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# Acute toxicological effects of zinc oxide nanoparticles in mice after intratracheal instillation

Dejun Wang[a,b], Haibo Li[c], Zihong Liu[a], Jingyang Zhou[b] and Tianliang Zhang[b]

[a]Department of Occupational and Environmental Health, School of Public Health, Peking University Health Science Center, Beijing, PR China; [b]Institute of Occupational and Environmental Health, Shandong Center for Disease Control and Prevention, Jinan, PR China; [c]College of Pharmacy, Third Military Medical University, Chongqing, PR China

**ABSTRACT**

**Background**: Zinc oxide nanoparticles (ZnO NPs) are increasingly being used in industry.
**Objective:** To evaluate the acute toxicology of ZnO NPs in Mice.
**Methods**: ZnO NPs were intratracheally instilled into mice at different dose (200, 400, 800 μg/kg), which was 1, 2, or 4 times of accumulative intake in one week according to the threshold limit value. Acute toxicity was assessed by animal mortality, organ/body weight ratios, hematology, blood biochemistry, and histopathology as well as by the determination of cells, proteins, and lactate dehydrogenase activity in bronchoalveolar lavage fluid.
**Results**: Exposure to ZnO NPs also resulted in bodyweight loss and a higher level of total cell number, total protein, and hydroxyproline content in BALF. Nitric oxide and malondialdehyde levels in the lung homogenates were also increased. In addition, inflammatory and hyperplastic changes in the lungs were observed.
**Conclusion:** Threshold limit value (5 mg/m$^3$) may unfit for ZnO NPs.

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Acute toxicological effects; zinc oxide nanoparticles; intratracheal instillation; murine model; lung fibrosis

## Introduction

A better understanding of the health effects of nanoparticles (Nps) is increasingly important given their continuous and widespread use. To date, most health-related nanotoxicity research has focused on the effects of respiratory tract exposure [1]. ZnO NPs is a new type of high-function fine inorganic product (particle size: 1–100 nm). ZnO NPs have been increasingly used in traditional industries, such as rubber, ceramic, chemical daily expenses, paints, catalysts, magnetic materials, national defense materials, electric conducting materials, and other fields, because of their special structural features and properties [2]. With the industrialization of nanotechnology, ZnO NPs exposure is increasingly common, especially in production and application processes. The respiratory tract is the major route for ZnO NPs exposure [3].

Recently, investigations focused on the *in vitro* and *in vivo* toxicity of ZnO NPs [4–6]. However, few studies reported its acute toxicity [7]. In these studies, only the threshold limit value (TLV) of zinc oxide (5 mg/m$^3$) recommended by the Occupational Safety and Health Administration (OSHA) is clear. However, other things, such as the general behavior, LD50, characterization, and general toxicology of ZnO nanoparticles, remain unknown [8]. Therefore, the assessment of the safety of ZnO NPs is meaningful because different sized particles might substantially cause different toxic effect. The objective of the present study was to explore the acute toxicity of ZnO NPs in mice by intratracheal instillation to assess respiratory nanotoxicity and provide scientific information for the TLV of ZnO NPs. Results can be used to regulate the health and safety of ZnO NPs in the workplace.

## Methods

### *Ethics statement*

Animal maintenance and experimental procedures were carried out in accordance with the National Institutes of Health Guidelines for the Use of Experimental Animals and were approved by the Biomedical Ethics Committee of Peking University (Approval Number: 2007067) [9]. To minimize animal suffering and distress, trained and skilled animal care personnel were involved in this research. All operations were performed under 1% sodium pentobarbital anesthesia to minimize pain. During this study, we monitored the health of the mice every 8–12 h and euthanized the mice when they lacked sustained purposeful response to gentle stimuli. Carbon dioxide was used for euthanasia.

**CONTACT** Dejun Wang wdjsd2015@163.com; Haibo Li lihaibo@tmmu.edu.cn
Department of Occupational and Environmental Health, School of Public Health, Peking University Health Science Center, Beijing, PR China; Institute of Occupational and Environmental Health, Shandong Center for Disease Control and Prevention, Jinan, PR China; College of Pharmacy, Third Military Medical University, Chongqing, PR China

### Laboratory animals

Fifty ICR male mice (specific pathogen-free mice were provided by the Experimental Animal Center, Peking University) weighing 23–27 g were used in the experiments. Each group consisted of 10 mice housed in stainless steel cages containing sterile paddy husk as bedding in ventilated animal rooms. The exposure groups included 200, 400, and 800 μg/kg group. A control group and a blank group were also included. The mice in the 200, 400, and 800 μg/kg groups were conducted with ZnO NPs in suspension; the control group was administered with phosphate buffer saline (PBS); and the blank group received no treatment. Mice were acclimated in a controlled environment (temperature: 22–24 °C, humidity: 60 ± 10% and light: 12 h light/dark cycle) with free access to water and commercial laboratory complete food.

### Characterization of ZnO NPs

ZnO NPs were supplied by a laboratory of the Faculty of Chemistry, Peking University. The size and morphology of ZnO NPs were investigated by transmission electron microscopy (TEM, JEM-200CX). The crystalline phase and particle size of ZnO NPs were analyzed by X-ray diffraction (XRD, D/MAX 2000). Brunauer-Emmett-Teller (BET, ASAP2010) technique was adopted to gain the specific surface area (SSA). The purity was analyzed by X-ray fluorescence (XRF, S4-Explorer).

### Preparation of ZnO NPs suspensions

ZnO NPs were stored and analyzed in a dry powder form until they were used in the animal experiments. The suspending agent for ZnO NPs was prepared according to the volume of inspiration based on the animal's body weight [10]. Stock suspensions (1 mg/ml) of particles were prepared in a phosphate-buffered saline (PBS) solution (sterile filtered). The suspending solutions containing ZnO NPs were treated by ultrasound for 10 min and vibrated for 2 min. Mice were subsequently exposed to these solutions via tracheoesophageal punctures in different doses.

### Exposure dose

The volume of inspiration of healthy adult humans is approximately 10 liters per min and this volume of inspiration would accumulate to 24 $m^3$ (10 L/min × 60 min × 40 h/1000) for an individual working 8 h per day in a 40 h work week. Assuming that 10% of the inhalable particles are deposited in the pulmonary region [11] and that a 60 kg man breathes in 10 L of air per minute, a man exposed to respirable dust at 5 mg/$m^3$per eight hour day would accumulate 2.4 mg per day and 12 mg per week (40 h per week). Given that the mass of breathing increases with body weight, a mouse weighing 25 g would inhale 5 μg/week. Combined with the results of the pre-test, we instilled mice with one, two or four times this amount. Stock suspensions (1 mg/ml) of particles were prepared in PBS (sterile filtered). Each suspension was sonicated for 10 min prior to exposure and characterization. Each stock solution was subsequently serially diluted into three concentrations of 0.4, 0.2, and 0.1 mg/ml. Each mouse was intratracheally instilled with 50 μl of the corresponding suspension.

### Animal treatment

The test samples containing 0, 5, 10, and 20 μg ZnO NPs in 50 μl of PBS were drawn into a disposable syringe along with the same volume air and rapidly propelled from the tubing and needled into the lungs of the mice. The neck incision was sutured and swabbed with medical alcohol. The mice recovered and resumed activity within 15 min after removing the intraperitoneal anesthetic injection. The incisions were healed within two days and were observed daily until the end of the experiment.

### Hematology

Blood samples taken from the angular veins of mice were measured after anticoagulation. The blood parameters were determined by an automatic hematology analyzer (MEK-6318K) and included white blood cell (WBC), red blood cell (RBC), hemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell volume distribution width (RDW), platelet count (PLT), mean platelet volume (MPV), lymphocyte percentage (LYM%), mononuclear cell percentage (MOD%), and granulocyte percentage (GRN%).

### Blood biochemical analysis

The serum was obtained after centrifugation at 4000 rpm for 10 min. The biochemical indices were determined by an automatic biochemical analyzer (7170A, Hitachi, Tokyo) and included alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), total bilirubin (TBIL), total bile acid (TBA), glucose (GLU), uric acid (UA), blood urea nitrogen (BUN), creatinine (CR), angiotensin-converting enzyme (ACE), alkaline phosphatase (AKP), and α-hydroxybutyrate dehydrogenase (HBDH).

### Biochemical and cytological evaluation of BALF

After cutting open the skin of the neck, stripping the subcutaneous tissue and baring the trachea, the animals were intratracheally instilled with 0.4 ml of PBS using a 1 ml syringe for reclaiming their fluids. This process

was repeated four times. The total cellular scores in BAL were counted by a cell counting plate under an optical microscope. The supernatant was obtained by centrifugation of BAL at 2000 rpm for 5 min. The total protein was assayed with the coomassie brilliant blue method. The alkaline phosphatase (AKP), hydroxyproline, and lactate dehydrogenase (LDH) were measured by a conventional chemical shade selection method.

### Measurement of NO and MDA in lung homogenates

Nitric oxide (NO) and malondialdehyde (MDA) levels in the lung homogenates were quantified using commercially available kits according to the manufacturer's instructions (Nanjing Jiancheng Co Ltd).

### Histopathology

On the seventh day after instillation of the test material, all animals were decapitated and executed to remove their lungs and tracheas. Their wet lung weights were measured and the top-left parts of the lungs were immediately fixed in 10% formalin for further histopathological examinations. The remaining parts of the lung were homogenated to analyze the malondialdehyde (MDA) and nitrogen monoxide (NO) contents. The lung tissue was embedded in paraffin blocks and was sliced and placed onto glass slides. After histological hematoxylin-eosin staining, the slides were observed using an optical microscope.

### Statistical analysis

The data were expressed as the mean ± standard deviation for statistical analysis. Nine animals were in 400 μg/kg group, and seven were in 800 μg/kg group on the third day; eight were in 400 μg/kg group, and two were in 800 μg/kg group on the fifth day; seven were in 400 μg/kg group, and one was in 800 μg/kg group on the seventh day. Thus, the body weight changes of mice after ZnO NPs exposure were not involved in the statistical analysis. Due to the small number of animals in the 800 μg/kg group at the end of the experiment, no statistical analysis of the hematology, clinical biochemistry, BAL, or histopathology parameters was completed. The comparison of the different ZnO nanoparticle dose groups was performed by ANOVA, and the mean differences among the group were conducted by Dunnett's T-test. The alpha level at which all of the tests were conducted was 0.05. Data were analyzed using SPSS10.0 software.

## Results

A TEM image of ZnO NPs is shown in Figure 1(A). ZnO NPs had the average width of 60 ± 20 nm and the average length of 100 ± 40 nm. The peak value for width is 50 nm and for length is 110 nm according to the size distribution (Figure 1(C) and (D)). The specific surface area of the ZnO NPs is 8.60 $m^2/g$ determined by BET analysis. XRD spectra show that ZnO NPs are hexagonal in shape with average particle size of 48 nm. The purity of the ZnO NPs analyzed by X-ray fluorescence is greater than 99.9%.

There were differences in the general behavior of mice in the 400 and the 800 μg/kg group compared to the other three groups. Mice in the exposure group had disheveled hair, decreased eating, drinking (Figure 2(A) and (B)) and defecation, dry stool, dyspneic respiration, and acrocyanosis. At the end of this experiment, three mice in the 400 μg/kg group and nine mice in the 800 μg/kg group died (Figure 2(A)). Therefore, only the tissues in the 800 μg/kg group were histopathologically analyzed. Our study also found that the LD50 in the intratracheal instillation of ZnO NPs (48 nm) was 493.85 μg/kg body weight.

One day after exposure, more body weight (BW) loss was observed in the treated groups compared with the control group. The degree of BW loss in the 800 μg/kg group was greater than that in the 400 μg/kg group (Figure 2(B)). Also the lung weight/BW ratios of all of the treated mice increased (Figure 3). In the 400 μg/kg group, the spleen weight/BW and brain weight/BW ratios significantly increased ($p < 0.05$); however, the ratios of the kidney weight/BW decreased ($p < 0.05$). No significance was observed in heart weight/BW and liver weight/BW among all groups.

There were several significant changes in hematologic parameters for mice exposed to ZnO NPs. Compared with the two control group, many significant increases in MOD%, MCV, MCH, RDW, and MPV were observed in the 200 μg/kg group ($p < 0.05$, Table 1); significant reductions in RBC and MCHC were also found in the 200 μg/kg and 400 μg/kg groups ($p < 0.05$, Table 1).

The biochemical parameters of the serum after ZnO NPs exposure are presented in Table 2. Plasma TBIL, AKP, and GLU of the 200 μg/kg group were significantly higher than those of the control groups ($p < 0.05$), and BUN and CR in the 200 μg/kg and 400 μg/kg groups also increased significantly ($p < 0.05$). However, there were no significant differences in any of the other parameters analyzed.

There was a dose-dependent increase in the total cell number in BAL of animals treated with ZnO NPs compared with the control groups (Figure 4(A)). We found a significant increase in the total protein in the BAL of the treated mice compared to that in the control group (Figure 4(B)). Although no difference in LDH level in the BAL was observed (Figure 4(E)), there was a dose-dependent increase in the AKP and hydroxyprolin in the BAL of the treated animals (Figure 4(C) and (D)). In addition, the levels of lung MDA and NO in the treated mice were also significantly increased compared with those in the control group (Figure 5(A) and (B)).

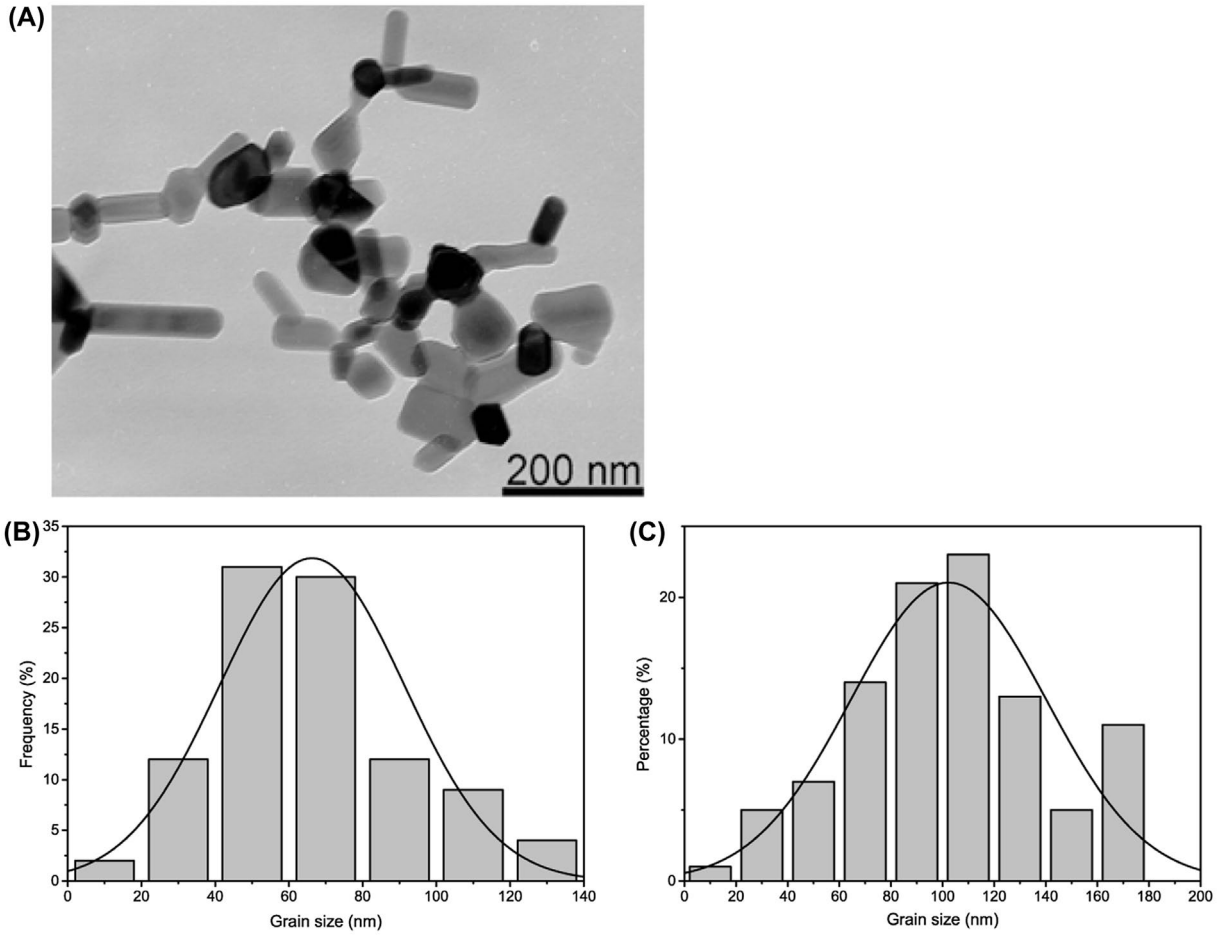


**Figure 1.** Characterization of ZnO NPs. (A) TEM image, (B) the width distribution, (C) the length distribution.

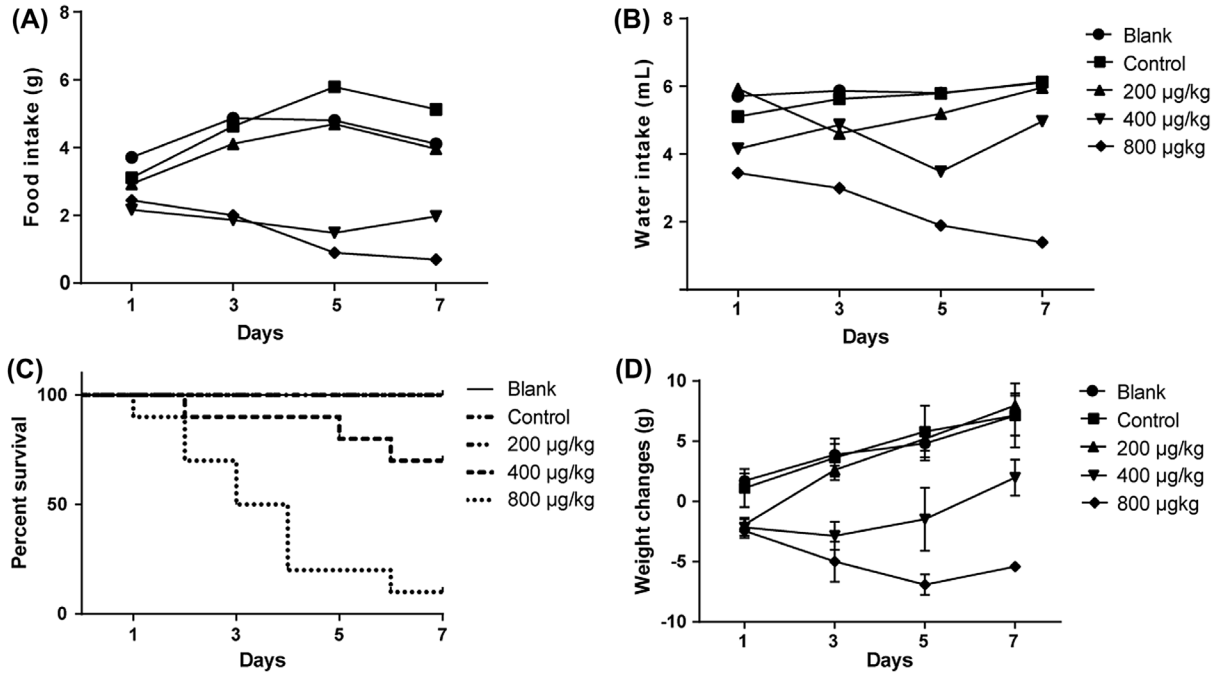


**Figure 2.** Average food intake (A), average water intake (B), survival (A), and weight change (B) after intratracheal instillation of ZnO NPs.

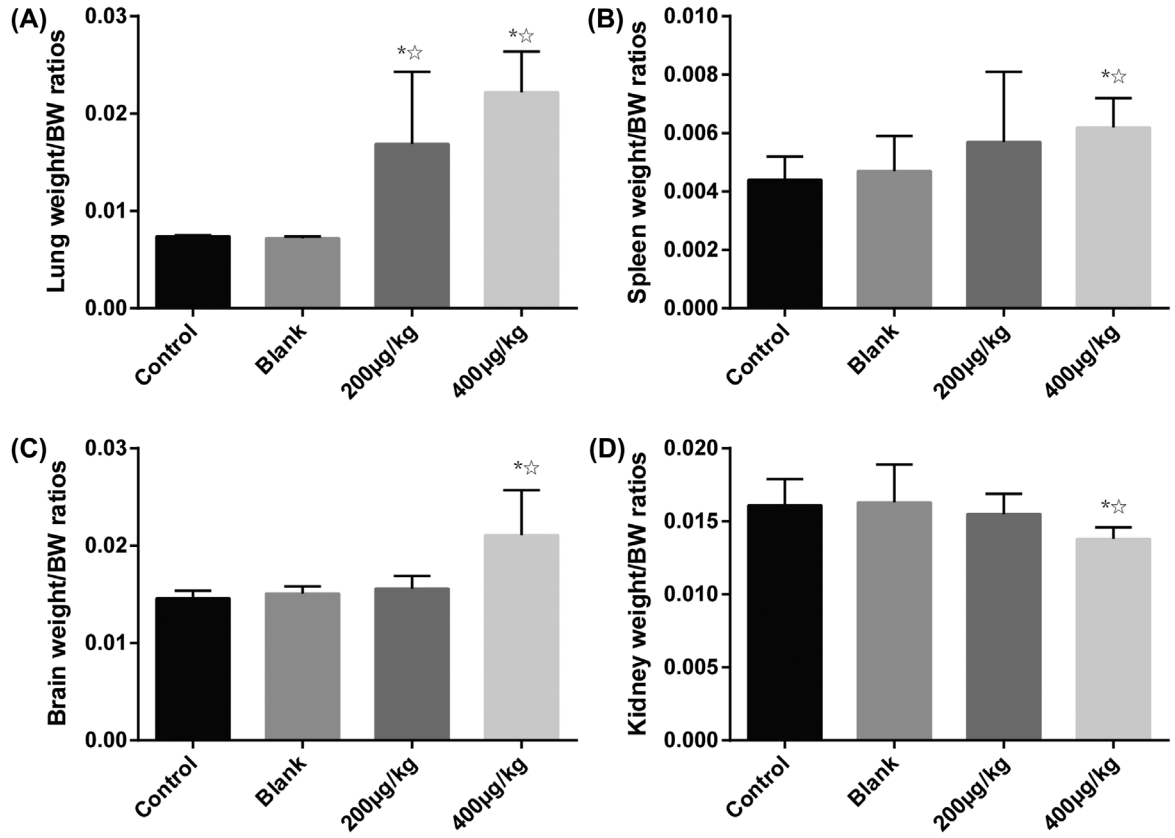


**Figure 3.** Analysis of organ weight/BW ratios 7 days after intratracheal instillation. Lung weight/BW ratios (A) in both the 200 μg/kg and 400 μg/kg groups increased significantly; spleen weight/BW ratios (B) and brain weight/BW ratios (C) in the 400 μg/kg group were significantly increased. Kidney weight/BW ratios (D) in the 400 μg/kg group decreased significantly. *$p < 0.05$ compared with the blank group, ☆$p < 0.05$ compared with the control group and ★$p < 0.05$ compared with the 200 μg/kg group.

**Table 1.** Hematology parameters in mice after intratracheal instillation of ZnO NPs.

| | Groups | | | |
|---|---|---|---|---|
| Indexes | Blank | Control | 200 μg/kg | 400 μg/kg |
| RBC($\times10^{12}$/L) | 7.84 ± 0.82 | 7.90 ± 0.47 | 6.68 ± 0.50*☆ | 6.86 ± 0.82*☆ |
| WBC($\times10^{9}$/L) | 7.02 ± 2.44 | 8.29 ± 2.44 | 5.20 ± 1.28 | 5.59 ± 2.83 |
| PLT($\times10^{9}$/L) | 432.91 ± 88.40 | 480.45 ± 114.67 | 596.72 ± 208.64 | 703.44 ± 197.33 |
| HGB(g/L) | 110.71 ± 10.52 | 110.30 ± 5.66 | 104.28 ± 5.58 | 106.02 ± 14.19 |
| LYM($\times10^{9}$/L) | 4.88 ± 1.86 | 5.53 ± 2.08 | 3.89 ± 0.83 | 3.41 ± 1.45 |
| MOD($\times10^{9}$/L) | 0.51 ± 0.25 | 0.56 ± 0.37 | 0.41 ± 0.25 | 0.82 ± 0.41 |
| GRN($\times10^{9}$/L) | 1.95 ± 0.96 | 2.30 ± 0.64 | 1.23 ± 0.69 | 1.33 ± 1.07 |
| MCV(fl) | 51.78 ± 3.17 | 52.08 ± 4.12 | 57.53 ± 3.23*☆ | 60.58 ± 4.56*☆ |
| MCH(pg) | 14.57 ± 0.62 | 14.39 ± 0.50 | 15.76 ± 0.41*☆ | 15.79 ± 0.50*☆ |
| MCHC(g/L) | 280.43 ± 18.35 | 277.64 ± 16.54 | 274.68 ± 12.89 | 258.37 ± 23.66☆ |
| RDW(%cv) | 16.20 ± 2.12 | 16.19 ± 2.08 | 22.25 ± 1.92*☆ | 24.46 ± 1.35*☆★ |
| HCT(%) | 39.89 ± 5.23 | 40.24 ± 3.80 | 38.87 ± 2.39 | 41.22 ± 4.76 |
| PCT(%) | 0.66 ± 0.16 | 0.54 ± 0.20 | 0.92 ± 0.33 | 1.22 ± 0.38 |
| MPV(fl) | 10.24 ± 0.98 | 10.54 ± 1.30 | 12.26 ± 1.13*☆ | 13.39 ± 1.09*☆★ |
| PDW(%) | 13.89 ± 0.85 | 14.30 ± 1.21 | 13.85 ± 1.22 | 14.59 ± 0.53 |
| LYM% | 69.95 ± 10.47 | 67.05 ± 8.31 | 71.79 ± 10.00 | 68.22 ± 15.01 |
| MOD% | 4.74 ± 2.44 | 6.54 ± 2.79 | 6.60 ± 2.07 | 10.31 ± 2.08*☆ |
| GRN% | 25.82 ± 9.63 | 26.99 ± 7.14 | 22.12 ± 8.98 | 21.99 ± 13.61 |

Notes: The data are expressed as the mean ± SD.
*$p < 0.05$ compared with the blank group; ☆$p < 0.05$ compared with the control group; ★$p < 0.05$ compared with the 200 μg/kg group.

Pathological changes were identified in the mice treated with ZnO NPs (Figure 6). Fibrosis in the alveolar wall and the collapsed alveoli occurred in the 200 μg/kg group. Obvious fibrosis and chronic inflammation were found in the alveolar wall when treated with 400 μg/kg ZnO NPs, which caused the thickness of the alveolar wall. The 800 μg/kg group showed diffused fibrosis and severe chronic inflammation. No gross pathological lesions in other organs were observed in any of the treatment groups.

## Discussion

Currently, the TLV of zinc oxide is 5 mg/m$^3$, as recommended by OSHA [8]. According to the TLV, 200, 400, and 800 μg/kg doses of ZnO NPs used in mice were

**Table 2.** Serum biochemistry parameters in mice after intratracheal instillation of ZnO NPs.

| Indexes | Groups | | | |
|---|---|---|---|---|
| | Blank | Control | 200 μg/kg | 400 μg/kg |
| TBIL(mmol/L) | 1.35 ± 0.13 | 1.43 ± 0.20 | 0.89 ± 0.16*☆ | 1.45 ± 0.61 |
| TBA (mmol/L) | 0.71 ± 0.44 | 0.69 ± 0.27 | 0.88 ± 0.41 | 1.67 ± 1.65 |
| UA (mmol/L) | 141.02 ± 65.96 | 134.23 ± 39.54 | 105.03 ± 50.24 | 103.73 ± 46.64 |
| BUN (mmol/L) | 5.90 ± 1.03 | 6.52 ± 0.93 | 7.92 ± 1.01*☆ | 8.42 ± 1.73*☆ |
| CR (mmol/L) | 39.84 ± 6.26 | 42.69 ± 4.22 | 53.33 ± 5.05*☆ | 50.87 ± 2.62*☆ |
| GLU (mmol/L) | 6.66 ± 1.14 | 6.93 ± 1.42 | 9.84 ± 1.35*☆★ | 6.31 ± 1.45 |
| LDH (U/L) | 999.01 ± 262.74 | 1109.32 ± 180.60 | 973.01 ± 231.74 | 946.70 ± 264.73 |
| AKP (U/L) | 87.04 ± 19.34 | 88.54 ± 21.21 | 113.94 ± 28.16*☆ | 100.87 ± 26.73 |
| ALT (U/L) | 19.10 ± 3.94 | 22.87 ± 5.23 | 30.72 ± 11.63 | 38.83 ± 11.64 |
| AST (U/L) | 62.55 ± 18.15 | 69.76 ± 9.55 | 87.51 ± 28.30 | 101.72 ± 53.67 |
| HBDH (U/L) | 427.80 ± 126.41 | 467.55 ± 93.27 | 416.41 ± 112.37 | 418.56 ± 133.84 |
| ACE (U/L) | 232.06 ± 18.47 | 234.88 ± 15.59 | 248.94 ± 12.56 | 238.44 ± 19.79 |

Note: The data are expressed as the mean ± S.D.
*$p < 0.05$ compared with the blank group; ☆$p < 0.05$ compared with the control group; ★$p < 0.05$ compared with the 200 μg/kg group.

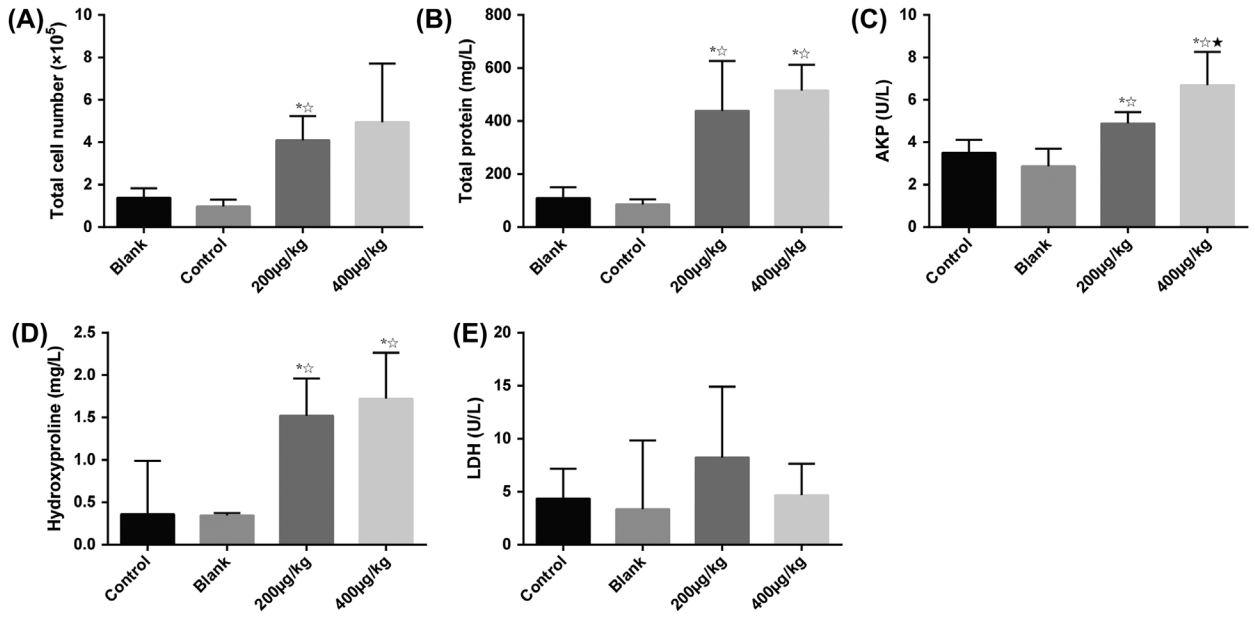


**Figure 4.** Total cell number (A), total protein levels (B) and biochemical indicators (C–E) in BALF of mice 7 days after ZnO NPs exposure. *$p < 0.05$compared with the blank group, ☆$p < 0.05$ compared with the control group, and ★$p < 0.05$ compared with the 200 μg/kg group.

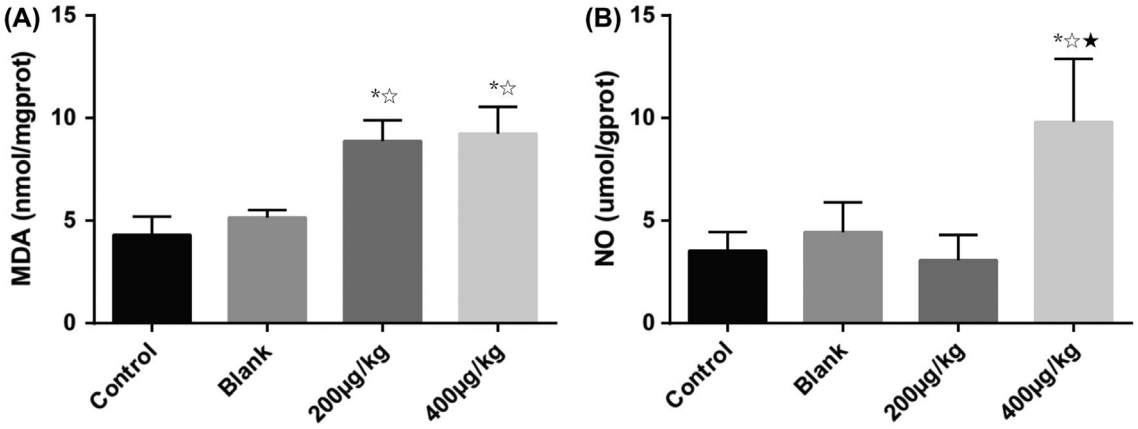


**Figure 5.** The concentration of MDA and NO in lung homogenates of mice after intratracheal instillation of ZnO NPs. *$p < 0.05$compared with the blank group, ☆$p < 0.05$ compared with the control group, and ★$p < 0.05$ compared with the 200 μg/kg group.

equal to 1, 2, or 4 times of cumulative intake, respectively, in a man who works consecutively for 8 h daily during a 40 h per week. Seven days after exposure, three mice in the 400 μg/kg group and nine mice in the 800 μg/kg group died. Body weights in the three exposed groups significantly decreased on the first day after exposure,

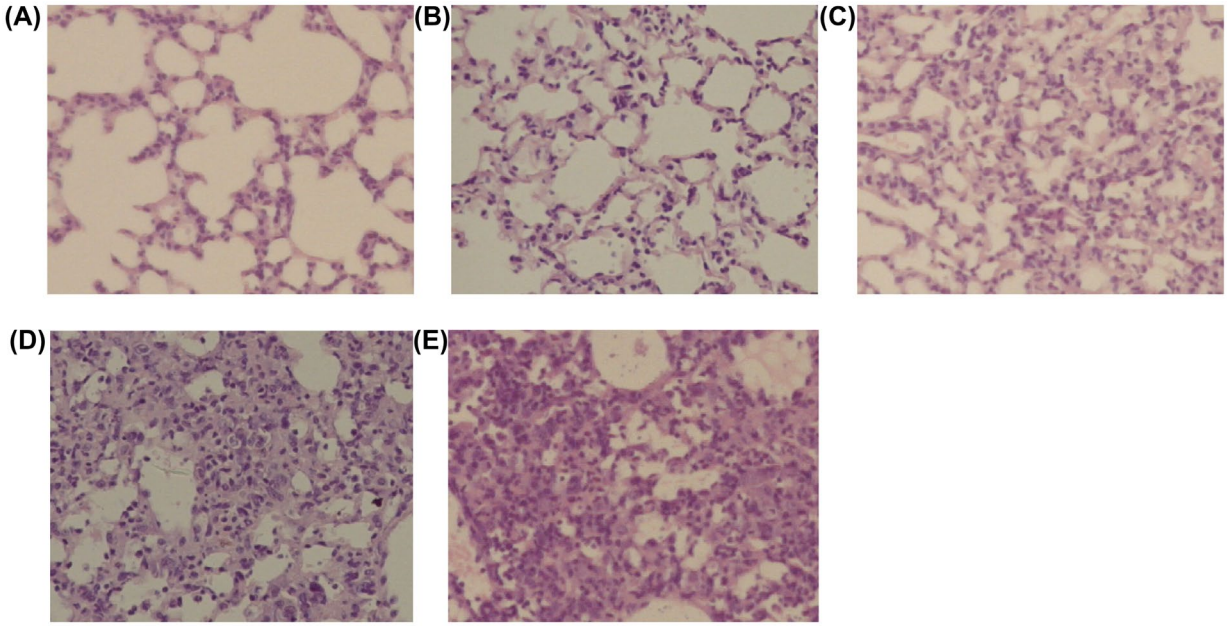


**Figure 6.** Representative lung histology of mice after intratracheal instillation of ZnO NPs (HE × 200). Lung section from a mouse in the blank group (A). Lung section from a mouse in the control group that received PBS (B). Lung sections from mice intratracheally instilled with 200 μg/kg (C), 400 μg/kg (D), and 800 μg/kg (E) doses of ZnO NPs.

and the loss of body weight in the 400 μg/kg and 800 μg/kg groups was greater than that in the two control groups five days after exposure. Although decreased eating and drinking may lead to a decline in body weight of mice, it does not necessarily result in death. Therefore, direct injury of ZnO NPs in mice may be more closely associated with the weight loss and death. Previous research has suggested that mental NPs can cross the blood brain barrier and have a direct detrimental impact on the central nervous system, potentially leading to anorexia and weight loss [12]. Moreover, we observed direct lung injury when treated with ZnO NPs in the current study, which was characterized by increased lung weight/BW ratios and obvious fibrosis in the alveolar wall. Several studies have reported that direct lung injury may lead to difficulty breathing, fatigue, weight loss, and eventually death [13]. Given that weight loss and death in mice administered with ZnO NPs was dose-dependent, we conclude that the weight loss and death are associated with the toxic effects of ZnO NPs.

Shiseido reported that the acute oral toxicity (LD50) of FINEX-50 ZnO (20 nm) was >2000 mg/kg body weight for male Sprague-Dawley rats [14]. However, a different study reported that ZnO NPs were one of the most toxic nanosized particles, with the lowest LD50 value among engineered metal oxide nanoparticles [15]. Our study found that the LD50 in the intratracheal instillation of ZnO NPs (48 nm) was 493.85 μg/kg body weight.

A number of studies have suggested that nanoparticles were not recognized or were devoured completely after entering into the lungs and were directly transferred into the circulation system by breaking through the alveolar wall [16–20]. Therefore, NPs could affect the hematological system. In this study, our results suggested that treated mice had giant cell anemia, possibly a result of DNA dyssynthesis caused by a lack of folic acid and/or vitamin $B_{12}$ resulting from a food intake decrease after exposure to ZnO NPs [21] The elevated levels of MPV revealed increased platelet destruction and compensation for the function of the bone marrow. Some epidemiological investigations have indicated that ultrafine particles can provoke adverse health effects in the cardiovascular system; these effects were associated with thrombosis caused by damage to the function of the endothelial system and affected red blood cells and platelets, thus influencing blood circulation [22]. In our study, PLT in the 200 and 400 μg/kg groups increased with MPV, with platelet levels in ZnO NPs-treated mice higher than those in the control groups as a result of bone marrow compensation, although ZnO NPs could cause platelet destruction. These results indicate that ZnO NPs may enter the blood from the lungs and further affect the body's cardiovascular system [22]. Elevated levels of MOD% in the 400 μg/kg group suggested that the mice in this group were in the acute phase of infection.

We used serum biochemical parameters to reflect the function of the liver, kidney, cardiac muscle, and other systems, and to monitor the response to the exogenous toxic exposure. The TBIL, TBA, ALT, and AST were sensitive for evaluating hepatic injury. In our experiments, the levels of ALT, AST, and AKP were higher in the 200 and 400 μg/kg groups compared with the control group. The level of TBA in the 400 μg/kg group was higher than that in other groups, suggesting that the hepatic function might be damaged after being intratracheally instilled

with ZnO NPs. Researchers have observed that ultrafine particles in the respiratory tract can permeate the blood-air barrier and then enter the liver, kidney, spleen, or other organs outside of the lung by passing through the recirculating system [11,22–24]. Some previous studies also reported that excessive Zn oral exposure may cause liver damage [26]. Based on these findings, the liver injury in our study might be caused by intratracheal instillation of ZnO NPs in mice.

The total protein, AKP, hydroxyproline, and LDH of the BAL were important indicators reflecting inflammation, a typeIIpneumocyte membrane injury, earlier lung fibrosis, and cytomembrane construction injury in the lung [26,27]. In this study, the total protein, AKP and hydroxyproline in the 400 μg/kg and the 200 μg/kg mice were significantly higher than those in the control mice. Although the LDH of the BAL did not significantly change in the treated groups, it showed an increasing trend compared to the control group. Based on our results, we conclude that ZnO NPs may play a role in damaging pulmonary macrophages, type II pneumocytes, and vascular endothelial cells, and initiating lung inflammation and fibrosis. MDA level is a known marker of oxidative stress [28]. In this study, the exposure groups showed increased MDA and NO levels compared to the non-treatment groups. Oxidative stress and free radicals induced by intratracheal instillation of ZnO NPs may play a role in the formation of pulmonary fibrosis.

There is controversy about whether material subdivided into nanoparticles increases the toxicity of the materials [10,29,30]. As early as 1985, Lam *et al.* discovered that ultrafine ZnO (U-ZnO) inhaled by guinea pigs at 5 mg/m$^3$ led to decreased lung function and inflammation of alveolar ducts and alveoli [31]. Other studies found that levels of total protein, AKP, and LDH in the BAL of the guinea pigs increased when exposed to U-ZnO at 5.9 mg/m$^3$ or 12.9 mg/m$^3$ [10,32]. Our results also indicated that 5 mg/m$^3$ of the TLV was unfit for ZnO NPs. Moreover, our study suggested that biochemical examinations of the BAL were effective and sensitive indicators for the quantitative assessment of the lung injury that was caused by exposure to a low level of ZnO NPs.

In summary, after intratracheal instillation of three different doses of ZnO NPs in mice, mortality, serum and BALF biochemistry parameters, hematology and histopathology were investigated 7 days after treatment. Mice died and acute toxicity symptoms such as decreased food and water intake were observed in the 400 and 800 μg/kg group. The LD50 of intratracheal instilled ZnO NPs (493.85 μg/kg) was calculated in mice. The mice treated with ZnO NPs showed significantly higher lung weight/BW ratios compared to control. The histopathological analysis also revealed that intratracheal instillation of ZnO NPs caused pulmonary fibrosis and inflammation. Hematological analysis and serum biochemical parameters suggested that ZnO NPs could induce giant cell anemia and hepatic damage. Based on above all, we concluded that 5 mg/m$^3$ of the TLV was unfit for ZnO NPs. Further evaluation for chronic toxicities after intratracheal instillation of ZnO NPs in animals is still needed.

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## Disclosure statement

No potential conflict of interest was reported by the authors.

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## ORCID

*Haibo Li* http://orcid.org/0000-0002-1873-1843
*Zihong Liu* http://orcid.org/0000-0001-5652-5998
*Jingyang Zhou* http://orcid.org/0000-0003-2436-4338
*Tianliang Zhang* http://orcid.org/0000-0003-2951-1317

## References

[1] Bakand S, Hayes A, Dechsakulthorn F. Nanoparticles: a review of particle toxicology following inhalation exposure. Inhalation Toxicol. 2012;24(2):125–135.
[2] Dastjerdi R, Montazer M. A review on the application of inorganic nano-structured materials in the modification of textiles: focus on anti-microbial properties. Colloids Surf B. 2010;79(1):5–18.
[3] Choi SJ, Choy JH. Biokinetics of zinc oxide nanoparticles: toxicokinetics, biological fates, and protein interaction. Int J Nanomed. 2014;9:261–269.
[4] Heng BC, Zhao X, Xiong S, et al. Toxicity of zinc oxide (ZnO) nanoparticles on human bronchial epithelial cells (BEAS-2B) is accentuated by oxidative stress. Food Chem Toxicol. 2010;48(6):1762–1766.
[5] Gao L, Yang ST, Li S, et al. Acute toxicity of zinc oxide nanoparticles to the rat olfactory system after intranasal instillation. J Appl Toxicol. 2013;33(10):1079–1088.
[6] Park HS, Shin SS, Meang EH, et al. A 90-day study of subchronic oral toxicity of 20 nm, negatively charged zinc oxide nanoparticles in Sprague Dawley rats. Int J Nanomed. 2014;9(2):79–92.
[7] Jacobsen NR, Stoeger T, van den Brule S, et al. Acute and subacute pulmonary toxicity and mortality in mice after intratracheal instillation of ZnO nanoparticles in three laboratories. Food Chem Toxicol. 2015;85:84–95.
[8] Osha.gov [Internet]. Washington, DC: Occupational Safety and Health Administration; [updated 2012 Sep 6]. Available from: https://www.osha.gov/dts/chemicalsampling/data/CH_277000.html

[9] National Research Council. Guide for the care and use of laboratory animals. 6th ed. Washington, DC: US Department of Health and Human Services; 1985.
[10] Sayes CM, Reed KL, Warheit DB. Assessing toxicity of fine and nanoparticles: comparing in vitro measurements to *in vivo* pulmonary toxicity profiles. Toxicol Sci. 2007;97(1):163–180.
[11] Geiser M, Kreyling WG. Deposition and biokinetics of inhaled nanoparticles. Part Fibre Toxicol. 2010;7:2. DOI: 10.1186/1743-8977-7-2
[12] Chen H, Dorrigan A, Saad S, et al. *In vivo* study of spherical gold nanoparticles: inflammatory effects and distribution in mice. PloS one. 2013;8(2):e58208. DOI: 10.1371/journal.pone.0058208
[13] Rathore M, Mohanty IR, Maheswari U, et al. Comparative *in vivo* assessment of the subacute toxicity of gold and silver nanoparticles. J Nanopart Res. 2014;16(4):2338. DOI: 10.1007/s11051-014-2338-x
[14] Shiseido. Single dose oral toxicity study of FINEX-50 ZnO in rats. Kanagawa: Safety Assessment Group, Quality Assessment Center, Shiseido Co. Ltd.; 2006
[15] Hu X, Cook S, Wang P, et al. In vitro evaluation of cytotoxicity of engineered metal oxide nanoparticles. Sci Total Environ. 2009;407(8):3070–3072.
[16] Shimada A, Kawamura N, Okajima M, et al. Translocation pathway of the intratracheally instilled ultrafine particles from the lung into the blood circulation in the mouse. Toxicol Pathol. 2006;34(7):949–957.
[17] Nemmar A, Vanbilloen H, Hoylaerts MF, et al. Passage of intratracheally instilled ultrafine particles from the lung into the systemic circulation in hamster. Am J Respir Crit Care Med. 2001;164(9):1665–1668.
[18] Naota M, Shimada A, Morita T, et al. Translocation pathway of the intratracheally instilled C60 fullerene from the lung into the blood circulation in the mouse: possible association of diffusion and caveolae-mediated pinocytosis. Toxicol Pathol. 2009;37(4):456–462.
[19] Takenaka S, Karg E, Kreyling WG, et al. Fate and toxic effects of inhaled ultrafine cadmium oxide particles in the rat lung. Inhalation Toxicol. 2004;16(Suppl 1):83–92.
[20] Chen J, Tan M, Nemmar A, et al. Quantification of extrapulmonary translocation of intratracheal-instilled particles in vivo in rats: effect of lipopolysaccharide. Toxicology. 2006;222(3):195–201.
[21] Vilter RW, Horrigan D, Mueller JF, et al. Studies on the relationships of vitamin B12, folic acid, thymine, uracil and methyl group donors in persons with pernicious anemia and related megaloblastic anemias. Blood. 1950;5(8):695–717.
[22] Donaldson K, Stone V, Seaton A, et al. Ambient particle inhalation and the cardiovascular system: potential mechanisms. Environ Health Perspect. 2001;109(Suppl 4):523–527.
[23] Zhu MT, Feng WY, Wang Y, et al. Particokinetics and extrapulmonary translocation of intratracheally instilled ferric oxide nanoparticles in rats and the potential health risk assessment. Toxicol Sci. 2009;107(2):342–351.
[24] Liberda EN, Cuevas AK, Qu Q, et al. The acute exposure effects of inhaled nickel nanoparticles on murine endothelial progenitor cells. Inhalation Toxicol. 2014;26(10):588–597.
[25] Wang B, Feng WY, Wang TC, et al. Acute toxicity of nano- and micro-scale zinc powder in healthy adult mice. Toxicol Lett. 2006;161(2):115–123.
[26] Li H, Zhang T, Liang GY, et al. *In vivo* evaluation of acute toxicity of water-soluble carbon nanotubes. Toxicol Environ Chem. 2011;93(3):603–615.
[27] Zheng J, Zhang G, Lu Y, et al. Effect of pulmonary surfactant and phospholipid hexadecanol tyloxapol on recombinant human-insulin absorption from intratracheally administered dry powders in diabetic rats. Chem Pharm Bull. 2010;58(12):1612–1616.
[28] Ho E, Karimi Galougahi K, Liu CC, et al. Biological markers of oxidative stress: Applications to cardiovascular research and practice. Redox Biol. 2013;1:483–491.
[29] Steinritz D, Möhle N, Pohl C, et al. Use of the Cultex® Radial Flow System as an in vitro exposure method to assess acute pulmonary toxicity of fine dusts and nanoparticles with special focus on the intra- and inter-laboratory reproducibility. Chem Biol Interact. 2013;206(3):479–490.
[30] Nel A, Xia T, Madler L, et al. Toxic potential of materials at the nanolevel. Science. 2006;311(5761):622–627.
[31] Lam HF, Conner MW, Rogers AE, et al. Functional and morphologic changes in the lungs of guinea pigs exposed to freshly generated ultrafine zinc oxide. Toxicol Appl Pharm. 1985;78(1):29–38.
[32] Cooper RG. Zinc toxicology following particulate inhalation. Indian J Occup Environ Med. 2008;12(1):10–13.