



Zinc oxide nanoparticles induce eosinophilic airway inflammation in mice



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HIGHLIGHTS

- ZnO nanoparticles induced eosinophilic airway inflammation in a murine asthma model.
- ZnO nanoparticles without allergen application induced the expression of Th2 cytokines.
- Zinc ions did not induce eosinophilia in the BALF of mice.

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ABSTRACT

Zinc oxide nanoparticles (ZnO NPs) have been widely used in industry. The metal composition of PM_{2.5} might contribute to the higher prevalence of asthma. To investigate the effects of ZnO NPs on allergic airway inflammation, mice were first exposed to different concentrations of ZnO NPs (0.1 mg/kg, 0.5 mg/kg) or to a combination of ZnO NPs and chicken egg ovalbumin (OVA) by oropharyngeal aspiration on day 0 and day 7 and then were sacrificed 5 days later. The subsequent time course of airway inflammation in the mice after ZnO NPs exposure was evaluated on days 1, 7, and 14. To further determine the role of zinc ions, ZnCl₂ was also administered. The inflammatory cell count, cytokine levels in the bronchoalveolar lavage fluid (BALF), and lung histopathology were examined. We found significant neutrophilia after exposure to high-dose ZnO NPs on day 1 and significant eosinophilia in the BALF at 7 days. However, the expression levels of the T helper 2 (Th2) cytokines IL-4, IL-5, and IL-13 increased significantly after 24 h of exposure to only ZnO NPs and then decreased gradually. These results suggested that ZnO NPs could cause eosinophilic airway inflammation in the absence of allergens.

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1. Introduction

Zinc oxide nanoparticles (ZnO NPs) are widely used in the manufacture of paints, coatings, cosmetics, sunscreens, and personal hygiene products as well as in biomedicine. The adverse health effects of zinc oxide were first described as metal fume fever in workers exposed to welding fumes or ZnO particles [1,2]. A previous study indicated that zinc exposure might be a cause of occupational asthma [3]. Adamson et al. found that among metals and metal

oxide nanoparticles present in air pollution, zinc might be a crucial factor in lung inflammation [4].

Atmospheric zinc is emitted from incinerators, motor vehicles, and various industries, and it exists primarily in an oxidized form (i.e., ZnO) [5]. The size of aerosolized ZnO particles is determined by the emission source, and most particles are sufficiently small to be inhaled [6]. In an animal study, inhalation of occupationally relevant ZnO NPs caused neutrophilic inflammation in the lungs of rats [7]. Infusing ZnO NPs into mice increased the concentrations of eotaxin and interleukin-13 (IL-13) in the bronchoalveolar lavage fluid (BALF), and eosinophils were recruited to the alveolar interstitium [8].

Asthma is a chronic inflammatory disease of the airways and is the most common chronic disease of children. As shown in related

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studies, asthma is a worldwide problem. The global prevalence rates of asthma range from 1% to 18% of the population of a given country [9]. The International Study of Asthma and Allergies in Childhood showed that the prevalence of asthma symptoms varies significantly worldwide, even within genetically similar groups [10]. This finding suggests that environmental factors could be responsible for some of this variation.

There has been abundant epidemiologic evidence that air pollution can exacerbate asthma [11–14]. However, whether air pollution contributes to asthma onset remains uncertain. The Southern California Children's Health cohort study suggested that exposure to air pollution might be related to new-onset asthma [15]. A cross-sectional epidemiological study in Germany suggested that the composition of particulate matter might contribute to the higher asthma prevalence in that area [16]. Although it remains unclear which component of PM is responsible for certain biological effects, it was shown that high ambient air PM_{2.5} zinc levels in combination with other components were associated with increased pediatric asthma morbidity [17].

Although many inflammatory pathways are involved in the development of asthma, eosinophils play an important role in the pathogenesis of the disease [18]. In an *in vitro* study, ZnO NPs had immunological effects on mouse antigen-presenting cells [19]. A previous animal study suggested that ZnO has an adjuvant effect on the Th2, but not the Th1, immune response [20].

Although epidemiological and animal studies have suggested that ZnO exposure might be related to asthma development, there have been no animal experiments to determine the causative role and pathological mechanisms of ZnO in asthma. We hypothesized that exposure to ZnO NPs would cause allergic airway inflammation in mice or would have an adjuvant effect. In the present study, we evaluated the effects of ZnO NPs on allergic airway inflammation.

2. Materials and methods

2.1. Animals

Seven-week-old female Balb/c mice (19–21 g) obtained from the National Laboratory Animal Center (Taipei, Taiwan) were maintained under a 12 h light/dark cycle at a constant temperature of $22 \pm 2^\circ\text{C}$ in $55 \pm 10\%$ relative humidity throughout the study. The animals were housed in plastic cages and had *ad libitum* access to LabDiet 5001 (PMI Nutrition International, Brentwood, MO, USA) food and water. The animals were acclimated for one week prior to initiation of the study. All of the animal experiments were approved by the Laboratory Animal Center of the National Taiwan University and Institutional Animal Care and Use Committee (IACUC), Taipei Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation (Taiwan).

2.2. Preparation of ZnO NPs and ZnCl₂ solutions

To minimize the aggregation of ZnO NPs, phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) (Gibco Life Technologies, USA) by weight was used as the vehicle control (VEH) as described in previous experiments [21]. ZnO NPs (average diameter <50 nm; average surface area 10.8 m²/g) were purchased from Sigma–Aldrich (St. Louis, MO, USA). ZnO NPs were weighed using an analytical mass balance and then suspended in VEH at a final concentration of 40 µg/mL (0.1 mg/kg; low-dose) or 200 µg/mL (0.5 mg/kg; high-dose). The suspensions were shaken and sonicated for 30 min to ensure adequate mixing and stored at 4 °C. To determine the free Zn²⁺ effect, ZnCl₂ was purchased from Sigma–Aldrich and was prepared in PBS to match the equivalent Zn²⁺ concentration in the 0.5 mg/kg dose of ZnO NPs. The ZnO

NPs and ZnCl₂ solution were sonicated for 10 min and then were vortexed before oropharyngeal aspiration.

2.3. Characterization of ZnO NPs

The prepared samples were shaken and filtered through 0.1 µm filters to remove any particulates from the samples and were warmed to room temperature for at least 1 h. The hydrodynamic diameter and zeta potential of the ZnO NPs were determined using Malvern dynamic light scattering (DLS; Malvern Zetasizer Nano ZS, UK) at the Center for Nano Science and Technology, National Taiwan University. To determine the ZnO NPs morphology, the prepared solutions were carbon coated to an average thickness of 10 nm using a sputter coater (Bio-Rad, UK). The morphologies of the ZnO NPs were observed using a Nova NanoSEM 230 (FEI, Hillsboro, OR, USA) at an accelerating voltage of 5 kV with a 3.0 spot size.

2.4. Experimental design

2.4.1. Experiment A. The role of different concentrations of ZnO NPs with and without OVA on airway inflammation

The mice were divided into the following six groups: VEH; OVA; low-dose ZnO NPs (0.1 mg/kg); high-dose ZnO NPs (0.5 mg/kg); low-dose ZnO NPs + OVA; and high-dose ZnO NPs + OVA. OVA derived from chicken egg is a frequently used allergen that induces a robust allergic inflammation [22]. On days 0 and 7, the mice were exposed to 50 µL of the appropriate solution by oropharyngeal aspiration under light anesthesia (Sevoflurane; Abbott Laboratories, UK). Briefly, each mouse was held vertically, and the tongue was gently pulled out of the mouth with forceps. The solution (50 µL) was pipetted to the oropharynx when the mouse's nose was closed. By forcing the mouse to breathe through the mouth, the solution was aspirated to the lower airways [23]. For the ZnO NPs + OVA group, Balb/c mice were treated with 50 µL of ZnO NPs 1 h before they were treated with 10 µg/50 µL OVA. The animals were sacrificed 5 days after the second treatment. Tissue samples, including blood, bronchoalveolar lavage fluid (BALF) and lung, were collected (Fig 1A).

2.4.2. Experiment B. Time course of inflammatory events following ZnO NPs and zinc ion exposure

To evaluate the time course of the consequent allergic inflammation and further determine the role of zinc ions on allergic airway inflammation, we conducted a separate experiment. The mice were sensitized with VEH, OVA, low-dose ZnO NPs (0.1 mg/kg), high-dose ZnO NPs (0.5 mg/kg), or ZnCl₂ on days 0 and 7 and then sacrificed at 24 h, 1 week, or 2 weeks after exposure (Fig 1B).

2.5. Determination of total serum IgE

We obtained blood samples from the facial veins of the mice. Total serum IgE was measured using a Mouse IgE ELISA Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Briefly, a predetermined concentration of capture anti-IgE Ab was coated onto ELISA plates, and the plates were incubated at 4 °C overnight. The solution was aspirated, and the plates were washed 3 times with buffer solution; subsequently, diluted serum (50×) was added, and the plates were incubated at room temperature for 2 h. The working detector solution was added, and the plates were incubated at room temperature for 1 h. Enzyme activity was evaluated using tetramethylbenzidine (TMB) and hydrogen peroxide as substrates and monitored on an ELISA reader (BioTek Instruments, Inc., VT, USA) at 450 nm with λ correction at 570 nm within 30 min of adding the stop solution. The absorbance readings were corrected to ng/mL using values obtained from standard curves.

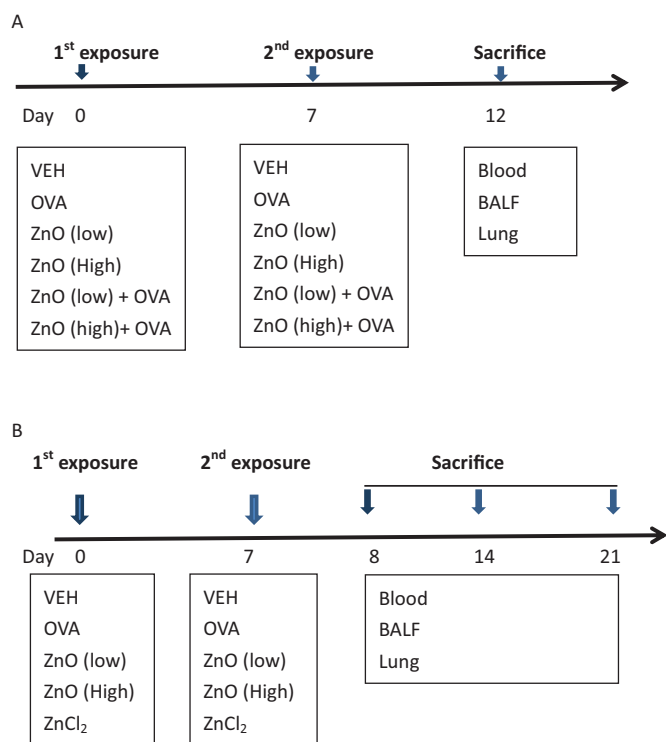


Fig. 1. Overview of the two experimental designs for ZnO NPs exposure in mice. (A) The mice were divided into the following six groups: 0.5% (wt) BSA in PBS (vehicle control, VEH); OVA (allergic control); low-dose ZnO NPs (0.1 mg/kg); high-dose ZnO NPs (0.5 mg/kg); low-dose ZnO NPs + OVA; and high-dose ZnO NPs + OVA. On days 0 and 7, the mice were exposed to 50 μ L of the appropriate solution by oropharyngeal aspiration under light anesthesia. The animals were sacrificed 5 days after the 2nd exposure. (B) The mice were exposed to VEH, OVA, low-dose ZnO NPs, high-dose ZnO NPs, or ZnCl₂ on days 0 and 7 and then sacrificed 24 h, 1 week, or 2 weeks after the 2nd exposure. Tissue samples, including blood, bronchoalveolar lavage fluid (BALF) and lung, were collected at each time point.

2.6. Preparation of bronchoalveolar lavage fluid (BALF)

The animals were sacrificed using an overdose of 0.1 mL sodium pentobarbital (200 mg/mL) by intraperitoneal injection. The trachea was clearly visualized after skin and soft tissue dissection, and then a 20 gauge cannula was inserted into the trachea. A single 1 mL syringe of PBS was used to lavage the lungs bilaterally. The lavage fluid was retrieved by gentle lavage. This procedure was repeated three times. The initially collected lavage fluid sample was cooled to 4 °C and centrifuged at 1500 $\times$ g for 5 min. We collected the supernatants and stored them at –80 °C for subsequent analysis of biochemical markers and cytokine levels.

2.7. Determination of inflammatory cell profiles in BALF

The cell pellet from the first lavage fluid was combined with the subsequent lavages to count inflammatory cells. Total cell counts were determined from fresh fluid specimens using a hemocytometer after trypan blue staining. Differential cell counts were measured on cytological slides, which were prepared with the use of an SP-1000 Slide Maker Stainer (Sysmex, Lincolnshire, IL, USA) and stained with ASK Liu's stain A and B (Tonyar Biotech, Taiwan). The slides were prepared and examined in a blinded manner by an experienced laboratory scientist.

2.8. Determination of inflammatory cytokines in BALF

Quantification of the Th proinflammatory cytokines IL-4, IL-5, IL-6, IL-10, IL-13, interferon- γ (IFN- γ) and tumor necrosis factor- α

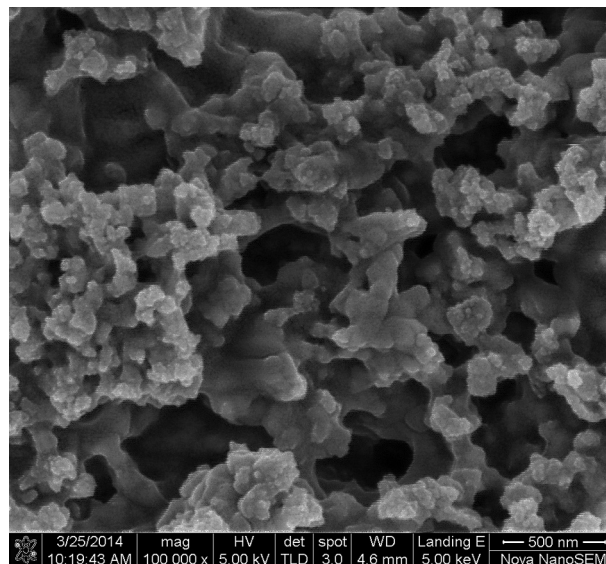


Fig. 2. Scanning electron microscopy (SEM) photograph of high-dose 50 nm ZnO NPs.

(TNF- α) in the BALF was performed with a cytometric bead array mouse enhanced sensitivity flex set system according to the manufacturer's instructions (BD BioSciences, San Diego, CA, USA). The sensitivity of these assays was 0.274 pg/mL.

2.9. Determination of total protein and lactic dehydrogenase levels in BALF

The total protein concentration was determined using a total protein assay kit (Bio-Rad Hercules, CA, USA) with BSA as the standard according to the manufacturer's protocol. LDH levels were determined with a Spotchem autoanalyzer SP-4410 (Arkray, Kyoto, Japan) at the Laboratory Animal Center, College of Medicine, National Taiwan University.

2.10. Lung histology

The left lung lobes were excised and fixed with 10% neutral phosphate-buffered formalin, embedded in paraffin, sectioned at 5 μ m thickness and stained with hematoxylin/eosin (H&E). The histological samples were examined in a blinded manner under light microscopy by an experienced histopathologist.

2.11. Statistical analysis

All of the results are expressed as the mean $\pm$ SEM. Differences between groups were evaluated by analysis of variance (ANOVA), and Student's *t*-test was used to distinguish between pairs of groups. All of the statistical analyses in this study were performed with SAS software (SAS Institute, Cary, NC, USA), version 9.3. The level of statistical significance was set at *p* < 0.05.

3. Results

3.1. Characterization of ZnO NPs

The hydrodynamic sizes of the 50 nm ZnO NPs suspended in VEH were 181.5 $\pm$ 18.8 nm (low-dose) and 360.0 $\pm$ 7.4 nm (high-dose). The zeta potentials of the ZnO NPs were –15.0 $\pm$ 1.5 mV (low-dose) and –16.7 $\pm$ 1.8 mV (high-dose). The representative morphology of the high-dose ZnO NPs in VEH is shown in Fig. 2. ZnO NPs in 0.5% (wt) BSA vehicle formed cubic agglomerates with several shape

Table 1

Total cell and eosinophil count in the BALF and total serum IgE levels 5 days after the 2nd oropharyngeal aspiration.

Group	n	Total cell (10 ³ /mL)	Eosinophil (10 ³ /mL)	IgE (ng/mL)
VEH	8	17.4 ± 1.9	0.1 ± 0.1	990.0 ± 202.8
OVA	6	20.8 ± 1.4	3.7 ± 2.2	1207.3 ± 296.3
ZnO (low)	5	70.8 ± 18.0*	2.6 ± 1	707.6 ± 176.3
ZnO (high)	8	59.4 ± 9.2*	26.1 ± 9.2*	1344.1 ± 279.1
ZnO (low) + OVA	5	82.8 ± 26.1*	45.8 ± 21.1*	923.4 ± 137.7
ZnO (high) + OVA	6	72.9 ± 11.9*	50.1 ± 10.6*	1596.2 ± 170.8

The mice were exposed to 50 μ L solutions by oropharyngeal aspiration on days 0 and 7, according to the study protocol in the Section 2. VEH: vehicle control. Values are the mean $\pm$ SEM.

* $p < 0.05$, compared with the VEH control group.

variations. The zeta potential results suggested that such agglomerates would remain stable. Our results were similar to those reported in a previous study [21].

3.2. Total cell and eosinophil count in BALF and total serum IgE levels

The inflammatory total cell and eosinophil count analysis of the BALF five days after the second exposure is shown in Table 1. The total cell count was significantly increased in the BALF of mice exposed to low-dose ZnO NPs, high-dose ZnO NPs, low-dose ZnO NPs + OVA, and high-dose ZnO NPs + OVA compared with the

control group. The eosinophil count was significantly elevated in the BALF of mice exposed to high-dose ZnO NPs, low-dose ZnO NPs + OVA, and high-dose ZnO NPs + OVA. In addition, eosinophilia was more prominent after exposure to the combination of ZnO NPs + OVA. Total serum IgE levels were not significantly increased following oropharyngeal aspiration of different doses of ZnO NPs or combinations of ZnO NPs and OVA (Table 1).

3.3. Time course of the inflammatory cell profile in BALF

Inflammatory cells in the BALF over time are shown in Fig. 3. The numbers of total cells and neutrophils increased 24 h after exposure to OVA, high-dose ZnO NPs or ZnCl₂ and then decreased gradually. The BALF neutrophilia was resolved by day 7 (Fig. 3A and B). The number of lymphocytes and eosinophils increased gradually and reached a maximum 7 days after exposure to high-dose ZnO NPs (Fig. 3C and D). The BALF eosinophilia lasted through day 14 in the high-dose ZnO NPs group. BALF eosinophilia was apparent at day 7 after the 2nd exposure to OVA and low-dose ZnO NPs, although it was not statistically significant (Fig. 3D).

3.4. Time course of the inflammatory cytokine profile in BALF

Cytokine expression in the BALF over time is shown in Fig. 4. The levels of IFN- γ , TNF- α , IL-4, IL-5, IL-6, and IL-13 increased significantly after high-dose ZnO NPs exposure, reaching a maximum at 24 h and then decreasing rapidly (Fig. 4A–F). The level of IL-5 in the

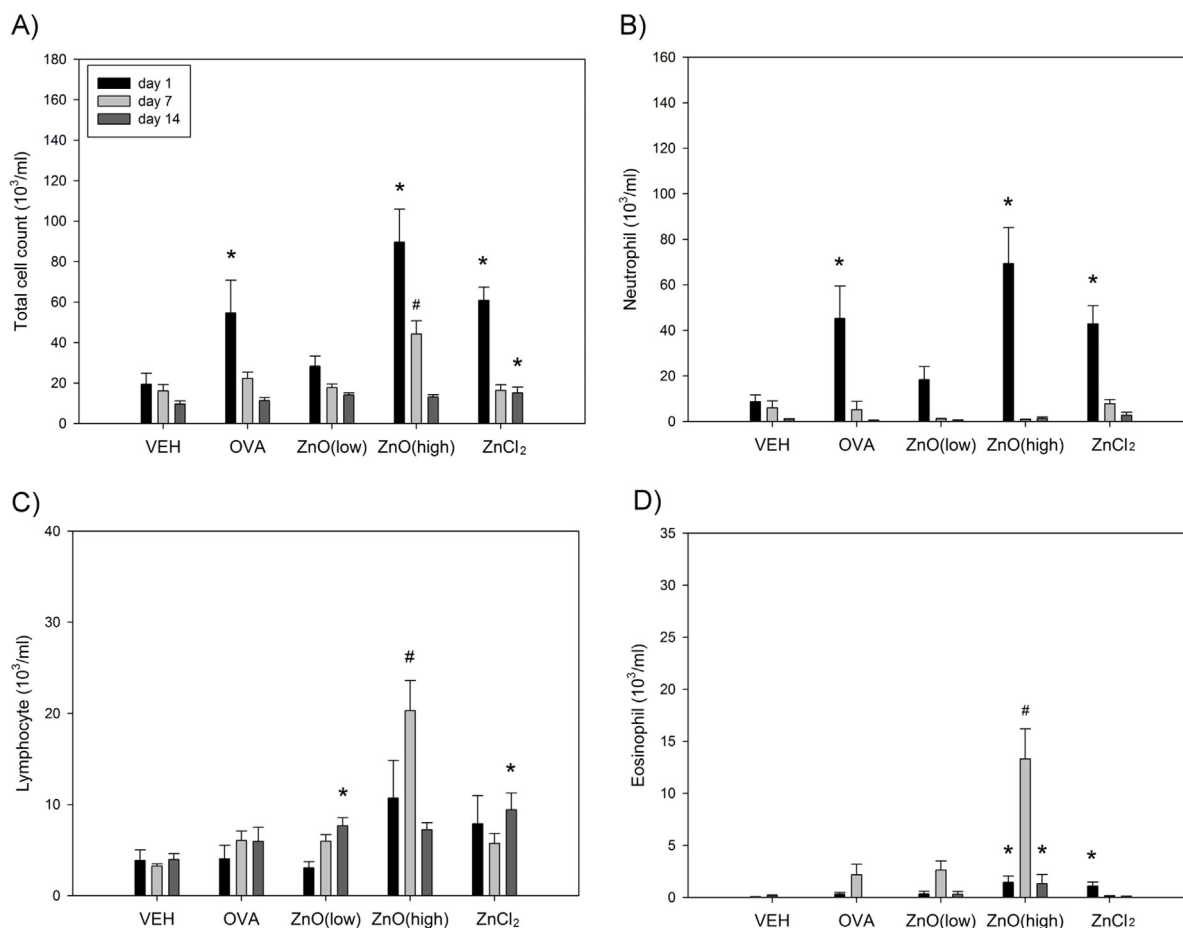


Fig. 3. Time course of the inflammatory cell profile in the BALF following the 2nd treatment. A: Total cells; B: Neutrophils; C: Lymphocytes; D: Eosinophils. The mice were exposed to 50- μ L solutions by oropharyngeal aspiration on days 0 and 7 as described in the study protocol in Section 2. VEH: vehicle control. The data are presented as the mean $\pm$ SEM ($n = 8$ for each treatment group). * $p < 0.05$, compared with the VEH group; # $p < 0.05$, compared with the other groups at the same time point.

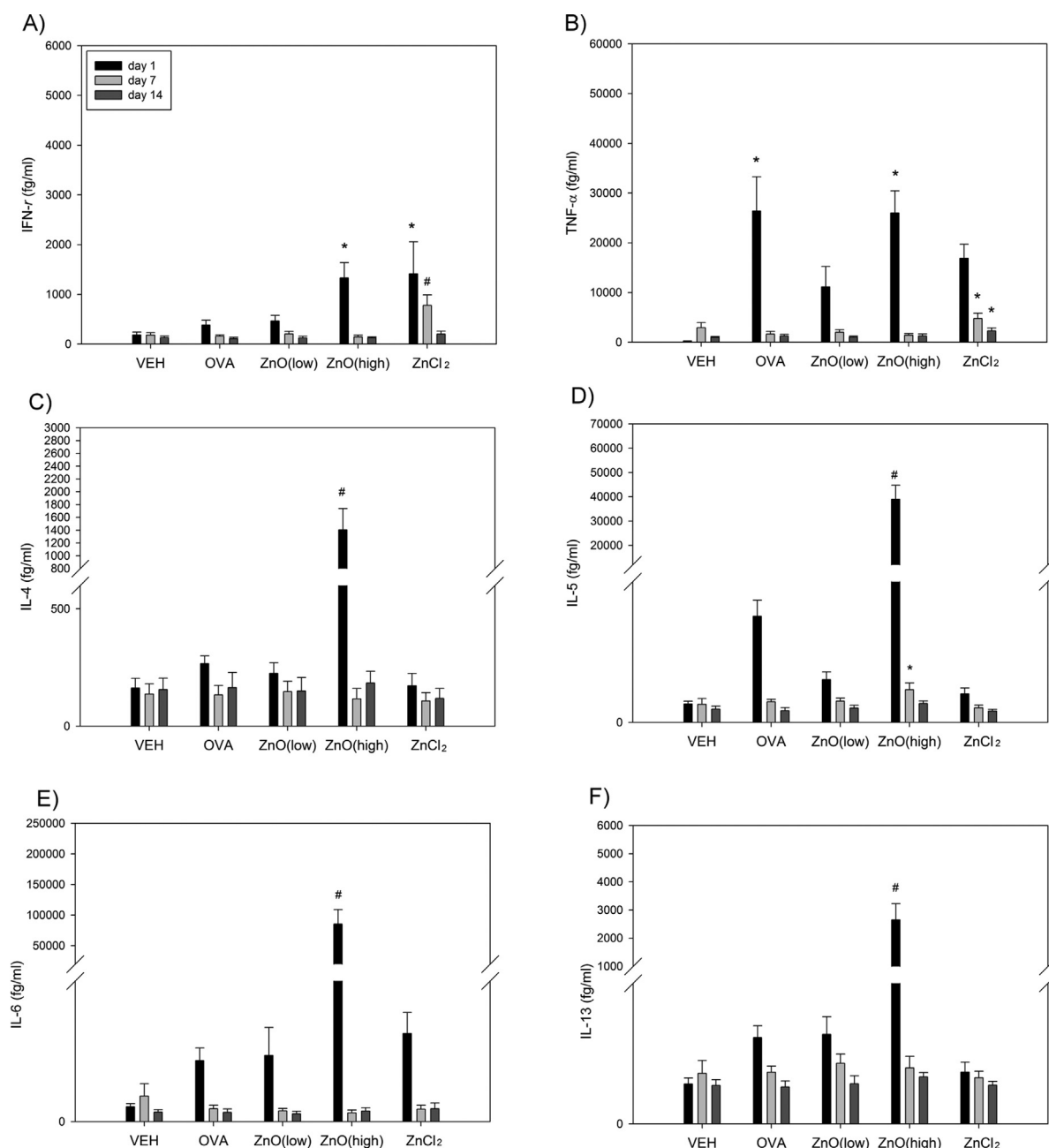


Fig. 4. Time course of the BALF cytokine levels following the 2nd treatment. A: IFN- γ ; B: TNF- α ; C: IL-4; D: IL-5; E: IL-6; F: IL-13. The mice were exposed to 50- μ L solutions by oropharyngeal aspiration on days 0 and 7 as described in the study protocol in the Section 2. VEH: vehicle control. The data are presented as the mean $\pm$ SEM ($n = 6-8$ for each treatment group). * $p < 0.05$, compared with the VEH control group; # $p < 0.05$, compared with the other groups at the same time point.

BALF remained significantly higher than that in the control group seven days after exposure to high-dose ZnO NPs (Fig 4D). Increases in BALF T helper cell type 1 (Th1) cytokines (IFN- γ and TNF- α) were observed after ZnCl₂ exposure (Fig 4A and B). An increase in IL-5, a BALF T helper cell type 2 (Th2) cytokine, was observed 24 h after OVA exposure, although this increase was not statistically significant (Fig 4D).

3.5. Time course of total serum IgE and total protein levels in BALF

Total serum IgE levels over time were not significantly increased after OVA or ZnO NPs exposure. Total BALF protein levels were significantly increased at 1 and 7 days after high-dose ZnO NPs exposure (Fig 5).

3.6. Histological analysis

Representative images of H and E-stained lung tissue are shown in Fig. 6. Microscopically, the lung tissue exhibited various pathological changes five days after aspirating high-dose ZnO NPs. Eosinophilic inflammation, airway epithelial cell regenerative proliferation, increased bronchial muscle wall thickness, alveolar interstitial fibrosis and infiltration of inflammatory cells into the alveolar spaces were the predominant observations. Representative images from the VEH group are presented in Fig. 6A. Aspirating OVA induced mild eosinophil infiltration in peribronchial interstitial tissues (Fig 6B). Exposure to low-dose ZnO NPs resulted in a mild increase in lymphocytes and scant eosinophils (Fig 6C). There were mild increases in lymphocytes and eosinophils in the low-dose ZnO NPs + OVA group (Fig 6E). After treatment with high-dose

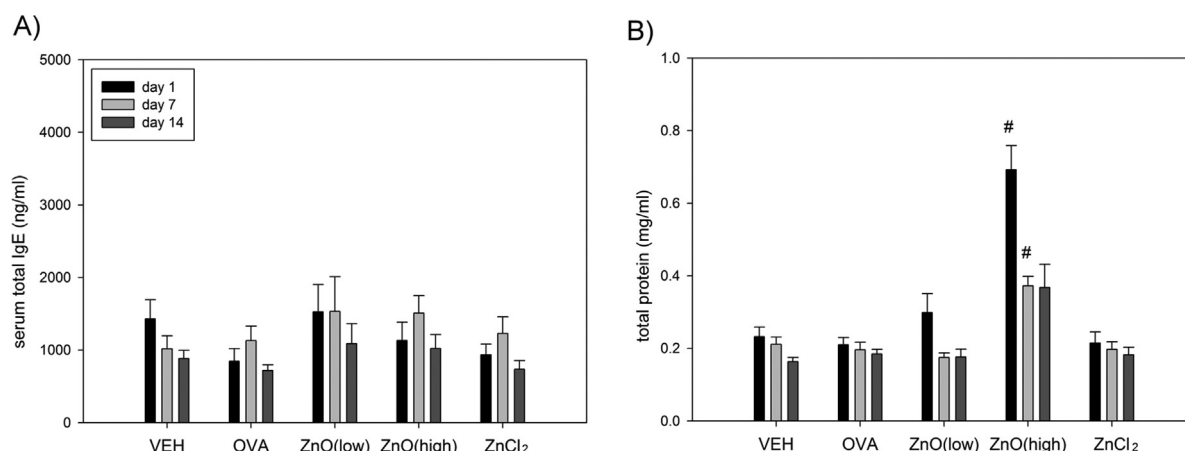


Fig. 5. Time course of total serum IgE and total protein BALF levels following the 2nd treatment. A: Total serum IgE; B: Total protein. The mice were exposed to 50- μ L solutions by oropharyngeal aspiration on days 0 and 7 as described in the study protocol in the Section 2. VEH: vehicle control. The data are presented as the mean $\pm$ SEM ($n = 8$ for each treatment group). [#] $p < 0.05$, compared with the other groups.

ZnO NPs or high-dose ZnO NPs + OVA, there were marked increases in eosinophil infiltration in peribronchial, peribronchiolar, and perivascular interstitial tissues and alveolar spaces, consistent with the cytological analysis (Fig 6D and F).

4. Discussion

In the present study, we observed significant eosinophilia in the BALF 5 days after exposing mice to high-dose ZnO NPs. The eosinophilia was more prominent upon exposure to a combination of ZnO NPs and OVA. Because ZnO NPs without OVA appeared to induce eosinophilic airway inflammation, additional experiments were performed using low or high doses of ZnO NPs to more accurately determine the time course of inflammatory events. To further determine the role of zinc ions in allergic airway inflammation, highly water soluble ZnCl₂ was also administered for comparison to ZnO NPs exposure. Our time course study showed a significant increase in the total number of inflammatory cells and neutrophils one day after exposure to OVA, high-dose ZnO NPs, or ZnCl₂, followed by a decrease 7 days later. The mice exposed to high-dose ZnO NPs showed significant eosinophilia and lymphocytosis in the BALF 7 days after exposure. We also found that IL-4, IL-5, IL-6, and IL-13 were produced early after exposure to ZnO NPs.

In our study, mice were exposed to doses of up to 20 μ g of ZnO NPs per mouse by oropharyngeal aspiration. This dose was estimated to be equivalent to the exposure of workers over 20 days of inhalation for 8 h/day at a concentration of 5 mg/m³ in an earlier single-walled carbon nanotube study [24]. Although the average concentration of Zn in ambient air is approximately 0.1–30.0 μ g/m³ [6], several hundred-fold higher concentrations might occur near industrial areas. Thus, the doses tested in our study were not unreasonable for human occupational and environmental exposure levels to ZnO NPs.

The OVA-induced murine asthma model demonstrated important features of allergic asthma, including increases in eosinophil counts and Th2 (IL-4, IL-5, IL-13) cytokines, which have been widely used to study the impact of environmental factors on asthma development [25]. Motorcycle exhaust particles (MEPs) induce airway inflammation, including infiltration of eosinophils, neutrophils, lymphocytes, and macrophages; enhancement of IL-4, IL-5, and IFN- γ levels in the BALF; and production of serum IgE in Balb/c mice [26]. An OVA-induced mouse model of allergic airway disease validated the results from an epidemiological study indicating that PM_{2.5} composition might be a causative factor in the increased prevalence and severity of allergic disease in an

industrial city [27]. In addition, the inhalation of ambient ultra-fine particles enhanced the secondary immune response to OVA in sensitized mice, suggesting that exposure to vehicle traffic exhaust might exacerbate allergic inflammation in asthmatic subjects [28]. Furthermore, ambient ultrafine particulate matter (UFP) had an adjuvant effect on allergic sensitization [29].

Because eosinophils play an important role in the pathogenesis of asthma, we modified the sensitization phase of the OVA-induced murine asthma model. The life span of eosinophils ranges from 2 to 5 days in the absence of stimulation. However, cytokines prolong eosinophil survival *in vivo* [30]. We initially evaluated eosinophilic airway inflammation 5 days after exposure. To evaluate the appearance of cytokines and eosinophils in the BALF over time, the mice were sacrificed 24 h, 7 days, or 14 days after exposure in separate experiments.

In our time course study, we observed that IL-4, IL-5, and IL-13 were produced early after exposure to ZnO NPs, and these molecules are key mediators of asthma development. Among these mediators, IL-5 exerts an important role in eosinophil proliferation, differentiation, maturation, migration, and survival [31]. IL-5 also played an obligatory role in airway remodeling in an OVA-induced murine asthma model [32]. In our study, eosinophils were difficult to find in the BALF 24 h after exposure but were easily detected 7 days after exposure. However, IL-5 was rapidly produced within 24 h after exposure and then decreased gradually. This observation was also noted by Trifilieff et al. when animals were given a single exposure of allergen in a challenge phase [33].

Cho et al. found that ZnO NPs induced eosinophilia in the BALF of rats after a single intratracheal aspiration. The effects were similar regardless of the source of ZnO NPs or the dispersal method, but micrometer-sized ZnO particles did not generate this response [34]. However, eosinophils were not found in the BALF 24 h after exposure to ZnO NPs, although they were present in the alveolar interstitium, and increased expression of eotaxin and IL-13 in the BALF of mice was also noted. The authors suggested that these molecules are the key mediators of the pathological effects of ZnO NPs exposure [8]. The results of our study support the finding that ZnO NPs cause pulmonary eosinophilia. However, Cho et al.'s study showed neither eosinophils in the BALF of mice nor a relationship between eosinophil counts and the production of IL-5 in the airways, which might have been due to the single exposure to 31 μ g/100 μ L ZnO NPs via aspiration without any prior sensitization and the single time point analyzed (24 h). In our study, the mice were sensitized with 10 μ g/50 μ L ZnO NPs on days 0 and 7 and were sacrificed at several time points.

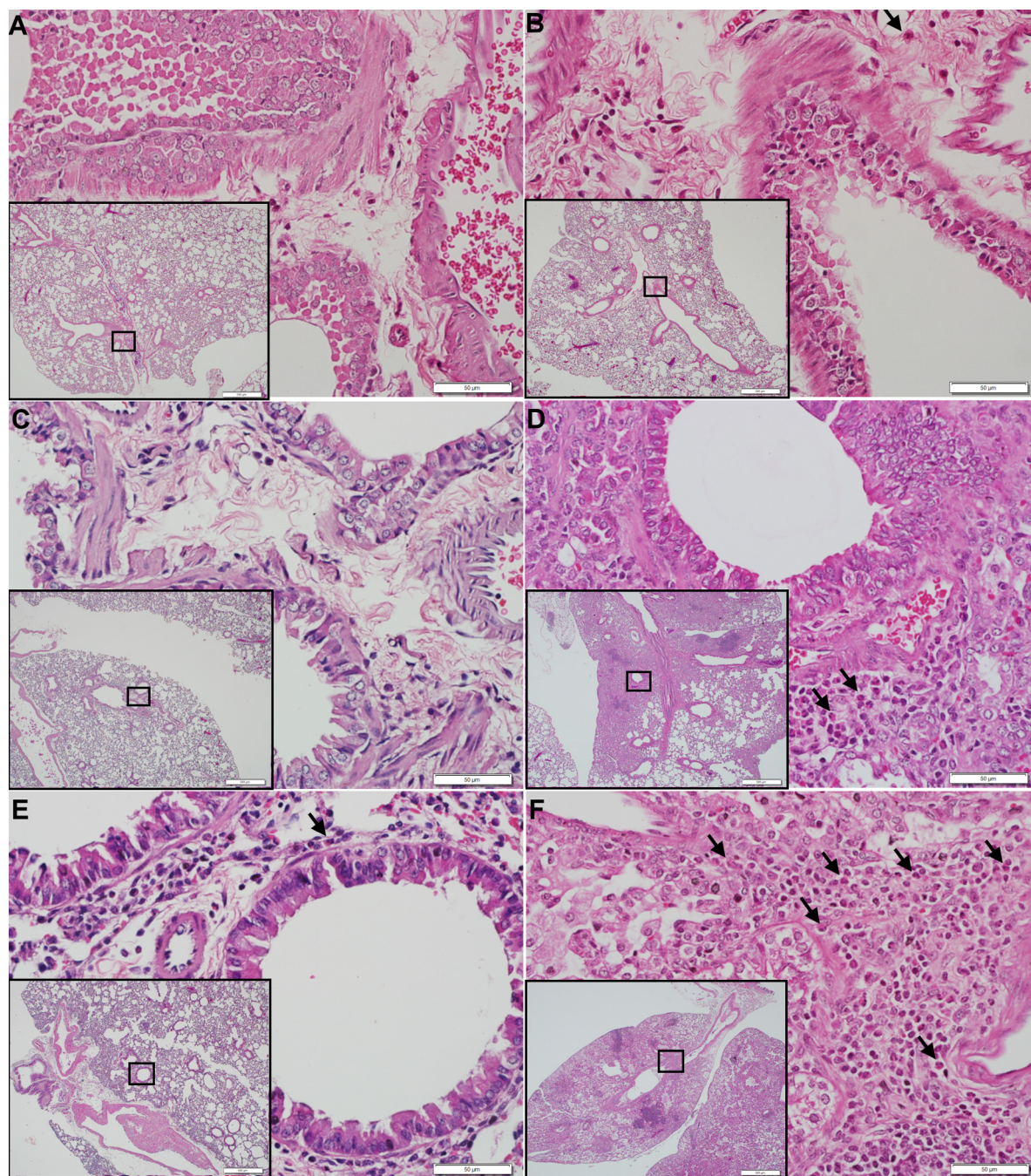


Fig. 6. Representative histology of lung tissue 5 days after the 2nd oropharyngeal aspiration. Lungs were excised and fixed with formaldehyde, and the slides were stained with hematoxylin and eosin (H&E). Scale bars: 50 μm at 400 $\times$ magnification and 500 μm at 40 $\times$ magnification (inset). The experimental groups are identical to those in Fig. 1A: (A) VEH; (B) OVA: mild increase in eosinophilic infiltration in the peribronchial areas; (C) low-dose ZnO NPs: mild increase in lymphocytes and scant eosinophils; (D) high-dose ZnO NPs: marked increase in eosinophil infiltration in peribronchial, peribronchiolar, and perivascular interstitial tissues; (E) low-dose ZnO NPs + OVA: moderate increase in lymphocytes and eosinophils; (F) high-dose ZnO NPs + OVA: marked increase in eosinophil infiltration in peribronchial, peribronchiolar, and perivascular interstitial tissues. The boxed part of the image at 40 $\times$ magnification is also presented at 400 $\times$ magnification. Arrows indicate eosinophils.

We failed to detect increased production of total serum IgE after exposure to OVA or ZnO NPs. We also observed only slight eosinophilia in the BALF following sensitization with OVA. To compare the effects of ZnO NPs on sensitization, oropharyngeal aspiration of low-dose OVA (10 μg) was used as a sensitizer in our study. Previous studies have been published using low-dose intranasal or oropharyngeal OVA sensitization, and these studies have reported similar results [27,35]. One possible explanation

is that the optimal sensitizing dose for antibody production is higher than the dose for airway inflammation and cytokine production [36]. Another explanation is that ZnO NPs elicit IgE-independent, cell-mediated, and mixed inflammatory reactions similar to low molecular weight (LMW) agents in the development of occupational asthma. In discussions of the possible mechanisms of LMW sensitizer-induced occupational asthma, the chemical structures, intensity and route of exposure, gene-environment

interactions (epigenetic mechanisms), hapten–protein conjugates (IgE-dependent), and mixed Th1/Th2 reactions have been proposed [37]. In our time-course study, elevated levels of Th1 and Th2 cytokines in the BALF were noted 24 h after exposure to ZnO NPs. However, only the production of IL-5 remained elevated one week after exposure. This mixed Th1/Th2 cytokine production was similar to the occupational asthma response caused by western red cedar [38].

To study the possible mechanism of the effects of ZnO NPs exposure on airway inflammation, Cho et al. suggested that pulmonary eosinophilia induced by ZnO NPs was mediated by soluble Zn^{2+} [8]. In addition, Zn^{2+} release from ZnO NPs was the most important factor in inducing oxidative stress [39]. However, another study conducted by Cho et al. found that ZnO NPs recruited eosinophils and neutrophils, but ZnO aqueous extract only induced mild neutrophilic inflammation [40], suggesting that ZnO NPs recruit eosinophils to the lungs. In our study, high levels of water soluble $ZnCl_2$ were used to study the role of Zn^{2+} in lung inflammation. We found neutrophilic but not eosinophilic lung inflammation associated with increased expression of the Th1 cytokines IFN- γ and TNF- α in mice treated with $ZnCl_2$, which are similar to the results from a study reported by Cho et al. It appears that Zn^{2+} released from ZnO NPs plays an important role in cell toxicity but not eosinophilic inflammation.

With the development of nanotechnology and large-scale consumer products containing nanoparticles, understanding the potential role of exposure to nanoparticles from occupational and environmental sources is of increasing concern. Animal studies have suggested that carbon nanotubes [41] and nano-structured titanium dioxide particles [42] promote allergic immune responses in mice. Brandenberger et al. found that engineered silica nanoparticles could act as adjuvants to enhance allergic airway disease in mice [43]. Hussain et al. showed that gold nanoparticles could aggravate airway hyperreactivity and inflammatory responses in TDI-sensitized mice [44]. These studies suggested that nanoparticles, such as ambient ultrafine particles, could modulate airway inflammation and could sensitize or exacerbate asthma. Our study found that ZnO NPs without OVA sensitization induced eosinophilic airway inflammation in mice. To the best of our knowledge, few studies have investigated the effect of inhaled NPs in the absence of allergens that might cause the development of allergic airway inflammation.

There are many cases in which the biological activities of nanoparticles are greater than those of micro-scale particles, even when they have similar chemical formulas [45,46], and this phenomenon has been reported for eosinophilic inflammation caused by ZnO NPs [34] and particulate matter [29]. It has been suggested that size is one of the key factors influencing the biologic effects of NPs. Similar mass particles of different sizes have different toxic effects on the lungs because smaller particles can be deposited in the distal airway, where they encounter different types of cells and can translocate across cell membranes and even into nuclei. Thus, nanoparticles might interact with biomolecules such as proteins, lipids and nucleic acids and modulate the immune response [47]. Palomaki et al. reported that ZnO NPs had a direct influence on antigen-presenting cells [19].

In the present study, ZnO NPs increased total BALF protein levels but did not increase LDH levels (Table A.1). Whether the elevation of BALF protein levels was caused by allergic inflammation, which induces plasma leakage, is not known at this time. Juang et al. identified differentially expressed proteins that mainly affected immune and inflammatory proteins in rat BALF after exposure to ZnO NPs [48]. Because there was no significant change in the BALF LDH levels, we believe that the dosage

of ZnO NPs in our experiments did not cause significant cell injury.

5. Conclusions

We found that ZnO NPs, but not zinc ions, induced eosinophilia and increased the production of Th2 cytokines. In particular, increased IL-5 and eosinophil infiltration are the major features of asthma. Although the precise mechanism by which ZnO NPs induced eosinophilic airway inflammation is not fully understood, the effects might be mediated partly through the enhanced lung expression of Th2 cytokines. Further studies are required to understand the mechanisms underlying this eosinophilic airway inflammation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jhazmat.2015.05.023>

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