶ In addition, Wang et al. reported that inhaled TiO_2 NPs can be translocated to olfactory bulb through the olfactory nerve system and elicited neurotoxicity.¹⁷

Therefore, it is necessary to investigate the systematic influence for scientific and full evaluation of biological effects of NPs. But till now, few studies investigated or assessed the systemic toxicity after TiO_2 NPs were inhaled or instilled, and which is essential and helpful for further understand of the potential hazard of NPs on human health. In this study, we focused on the systematic influence of 3 nm TiO_2 NPs after repeated intratracheal instillation. The changes of coefficients of tissues to body weight, biochemical parameters, histopathology, and distribution of TiO_2 NPs in tissues were assayed.

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2. MATERIALS AND METHODS

2.1. TiO₂ NPs

The synthesis and characterization of 3 nm TiO₂ NPs in this study were performed as described by Li's report.¹¹

2.2. Animals

The animals used in this work were 7-week-old male Kunming mice (The Slack Experimental Animal Center, Shanghai, China) which were held in clean cages by four per cage. The animal facility was maintained under a 12-h light–dark cycle at a temperature of 21–23 °C, and a relative humidity of 40–60%. All mice were supplied with sterilized food and water. They were acclimated for 7 days prior to instillation. The average animal weight was 28 g at the beginning of the study. The animal program is fully accredited by the national related regulations.

2.3. Intratracheal Instillation

The TiO₂ NPs, in stable colloidal state, were diluted into 1 mg/ml with Millipore ultrapure water. The suspension was ultrasonicated for 15 min before intratracheal instillation.

Thirty-nine mice were randomly divided into three groups (control group, Millipore water group, and TiO₂ group), thirteen in each group. All the mice were intraperitoneally anesthetized with 0.2 ml 0.5% pentobarbital sodium solution. Then the mice were intratracheally instilled with 0.1 ml Millipore ultrapure water and 0.1 ml TiO₂ NPs suspension, respectively. The procedure was repeated once a week for consecutive four weeks.

2.4. Evaluation of Biochemical Parameters in BALF

Five mice randomly chosen from each group were anaesthetized by intraperitoneal injection of 0.3 ml 0.5% pentobarbital sodium solution and sacrificed via the abdominal aorta at 28 days post-instillation. Bronchoalveolar lavage was performed on them by cannulating the trachea and lung lavaging two times with 2 ml physiological saline (37 °C). The BALF were collected and centrifuged (400 × g, 10 min, 4 °C), and biochemical parameters (total protein, alkaline phosphatase (ALP), acid phosphatase (ACP) and lactate dehydrogenase (LDH)) in supernatant were analyzed with commercial reagent kits which were obtained from Nanjing Jiancheng Bioengineering Institute.

2.5. Evaluation of Biochemical Parameters in Brain Homogenate

Five mice randomly selected from other eight mice in each group were anaesthetized with 0.3 ml 0.5% pentobarbital sodium solution. Brain lavage was performed as follow: the right atrium of mice was incised and lavage fluid

(37 °C) was injected into left ventricle until the lavage fluid effused from right atrium became colorless. The perfusion pressure was about 100 mmHg. After perfusion, brains were excised and homogenated. The brain homogenates were centrifuged (10000 × g, 10 min, 4 °C), and then superoxide anion, hydroxyl radicals, peroxide and Malondialdehyde (MDA) in supernatants were analyzed with reagent kits which were purchased from Nanjing Jiancheng Bioengineering Institute.

2.6. Histopathological Examination

The lungs, brains, livers, kidneys, and spleens of the remained mice in each group were excised and fixed with 4% formalin, then embedded in paraffin, sectioned coronally, and mounted on glass microscope slides. Sections were stained with hematoxylin-eosin and examined by light microscopy.

3. RESULTS AND DISCUSSION

Previous study indicated that exposure of TiO₂ NPs (15–25 nm) at low dose (e.g., 110 µg/m³) didn't lead to significant lung toxicity,¹³ whereas high dose (e.g., 250 mg/m³) resulted in lung over-loading.¹⁸ Pulmonary pathological changes emerged under high dose situation didn't accurately reflect the real toxicity of TiO₂. Drew et al. reported that single instillation of high dose (2 and 20 mg per rat) of glass fibers produced an artfactual glaucomatous lesion in the rat lung, whereas repeated exposure to low doses of fibers (0.1 mg per rat) resulted in a fiber homogeneous distribution pattern and response similar to that after inhalation.¹⁹

Furthermore, it is conceivable that giving multiple intratracheal instillations of small quantities of particles is more close to natural inhalation exposure than giving a single instillation of a large amount.^{20–21} In view of this, we adopted repeated instillation method (once weekly for 4 consecutive weeks) with a single dose of 3.3 mg/kg.

3.1. Changes in Permeability of Alveolar-Capillary Barrier

After four weeks instillation, histopathology examination of lungs in TiO₂ group showed that enormous macrophages aggregated and the alveolar walls were destroyed severely. In particular, there was almost no complete alveolar structure in focal lesions where characterized by alveolar metaplasia and interstitial fibrosis (Figs. 1(C, D)). ACP and ALP activity in BALF of TiO₂ group increased significantly than control group (with $p < 0.05$ and $p < 0.01$, respectively) (Fig. 2). The changes of biochemical parameters in BALF suggested that instilled TiO₂ NPs resulted in the injury of alveolar epithelial

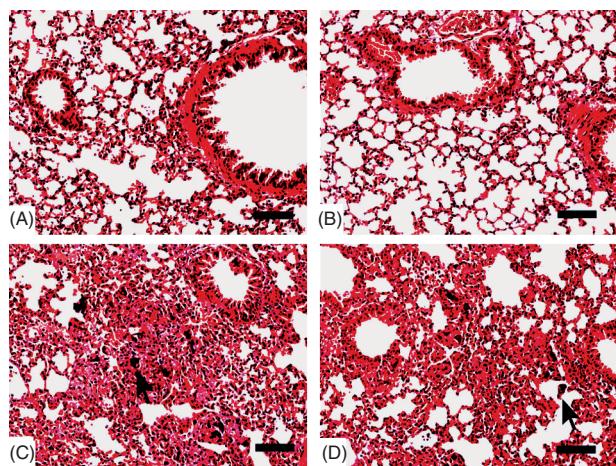


Fig. 1. The pathological changes of lung. (A) presents control group; (B) presents Millipore water group; (C) and (D) presents TiO_2 group. Bar = 100 μm .

cells and promotion of the permeability of alveolar-capillary barrier, and indicated the possibility TiO_2 penetrated capillary–blood barrier into blood circulation. To testify this hypothesis, we detected the amount of Ti in blood at 3 days post-instillation with the dose of 4 mg/kg by inductively coupled plasma mass spectrometry (ICP-MS). The significant increase of Ti in blood and brain not only proved that they could penetrate alveolar-capillary barrier and enter blood circulation, but also could further penetrate blood-brain barrier (Fig. 3). It was very likely that TiO_2 NPs entered the blood circulation via destroying pulmonary structure and promoting the permeability of alveolar-capillary barrier.

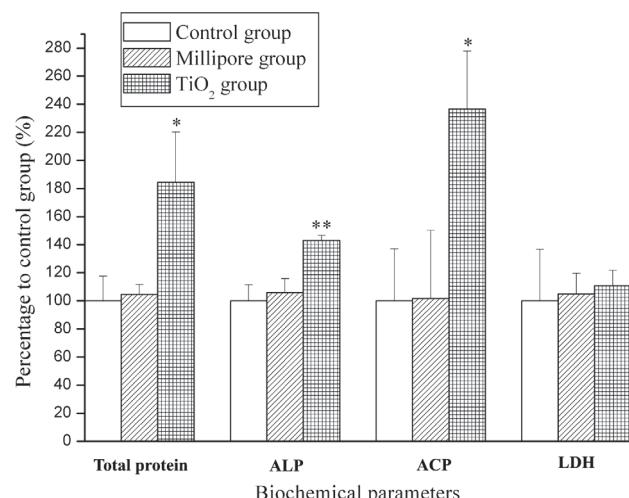


Fig. 2. The changes of total protein level, ALP, ACP, and LDH activity in BALF. The percentage of the biochemical parameters in the TiO_2 group to control group was adopted as the unit in the above chart. The ratio presents mean \pm SD. ($n = 5$) *presents significant difference at $p < 0.05$ level. **presents significant difference at $p < 0.01$ level.

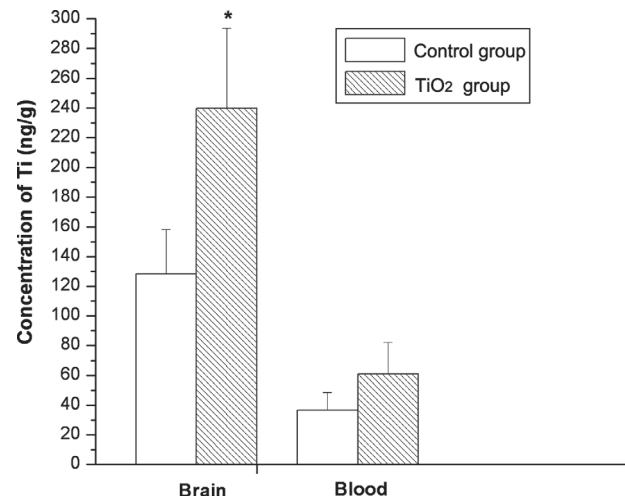


Fig. 3. The amount changes of Ti detected by ICP-MS at 3 days post instillation at one dose of 4 mg/kg.

3.2. The Systemic Influence

The weights of mice in three groups were weighed on day 7, 14, 21, and 28 post-instillation. And at the endpoint of experiment, the lungs, brains, livers, kidneys, and spleens of unlavaged mice were excised and weighed respectively. The growth rate of weight in TiO_2 group was almost in accordance with other two groups during the first week. In the following three weeks, however, the body weight increased in TiO_2 group was obviously less than the control group ($p < 0.05$) (Fig. 4). This indicated that repeated intratracheal instillations of TiO_2 NPs would inhibit the growth and development of mice. In addition, the viscera index of brain; kidney, lung and spleen in TiO_2 group have a significant difference than control group (Fig. 5). It can be regarded that organs injuries contribute greatly to the

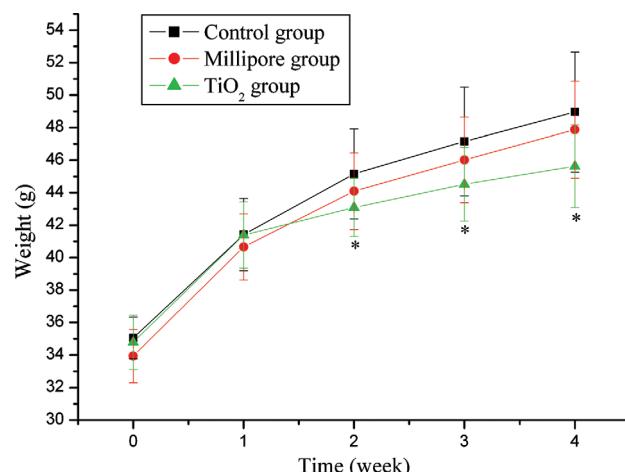


Fig. 4. Mice weight changes induced by 3 nm TiO_2 during 4-week intratracheal instillation. The mice weights in the three groups were measured on days 7, 14, 21, and 28. Each point represents mean \pm SD. ($n = 13$) *presents significant difference at $p < 0.05$ level.

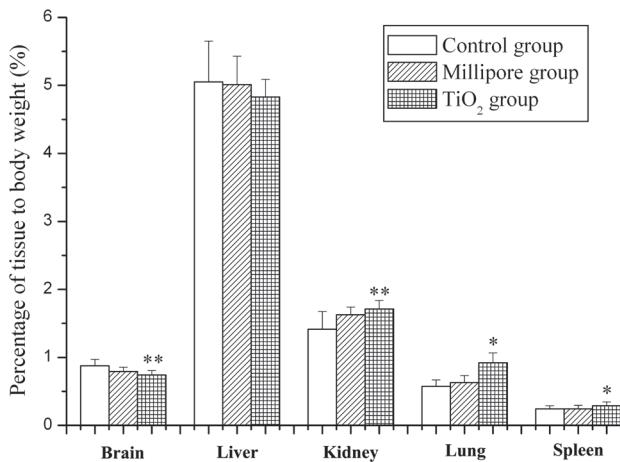


Fig. 5. The percentage changes of tissue to body weight in the control group, Millipore water group and TiO_2 group. The ratio presents mean \pm SD. ($n = 5$) *presents significant difference at $p < 0.05$ level. **presents significant difference at $p < 0.01$ level.

weight inhibition. In short, TiO_2 NPs led to a comprehensive influence on mice.

In order to understand the systematic influence after intratracheal instillation for four weeks, the histopathological examination of liver, kidney and brain in three groups were administrated. And for liver, there were some liver cells steatosis and inflammatory cells aggregation (Fig. 6(C)). In addition, some protein-like material

exudates in hepatic lobule (Fig. 6(D)). For certain areas, obvious hepatocellular necrosis and exudation of blood cells were observed (Fig. 6(E)). But no significant histopathological changes appeared in Millipore water group.

There were many principal characteristics such as protein-like effusion and some inflammation cells aggregation in renal medulla (Fig. 7(E)), blood cell effusion in renal tubule and destruct of part renal tubule structure (Fig. 7(F)). But no significant histopathologic changes appeared in the Millipore water group.

Wang et al., found that TiO_2 NPs could be translocated into the central nervous system and cause potential lesion of brain after nasal instillation.²²⁻²⁴ In this work, the histopathological examination of brain showed that some protein-like material exudates and inflammatory cells aggregation in the cerebral medullar (Fig. 8(E)), and protein-like effusion in hippocampus (Fig. 8(F)); also typical of “cuff-like” lesion appearance (Fig. 8(G)) and a lot of neuronal necrosis (Fig. 8(H)) were observed in TiO_2 group after four weeks’ instillation. TiO_2 NPs did induce brain injury after they entered body by intracheall instillation.

To further understand the injury mechanism of TiO_2 NPs on brain, the amount of Ti in brain of mice was measured by ICP-MS, after single instillation of TiO_2 with the dose of 4 mg/kg. The results indicated that the amount of Ti in brain reached 240 ng/g, which is about 2 times of the control group (Fig. 3). It is worth pointing out that the amount of Ti in brain was 4 times of that in blood in TiO_2 group, whereas the amount of Ti in blood in TiO_2

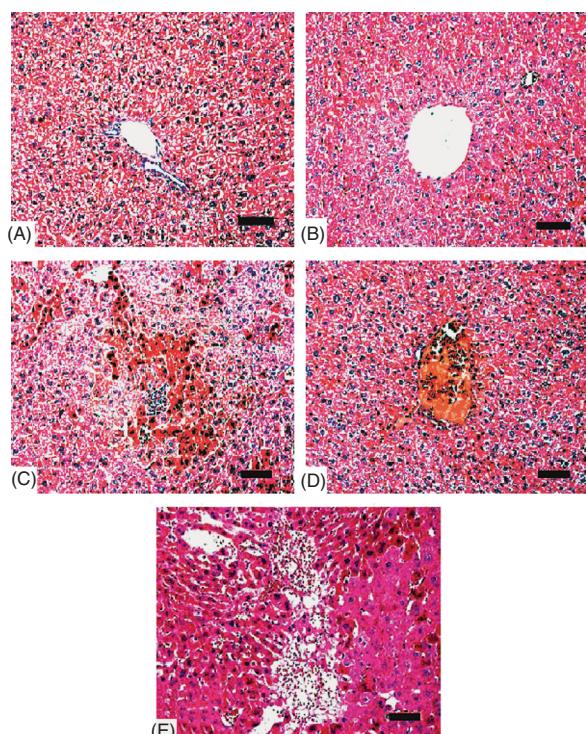


Fig. 6. The pathologic changes of liver. (A) presents control group (B) presents Millipore water group; (C), (D) and (E) present TiO_2 group. bar = 100 μm .

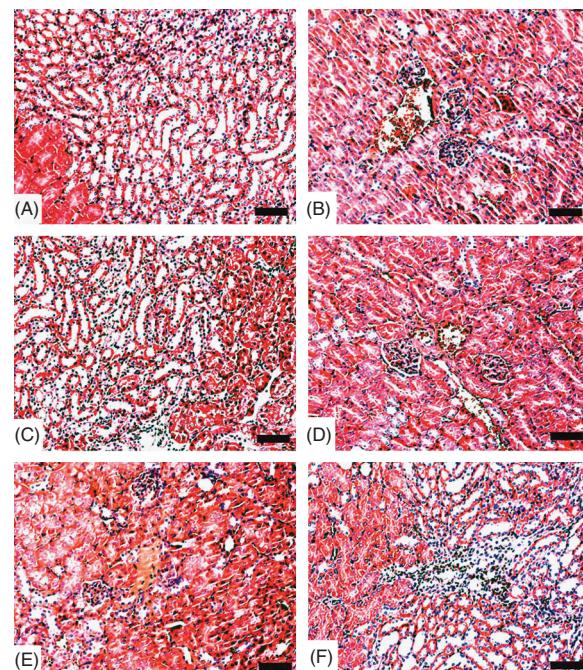


Fig. 7. The pathological changes of kidney. (A) and (B) present control group; (C) and (D) present Millipore water group; (E) and (F) present TiO_2 group. Bar = 100 μm .

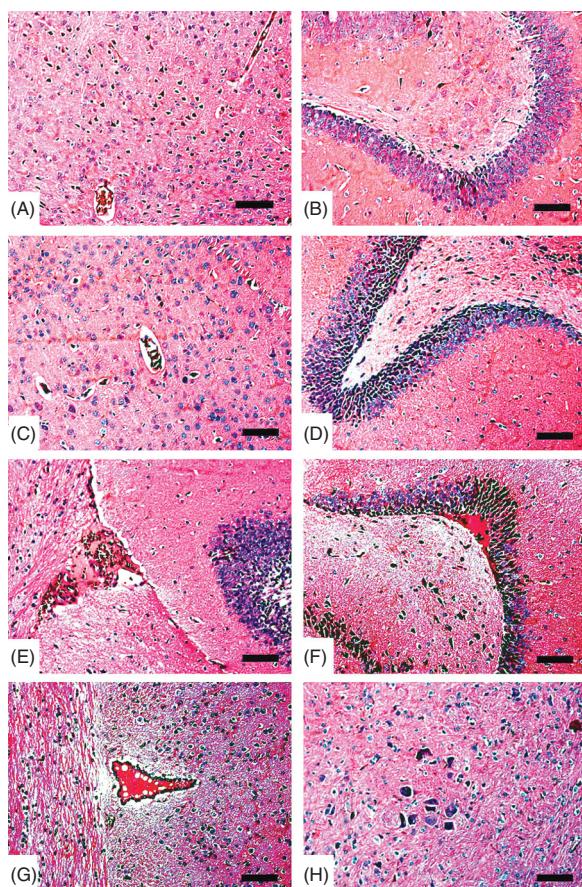


Fig. 8. Brain pathological change. (A) and (B) present control group; (C) and (D) Millipore water group; (E), (F), (G) and (H) present TiO_2 group. Bar = 100 μm .

group was only 1.7 times of that in control group. The results suggested that TiO_2 entered blood could be rapidly translocated to most organs or tissues, and a part of them penetrate BBB and get access to brain. The TiO_2 NPs in blood were cleared gradually, and accumulated in brain, and which maybe induce the injury of brain. It was also notable that the amount of Ti in brain of control group was also higher than that in blood (Fig. 3). Considering the possible resource of Ti in control group, we measured the amount of Ti in mice feed and found that it was as high as 9.1×10^3 ng/g mice feed. The result implied that it is essential to supply animal with feed without Ti for evaluating the biological effects of TiO_2 NPs reasonably.

Zhang et al. hold that NPs could induce free radicals then lead to biological toxicity through oxidative stress.²⁵ Gurr et al. also reported that TiO_2 NPs can induce toxicity in many categories of cells through oxidation pathways.^{21,26-28} In this work, brains of five mice in each group were homogenated, and the level of superoxide anion, hydroxyl radicals, peroxide, and MDA in supernatant were measured. The level of these four parameters in TiO_2 group had a significant increase as compared with that in control group ($p < 0.01$), and there was

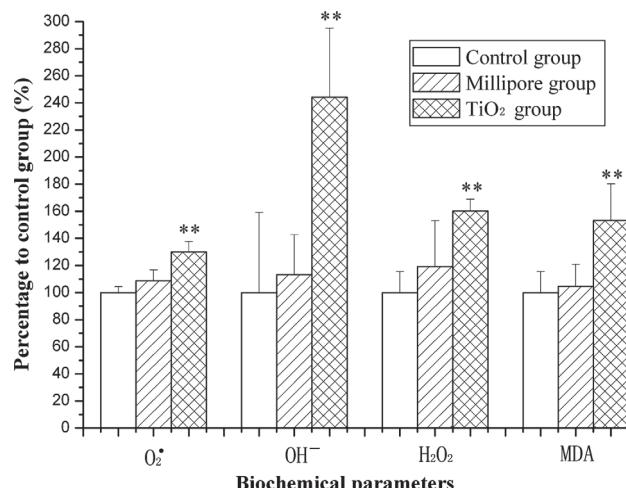


Fig. 9. The changes of superoxide anion, hydroxyl radicals, catalase (CAT), and malondialdehyde (MDA) in the supernatant of brain homogenate in the control group, Millipore water group and TiO_2 group, respectively. The percentage that the biochemical parameters in the experimental group to control group was adopted as the unit in the portrayed graph, showing biochemical parameters. The ratio presents mean \pm SD. ($n = 5$) *presents significant difference at $p < 0.05$ level. **presents significant difference at $p < 0.01$ level.

no obvious difference between Millipore water group and control group (Fig. 9). The changes of biological parameters in brain further indicated that the brain injury induced by TiO_2 NPs might through oxidative stress response.

4. CONCLUSIONS

In summary, the intratracheally instillation of 3 nm TiO_2 NPs leads lung inflammation and some other systemic influence. The results of this work suggest that the assessment of any toxicologic and environmental side effects must go with the development of novel nanoparticles for diagnosis, imaging, drug delivery, and industrial purposes. Of course, further experimental studies are needed to determine the mechanisms behind these observations.

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