Nanoparticles in the lungs of old mice: Pulmonary inflammation and oxidative stress without procoagulant effects

Katrien Luyts^{a,1}, Sofie Van Den Broucke^{a,1}, Bianca Hemmeryckx^b, Katrien Poels^c, Hans Scheers^a, Lidia Casas^a, Jeroen Vanoirbeek^{a,c}, Benoit Nemery^a, Peter H.M. Hoet^{a,*}

^a Department of Public Health and Primary Care, Occupational and Environmental Toxicology, KU Leuven, Leuven, Belgium

^b Department of Cardiovascular Sciences, Center for Molecular and Vascular Biology, KU Leuven, Leuven, Belgium

^c Department of Public Health and Primary Care, Laboratory for Occupational and Environmental Hygiene, KU Leuven, Leuven, Belgium

HIGHLIGHTS

- Health effects due to ultra-fine (nanoparticles) might be aggravated in elderly.
- We exposed 18-month-old C75Bl/6 mice during 5 consecutive weeks.
- The NPs caused pulmonary toxicity in these 18-month-old mice (without oxidative stress).
- Coagulation factor VIII was greatly increased up to 8 weeks after the subacute NP exposure.
- ZnO NPs induced a fibrosis-like pathology.

GRAPHICAL ABSTRACT

ARTICLE INFO

Article history:

Received 26 March 2018

Received in revised form 22 June 2018

Accepted 24 June 2018

Available online xxxx

Keywords:

Old mice

Pulmonary inflammation

Oxidative stress

Hemostasis parameters

Fibrosis

ABSTRACT

Pulmonary exposure to nanoparticles (NPs) has been shown to induce pulmonary as well as cardiovascular toxicity. These effects might be enhanced in elderly subjects as a result of a compromised immunity and/or declined organ functions. To study the adverse in vivo effects of NPs in a model for the elderly, we exposed 18-month-old C75Bl/6 mice to multi-walled carbon nanotubes (MWCNTs) or ZnO NPs by intratracheal instillation once a week during 5 consecutive weeks. Pulmonary and hemostatic toxicity was determined 24 h (T1) and 8 weeks (T2) after the last administration.

Both NP types significantly increased the pulmonary macrophages at both time points. The MWCNTs and ZnO NPs also induced a pulmonary influx of neutrophils, which was even larger at T2 compared to T1. All NPs induced only a modest increase of pulmonary IL-1 β , IL-6 and KC levels. Both types of NPs also increased blood neutrophils. Red blood cells were not significantly affected. Both NPs significantly increased coagulation factor VIII levels at both time points. Histological analysis revealed the presence of MWCNTs in the alveolar macrophages up to 8 weeks after the last administration and the ZnO NPs induced a pronounced alveolar inflammation.

Abbreviations: aPTT, activated partial thromboplastin time; BAL, broncho-alveolar lavage; BSA, bovine serum albumin; CRP, C-reactive protein; DAB, 3,3'-diaminobenzidine; ENPRA, engineered nanoparticle risk assessment; GSH, reduced glutathione; GSSG, oxidized glutathione; HBSS, Hank's balanced salt solution; IFN- γ , interferon gamma; IL, interleukin; KC, cytokine-induced neutrophil chemo-attractant; LAL, limulus amoebocyte lysate; LPS, lipopolysaccharide; MCV, mean corpuscular volume; MPV, mean platelet volume; MWCNT, multi-walled carbon nanotube; NP, nanoparticle; PT, prothrombin time; RBC, red blood cell; T1, time point 1 = 24 h after the last instillation; T2, time point 2 = 8 weeks after the last instillation; TNF- α , tumor necrosis factor alpha; WBC, white blood cell; ZnO, zinc oxide.

* Corresponding author at: KU Leuven, Herestraat 49, O&N1-706, 3000 Leuven, Belgium.

E-mail address: peter.hoet@kuleuven.be (P.H.M. Hoet).

¹ Authors equal contribution.

In these 18-month-old mice, NPs caused pulmonary inflammation (without evidence of oxidative stress) accompanied by large increases in coagulation factor VIII up to 8 weeks after the last NP exposure. The persistence of the MWCNTs in the lungs resulted in translocation from the lungs to the left heart and the ZnO NPs induced a fibrosis-like pathology.

© 2018 Elsevier B.V. All rights reserved.

1. Introduction

Nanotechnology is currently applied in various industries for manufacturing novel consumer products, such as cosmetics, clothing or sporting goods, ... Nanomaterials are also under study for use in medicine (Qian et al., 2017; Saptarshi et al., 2015). Almost half of the nanoparticle (NP)-containing products originate from the USA (Nanotechnology, n.d.). The use of NP-containing products may lead consumers to be exposed to NPs released from the product matrix due to the natural wear and tear. Inhalation is the most likely route of exposure, although oral uptake is also possible (Upadhyayula et al., 2014).

Despite the numerous benefits of these novel applications, a lot of research suggests toxicity after NP exposure. Several animal studies have shown pulmonary toxicity after the inhalation, instillation or aspiration of different types of NPs such as acute lung injury, increased risks of pulmonary fibrosis and cancer (Card et al., 2008; Oberdorster et al., 2005). It is generally believed that the mechanisms underlying this toxicity are inflammation and oxidative stress (Marano et al., 2010). However, the targets of toxicity induced by NP inhalation are not limited to the lungs, systemic effects and cardiovascular toxicity should also be considered. In animal models, the pulmonary administration of engineered NPs has been shown to induce endothelial dysfunction, promote atherosclerosis and to have pro-coagulant effects (Geys et al., 2008; LeBlanc et al., 2010; Nemmar et al., 2005; Nurkiewicz et al., 2008). Possible pathways linking the pulmonary and cardiovascular compartments consist of 1/ translocation of particles from the lungs to the systemic circulation thus exerting direct effects on the heart, blood and vasculature; 2/ the release of mediators (pro-inflammatory cytokines, oxidatively-modified macromolecules, vasoactive proteins, and prothrombotic factors) from the lung to the cardiovascular compartment and 3/ the stimulation of sensory nerves in the lung thus altering cardiac rhythm and function (Shannahan et al., 2012; Brook et al., 2010).

So far, most studies have used young healthy animals to assess the NP effects. However, some groups within the general population may be more susceptible to the deleterious effects of NPs and suffer greater damage from the same exposure than others. This includes children, people with cardiovascular disease (congestive heart failure, chronic coronary artery disease), chronic respiratory diseases, diabetics and the elderly (>65 years) (Goldberg et al., 2000; Gouveia and Fletcher, 2000; Jacobs et al., 2010; Jacobs et al., 2012). Increased sensitivity in older age can be explained by alterations in the neuroendocrine network, compromised immunity and a general decline in cellular and organ functions. Therefore, NPs may exhibit unusual absorption, distribution, metabolism and excretion profiles; and impaired protective/repair functions in elderly subjects may lead to worse toxic consequences than for healthy populations. Also, weaker anti-oxidative and immune functions may magnify toxicity (Li et al., 2014).

In this study, we focus on the elderly because the incidence of thrombotic cardiovascular disease increases with age (Tracy and Bovill, 1992). Epidemiological research has consistently demonstrated associations between episodes of increased air pollution and daily morbidity and mortality. Short-term, as well as long-term exposures to increased levels or air pollutants have been associated with cardiac arrhythmias, increases in blood pressure, subclinical atherosclerosis, deep vein thrombosis (Pope III and Dockery, 2006). Although limited epidemiological evidence data is available for ultrafine particles (<0.1 µm), experimental evidence indicates that this size fraction poses a

high risk for the cardiovascular system (Brook et al., 2010). Age-related changes occur in the hemostatic system which involves platelets, coagulation and fibrinolytic factors, and in the vasculature where sclerotic changes contribute to an increased incidence of thrombosis in the elderly (Kiechl and Willeit, 1999). In other words, the blood of the elderly is in a state of hypercoagulability due to an increased platelet activity and increased plasma levels of several coagulation factors without a proportional increase in anticoagulant factors (Balleisen et al., 1985; Sagripanti and Carpi, 1998). Increased levels of coagulation factor (F) VII, FVIII and fibrinogen have been shown to be associated with an increased risk for thrombotic disease (Koster et al., 1994).

To evaluate the pulmonary and hemostatic toxicity of NPs in a model with relevance to the elderly population, we repeatedly exposed 18-month-old mice to multi-walled carbon nanotubes (MWCNTs) or zinc oxide (ZnO) NPs. Either 24 h or 8 weeks after the last administration, pulmonary inflammation and oxidative stress were determined together with blood cell counts and several hemostasis parameters.

2. Materials and methods

2.1. Animal model

Eighteen-month-old male C57Bl/6 mice weighing, on average, 32.1 g at the start of the experiment were purchased from Janvier (Le Genest-Saint-Isle, France).

All animals were kept in micro-isolation cages in a temperature- and light-controlled (12-hour night/day cycle) environment and had free access to drinking water and standard chow (KM-04-k12, Muracon, Carfil, Oud-Turnhout, Belgium; 13% kcal as fat, caloric value 10.9 kJ/g) ad libitum. All animal procedures were approved by the Ethical Committee of the Katholieke Universiteit Leuven and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996).

2.2. Nanomaterials and preparation of exposure suspensions

The MWCNTs (Table 1) were purchased from Arkema Graphistrength C100 (NM402; diameter 30 nm, length 20 µm), ZnO NPs manufactured by BASF Z-Cote (NM 110; zinkite, uncoated, 100 nm) were received from the European Joint Commission Joint Research Centre (Ispra, Italy). A detailed characterization has been published earlier (Kermanizadeh et al., 2013; Luyts et al., 2014).

All NPs were weighed and dispersed in sterile distilled water containing 2 vol% mouse serum (dispersion medium) to create a stock concentration of 2.56 mg/ml. The mouse serum was obtained from healthy mice via puncture of the inferior vena cava. The blood was collected in Minicollect serum tubes, left at room temperature for 30 min and centrifuged at 2000g during 10 min. The supernatant (serum) was collected and tested to be free of LPS (LAL assay). In sterile conditions, the mouse serum was diluted to 2 vol% in Baxter water. The NP stock suspensions were sonicated using a Microson™ ultrasonic cell disruptor (Misonix, Newtown, USA), equipped with a 1/8" disruptor horn for 16 min on ice/water. Thereafter, the stock concentrations were diluted to obtain the final concentrations for exposure: 512 µg/ml MWCNT (corresponds to 25.6 µg/instillation) and 256 µg/ml ZnO (12.8 µg/instillation). All NP suspensions were used within 1 h after sonication.

Table 1

List of engineered NMs investigated, with the original source codes, the nominal sizes and properties as provided by the supplier.

Material	Label name	Size	CAS-number	Source
MWCNT	Multi-walled carbon nanotubes	Entangled, diameter 30 nm, length 20 µm	7782-42-5, EINECS 231-955-3	Arkema Graphistrength C100
ZnO NP	Zinc oxide	Uncoated 100 nm	1314-13-2, EINECS 215-222-5	BASF Z-Cote

CAS: Chemical Abstracts Service Registry Numbers; EINECS: European Inventory of Existing Commercial Chemical Substances; MWCNT: multi-walled carbon nanotube; NP: nanoparticle.

2.3. Study design (experimental lay-out)

All mice were deeply anesthetized using 5% isoflurane (Surgivet® model 100 vaporizer). Using a 20 G catheter, they were intratracheally instilled once a week, during 5 consecutive weeks. The first dose was administered at the age of 18 months. Mice received 25.6 µg MWCNT per instillation or 12.8 µg ZnO NPs per instillation. Each administration consisted of a volume of 50 µl followed by a bolus of 0.3 ml air. Two control groups were included: one group (named 'control') received 5 instillations of vehicle (dispersion medium), and one sham control (named 'sham') received no instillations, but was manipulated like the other animals (including anesthesia and intratracheal intubation). The mice were allowed to recover from the instillation before being returned to their cages. Mice were sacrificed either 24 h after the last administration (T1, age of 19 months), or 8 weeks after the last administration (T2, age of 21 months).

The mice were sacrificed using an overdose of pentobarbital. Blood was immediately sampled from the retro-orbital plexus and collected in a tube containing citrate. The final concentration of citrate in the blood was 0.38%. Thereafter, the thorax was opened and the left lung was ligated. A broncho-alveolar lavage was performed on the right lung, consisting of three flushes of 0.45 ml NaCl 0.9%. The lavage fluid was kept on ice until further processing. Finally, the heart-lung block was removed, the right lung was inflated with air using a syringe (until full expansion as assessed visually) and the heart-lung block was placed in 4% formaldehyde for histology. The left lung was snap frozen in liquid nitrogen and stored at −80 °C for cytokine and GSSG/GSH measurements.

2.4. Blood samples

Immediately after sampling, a small portion of the blood was diluted 5-fold (in saline) to perform cell counts using the Cell-Dyn® 3200 R counter (Abbott Diagnostics, Louvain-la-Neuve, Belgium). The remaining part of the blood was immediately centrifuged twice at 16,100g, for 10 min at room temperature. The plasma was stored at −80 °C for measurements of the activated partial thromboplastin time (aPTT), the prothrombin time (PT) and plasma levels of coagulation factors FVII, FVIII and fibrinogen.

2.5. Broncho-alveolar lavage (BAL)

The lavage fluid was centrifuged at 400g, for 10 min at 4 °C. The supernatant was stored at −80 °C. The cell pellet was resuspended and a total cell count was performed using a Bürker cell counter. Cells were spun onto microscope slides at 300g for 6 min (Cytospin 3, Shandon, TechGen, Zellik, Belgium) and stained using the DiffQuick® method. For each sample, the proportions of macrophages, neutrophils, eosinophils and lymphocytes were counted.

2.6. Cytokine release

The lung tissue was homogenized in 0.5 ml of 1% BSA (in HBSS-) and then centrifuged at 16,100g, 4 °C for 10 min. The supernatant was frozen at −80 °C until cytokine measurement using the MSD mouse pro-

inflammatory 7-plex (IFN-γ, IL-1β, IL-6, IL-10, IL-12p70, KC/GRO, TNF-α) according to the manufacturers' instructions (Meso Scale Discovery, Gaithersburg, Maryland).

2.7. Glutathione determination

The right lung lobes were homogenized on ice in cold 40 mM N-ethylmaleimide (20 ml/g tissue, for preventing rapid oxidation of GSH) and then centrifuged at 14,000g for 15 min at 4 °C. The supernatant was transferred to a new Eppendorf tube and 5% metaphosphoric acid was added (1/5th of the supernatant volume, final concentration is 1% metaphosphoric acid), mixed and again centrifuged at 14,000g during 15 min at 4 °C. The supernatant was stored at −80 °C until measurements. The measurements of the reduced (GSH) and oxidized (glutathione disulfide, GSSG) forms of glutathione were performed by LC/MS using a method developed in-house, partially based on Guan et al. (2003) as follows. The LC/MS analysis was conducted on a Waters Acquity UPLC coupled to a Micromass MS Technologies Quattro Premier mass spectrometer using electron spray ionization (ESI). The LC separation was done on a Waters Acquity UPLC BEH C18, 50 mm × 2.1 mm, 1.7 µm column, held at a temperature of 40 °C. The mobile phase used for the LC separation was a mixture of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The flow rate was 0.35 ml/min and the total run time was 4 min. Ten µl aliquots were injected per sample. The analyses were performed in the ESI+ mode and a multiple reaction monitoring (MRM) methodology was used with argon as the collision gas.

Data were normalized to protein content measured by the Bradford method using bovine serum albumin as the standard. The ratio of GSSG to total glutathione was then calculated.

2.8. Histology

The right lungs were embedded in paraffin, dehydrated in ethanol (70%–96%–100%) and cleared in xylene. Sections were cut using a HM360 microtome (Microm, Walldorf, Germany) at 7 µm thick sections and were deparaffinized in xylene, followed by decreasing series of ethanol concentrations and distilled water. To remove the artifacts (can result in black spots) induced by formaldehyde, ammonium was added to the 70% ethanol in the rehydration step before the staining. The sections were stained for hematoxylin & eosin.

2.9. Statistical analysis

The associations between each biological endpoint and treatment groups (i.e. type of NP administered; time of measurement after administration), were studied in three-way ANOVA models, separately for MWCNT and ZnO NPs. Data from the 'control' group were used for comparison with both MWCNT and ZnO NP administration. Three-way interaction terms and pairwise two-way interaction terms were inserted in the initial models. For each endpoint, non-significant interaction terms or main effects ($p > 0.05$) were manually removed from the model in a stepwise procedure. In addition to the one-by-one analysis of BAL cell types and WBC types, we created overall indicators of BAL cell and WBC augmentation, based on the first component of a principal component analysis (PCA) of BAL cell types and WBC types,

respectively. Results are reported as the percentage of the control. Statistical analyses were performed using SAS software, version 9.3 (SAS Institute, Cary, NC, USA) and all tests were two-sided with $\alpha = 0.05$.

3. Results

3.1. Broncho-alveolar lavage

The total numbers of cells in BAL fluid were significantly increased in the MWCNT and ZnO NP exposed mice compared to the control group ($p < 0.0001$), at both time points (Fig. 1A). The macrophages made up the largest cell fraction in the BAL fluid and followed the same pattern ($p < 0.0001$) as the total BAL cells (Fig. 1B). Exposure to MWCNTs and ZnO NPs significantly increased the BAL neutrophils at T1 ($p = 0.002$, Fig. 1C), and this response was more pronounced at T2, when the BAL neutrophils were increased 9-fold after MWCNTs and 23-fold after ZnO NPs.

3.2. Lung cytokines

Levels of IFN- γ , IL-10, IL-12p70 and TNF- α were below the lower limits of detection. Exposure to all NPs non-significantly increased pulmonary IL-1 β (Fig. 2A) and KC (Fig. 2C) levels compared to the control at T1. However, at T2, 8 weeks after the last exposure, all mice except the ZnO NPs exposed mice, had significantly higher pulmonary IL-1 β levels than the mice at T1 ($p < 0.05$). Only exposure to ZnO NPs induced a non-significant increase in pulmonary IL-6 levels (162-fold) compared to the control, at T1 (Fig. 2B).

Fig. 1. Total numbers of BAL cell, macrophages and neutrophils of old mice exposed to MWCNTs or ZnO NPs. Data are shown as mean $\pm$ SD, N = 4–8. ***p < 0.01 compared to control and ***p < 0.001 compared to control, Δ p < 0.05 T2 compared to T1 within the same NP exposed group.

Fig. 2. Concentrations of IL-1 β , IL-6 and KC measured in lung homogenates of old mice exposed to MWCNTs or ZnO NPs. Data are shown as mean $\pm$ SD, N = 4–8. Δ p < 0.05 compared to the same dose group at T1. $\Delta\Delta$ p < 0.01 compared to the same dose group at T1.

3.3. Pulmonary oxidative stress

No significant changes were found in total GSH or GSSG/GSH ratio after treatment with MWCNTs. NP-exposure increased the pulmonary total glutathione concentrations compared to the vehicle control at both time points (Fig. 3A). In both the control and NP-exposed mice, total glutathione levels tended to be higher at T2 than at T1, but these differences were non-significant. The GSSG/GSH ratio was not changed in the NP-exposed mice compared to the control (Fig. 3B). Eight weeks after the last exposure, the GSSG/GSH ratio was slightly increased compared to T1.

3.4. Blood cell counts

MWCNT-exposure increased the number of blood leukocytes 1.4-fold at T1 and 1.7-fold at T2 compared to the control ($p = 0.017$, see Fig. 4A). At T2, leukocyte numbers were significantly increased compared to T1, in all mice. MWCNT-exposure increased the blood neutrophils by 1.7-fold at T1 and 1.4-fold at T2, compared to the control. ZnO NP-exposure increased the blood neutrophils 2.2-fold at T1 and 1.2-fold at T2, compared to the control ($p = 0.008$, Fig. 4B).

NP-administration slightly (and non-significantly) decreased the number of red blood cells compared to control at T1 (by 11% for the MWCNTs and 7% for the ZnO NPs). At T2, no differences were observed between the NP-exposed groups and the control.

Fig. 3. Total glutathione and GSSG/GSH ratio measured in lung homogenates of old mice exposed to MWCNTs or ZnO NPs. Data are shown as mean $\pm$ SD, N = 4–8.

Fig. 4. White blood cells, neutrophils and red blood cells measured in the blood of old mice exposed to MWCNTs or ZnO NPs. Data are shown as mean $\pm$ SD, N = 4–8. * p < 0.05 compared to control, ** p < 0.01 compared to control, $\Delta\Delta$ p < 0.01 at T2 compared to the same dose group at T1, $\Delta\Delta\Delta$ p < 0.001 at T2 compared to the same dose group at T1.

3.5. Hemostasis parameters

At T1, NP-administration induced a small (non-significant) decrease in PT numbers (10% for both MWCNTs and ZnO NPs) compared to control (see Fig. 5A). However, at T2, significant increases in PT were observed compared to the T1 (by 24% and 12% for MWCNTs and ZnO NPs, respectively, $p = 0.0004$). No significant changes were noted for plasma fibrinogen concentrations at T1 (Fig. 5B). At T1, the plasma levels of Factor VII increased by 16% and 12% compared to the control after the administration MWCNTs and ZnO NPs, respectively (Fig. 5C). At T2, these levels returned to baseline (particle $\times$ time interaction p < 0.05). The largest effects were obtained for plasma FVIII concentrations with increases of 58% and 44% at T1 and 45% and 58% at T2, after MWCNTs and ZnO NPs, respectively (Fig. 5D).

Fig. 5. Prothrombin time, fibrinogen concentration, coagulation factors FVII and FVIII measured in plasma of old mice exposed to MWCNTs or ZnO NPs. Data are shown as mean $\pm$ SD, N = 4–8. *** p < 0.001 compared to control, $\Delta\Delta$ p < 0.01 at T2 compared to the same dose group at T1, $\Delta\Delta\Delta$ p < 0.001 at T2 compared to the same dose group at T1.

3.6. Lung histology (Fig. 6)

No tissue injury was observed in the lungs of sham and vehicle control groups. Nevertheless, both the sham and vehicle control groups had inflammatory cells in the alveolo-bronchiolar and venous regions, as well as inflammatory cells in the lymph follicles, mainly at T1 (Table 2). The MWCNTs were clearly visible in the alveolar macrophages, even at T2, but this was not accompanied by interstitial

inflammation. The ZnO NPs induced the most pronounced inflammation, mainly in the alveolar septa, as reflected in the scores (Table 2).

3.7. Correlation between the pulmonary and cardiovascular compartment

When considering the data of all groups together, we observed a positive correlation between the GSSG/GSH ratio and PT (Pearson correlation coefficient, PCC: 0.40135, $p = 0.0169$). However, clearer

Fig. 6. Lung histology in old mice exposed to MWCNTs or ZnO NPs. No abnormalities are shown for the control groups. The MWCNT are visible in the alveolar macrophages at both time points. ZnO NP administration induced the most pronounced inflammation in the alveolar septa at both time points. Hematoxylin and eosin stain, $\times 100$ magnification.

Table 2
Histological scores on the H&E stained lung sections.

Group	Time point	Presence of alveolar macrophages	Inflammatory cells in			Presence of follicles (lymphocytes)
			Alveoli	Alveolo-bronchiolar	Veins	
Sham	T1	0 ± 0	0 ± 0	0.80 ± 0.80	0.90 ± 0.40	0 ± 0
	T2	0 ± 0	0 ± 0	0.50 ± 0.50	0.50 ± 0.50	0 ± 0
Control	T1	0.10 ± 0.40	0 ± 0	0.50 ± 1.10	0.40 ± 0.40	1.00 ± 1.20
	T2	0.10 ± 0.40	0 ± 0	0.40 ± 0.40	0.60 ± 0.80	0.30 ± 0.50
MWCNTs	T1	0.50 ± 1.20*	0 ± 0	0.50 ± 0.40	0.40 ± 0.40	0 ± 0
	T2	0.30 ± 0.80*	0 ± 0	1.20 ± 0.40	1.20 ± 0.40	0.20 ± 0.40
ZnO NPs	T1	0.40 ± 0.90*	2.00 ± 1.40***	1.00 ± 1.00	1.70 ± 1.00	0.40 ± 0.90
	T2	0.70 ± 1.00*	0.80 ± 1.20***	1.00 ± 0.00	1.10 ± 0.50	0.40 ± 0.50

N = 3–7, T1: 24 h after the last administration, T2: 8 weeks after the last administration. Scores of 0–3 were given for each parameter per lung section. Scores are depicted as mean ± standard deviation. *p < 0.05 compared to control at the same time point; ***p < 0.001 compared to control at the same time point.

correlations were apparent when NP treatments were considered separately. In the MWCNT exposed mice, the total pulmonary macrophages (PCC: 0.61, $p = 0.03$) and IL-1 β levels (PCC: 0.70, $p = 0.01$) were positively correlated with coagulation factor VIII. In the ZnO NP exposed mice the total BAL cells (PCC: 0.75, $p = 0.01$), total BAL neutrophils (PCC: 0.71, $p = 0.02$) and pulmonary IL-6 (PCC: 0.84, $p = 0.0024$) were positively correlated with coagulation factor VIII. Also, the pulmonary GSSG/GSH ratio was positively correlated with PT (PCC: 0.74, $p = 0.02$).

4. Discussion

We used 18-month-old C57Bl/6 mice to study the pulmonary and hemostatic toxicity after subacute administration of MWCNTs or ZnO NPs. Limited data is available in the literature for such old mice.

The studied nanomaterials induced different inflammatory profiles in the lungs. The MWCNTs increased the total amount of macrophages and neutrophils in the BAL. The fibers were clearly visible in the alveolar macrophages up to T2, thus reflecting their persistent nature.

Compared to MWCNTs, the ZnO NPs induced a pronounced inflammation in the alveolar septa, thus suggesting an early fibrosis-like pathology with an increase in pulmonary IL-1 β , IL-6 and KC levels.

Other in vivo studies have shown that CNTs are able to cause pulmonary inflammation which can persist up to 1 month after the administration (Park et al., 2011; Shvedova et al., 2008). The pulmonary inflammation induced by the CNTs consists predominantly of an influx of macrophages and neutrophils (Shvedova et al., 2008; Teeguarden et al., 2011). These cells have been shown to be capable of breaking down CNTs by means of chemical degradation, e.g. by hydrogen peroxide released during the oxidative burst and by enzymatic catalysis e.g. by myeloperoxidase released by neutrophils (Wei et al., 2009; Kotchey et al., 2012). Since neutrophils are short-lived cells, the presence of the macrophages mainly determined the CNT kinetics. Recently, Kagan et al. reported another pathway involved in the “digestion” of CNTs from the lung, namely the superoxide/peroxynitrite-driven oxidative pathways of macrophages (Kagan et al., 2014).

The deposition of nanoparticles in vascular tissue in animals and man was recently reported by Miller et al., showing a clear association between deposition and vascular disease (Miller et al., 2017). In our mice, blackening of the endothelium of the left atrium of the heart was noted (data not shown, see Supplement Fig. 1). Although we excluded histological artifacts, we could not ‘prove’ the presence of MWCNT in these tissues; therefore this result needs to be interpreted with caution.

The alveolo-capillary barrier might be compromised by pulmonary inflammation, thus facilitating the translocation of particles or fibers from the alveoli to blood. The left atrium of the heart is the first extra-pulmonary organ that the blood from the pulmonary circulation encounters, so finding MWCNTs in this area should not be surprising. The study performed by Mercer et al. also reported translocation of

MWCNTs to the heart, 336 days after pulmonary administration (Mercer et al., 2013).

The ZnO NPs used in this study induced effects that can be associated with early pulmonary fibrosis after subacute exposure to a cumulative dose of 64 $\mu\text{g}/\text{mouse}$. Jain et al. reported a fibrotic response after co-exposure of ZnO NPs with toluene, even at their respective permissible exposure level (5 mg/m^3 for ZnO and 200 ppm for toluene) (Jain et al., 2013). We observed that the ZnO NPs induced a pronounced pulmonary cellular influx but only with a modest increase of IL-1 β , IL-6 and KC concentrations. Others found that repeated exposure to ZnO NPs (5 consecutive days) did not induce pulmonary inflammation, whereas a single exposure did (Chen et al., 2015). Also, in humans, the syndrome known as metal fume fever, an acute inflammatory response induced by the inhalation of metal fumes, resolves in 1–4 days after exposure (Vogelmeier et al., 1987). Zinc ions have been shown to dissolve from ZnO NPs creating locally high zinc concentrations. A zinc excess suppresses several immunological functions and, therefore, it is possible that the pattern of exposure to ZnO NPs used here (5 administrations, each separated by one week) suppressed pulmonary immunity leading to a reduced clearance of the NPs and the limited cytokine secretion (Good, 1989; Ibs and Rink, 2003). The damage induced by the NPs and the diminished repair mechanisms could be responsible for the observed fibrosis-like pathology.

The subacute pulmonary exposure to MWCNTs and ZnO NPs significantly increased the blood neutrophils at both time points, in addition to a significant neutrophil influx in the lungs. The pulmonary inflammation is likely to cause the observed effects in the circulation, which is also reflected by the significant correlations between the cellular influx in BAL and the pulmonary cytokines, on the one hand, and the coagulation factor VIII for both NPs on the other hand. The release of pro-inflammatory cytokines from the lungs into the circulation could lead to a systemic innate immune response known as the acute phase response. IL-1, IL-6 and TNF- α as well as the release of oxidatively-modified macromolecules are known to stimulate the liver to synthesize and release specific acute phase proteins (Cray et al., 2009). For example, Niwa et al. demonstrated increased serum levels of IL-6, MPC-1 and C-reactive protein (CRP), with only a mild pulmonary inflammation after inhalation exposure to carbon black (Niwa et al., 2008). Mutlu et al. linked increased levels of circulating pro-inflammatory cytokine levels to decreased clotting times and increased levels of fibrinogen and coagulation factors II, VIII and X after exposure to ambient particulate matter (Mutlu et al., 2007). On the other hand, Emmerechts et al. observed increased platelet numbers, von Willebrand factor, soluble P-selectin and microvesicles in (20 months) old mice subchronically exposed to polluted air, in the absence of pulmonary inflammation (Emmerechts et al., 2012).

The release of acute phase response proteins such as CRP, fibrinogen and FVII from the liver into the circulation also promotes a prothrombotic state and risk factors for myocardial infarction (Donaldson et al., 2001). However, the mechanisms by which CRP contributes to NP-induced cardiovascular disease is not clear and it is also

not known if other acute phase proteins play a role in modulating the cardiovascular alterations following NP exposure. Possibly, the acute phase proteins contribute in the removal of the circulating cytokines and oxidatively-damaged molecules. The acute phase proteins are, nevertheless, a good marker for the systemic effects after NP inhalation.

4.1. Additional analysis comparing young and old mice

To place the current results into perspective we have compared the data of the old mice to data of 8-week-old mice described in an earlier study of our research group (Luyts et al., 2014). We merged the relevant data of the datasets of the present study with that of our previous work and compared the measured endpoints in both age groups (8 week versus 18 month old mice) using Wilcoxon tests. To assess a potential effect modification of age on the associations between the health measurements and the exposure, we run generalized linear models (GLM) including the interaction term between the exposure-time variable and the age group (old vs young).

Overall, statistically significant differences (p -values < 0.05) were observed between young and old mice in most of the endpoints. The responses for BAL cells, red blood cells, IL 1 β , MCV, PT, and aPTT were higher in the young mice, whereas responses for blood leukocytes, glutathione, MPV, platelets, Fibrinogen, FVII and FVIII were higher in the old mice. No statistically significant differences were observed for BAL neutrophils, KC, ratio and blood lymphocytes. Comparing the results in young mice with those in old mice, the differences between treated and not treated mice is in general larger for old mice. Notably, an increase at T2 of BAL-neutrophils, and at both time-points higher IL-6 levels, WBC and neutrophils counts in blood and an increase in clotting factor F-VIII were strikingly different between exposed young and old mice.

In the GLM including the interaction terms, we observed statistically significant interactions (p -values < 0.05) for BAL neutrophils, IL-1 β , GSSG/GSH ratio, WBC, blood neutrophils, blood lymphocytes, eosinophils, and basophils, aPT and FVIII, suggesting that age may modify the effect of the treatment on these specific endpoints.

5. Conclusion

To conclude, in 18-month-old mice that were subacutely exposed to MWCNTs and ZnO NPs we observed moderate pulmonary toxicity without oxidative stress which was accompanied by hemostatic toxicity where coagulation factor VIII was greatly increased up to 8 weeks after the subacute NP exposure. Moreover, several parameters of pulmonary inflammation were significantly correlated with the observed procoagulant effects.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2018.06.301>.

Acknowledgements

This work was supported by the agency for Innovation through Science and Technology (agentschap voor Innovatie door Wetenschap en Technologie, IWT – 101061), and by the project “Engineered Nanoparticles Risk Assessment” (ENPRA, EU FP7).

References

Balleisen, L., Bailey, J., Epping, P.H., Schulte, H., van de Loo, J., 1985. Epidemiological study on factor VII, factor VIII and fibrinogen in an industrial population: I. Baseline data on the relation to age, gender, body-weight, smoking, alcohol, pill-using, and menopause. *Thromb. Haemost.* 54 (2), 475–479 (Aug 30).

Brook, R.D., Rajagopalan, S., Pope, C.A., Brook, J.R., Bhatnagar, A., Diez-Roux, A.V., et al., 2010. Particulate matter air pollution and cardiovascular disease: an update to the scientific statement from the American Heart Association. *Circulation* 121 (21), 2331–2378 (Jun 1).

Card, J.W., Zeldin, D.C., Bonner, J.C., Nestmann, E.R., 2008. Pulmonary applications and toxicity of engineered nanoparticles. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 295, L400–L411 (1040–0605 (Print), Sep).

Chen, J.-K., Ho, C.-C., Chang, H.-C., Lin, J.-F., Yang, C.S., Tsai, M.-H., et al., 2015. Particulate nature of inhaled zinc oxide nanoparticles determines systemic effects and mechanisms of pulmonary inflammation in mice. *Nanotoxicology* 9 (1), 43–53 (Feb).

Cray, C., Zaia, J., Altman, N.H., 2009. Acute phase response in animals: a review. *Comp. Med.* 59 (6), 517–526 (Dec).

Donaldson, K., Stone, V., Seaton, A., MacNee, W., 2001. Ambient particle inhalation and the cardiovascular system: potential mechanisms. *Environ. Health Perspect.* 109 (Suppl 4), 523–527 (Aug).

Emmerts, J., De Vooght, V., Haenen, S., Luyen, S., Van Kerckhoven, S., Hemmeryckx, B., et al., 2012. Thrombogenic changes in young and old mice upon subchronic exposure to air pollution in an urban roadside tunnel. *Thromb. Haemost.* 108, 756–768 (Oct).

Geys, J., Nemmar, A., Verbeke, E., Smolders, E., Rato, M., Hoyleaerts, M.F., et al., 2008. Acute toxicity and prothrombotic effects of quantum dots: impact of surface charge. *Environ. Health Perspect.* 116, 1607–1613 (Dec).

Goldberg, M.S., Bailar, J.C., Burnett, R.T., Brook, J.R., Tamblin, R., Bonvalot, Y., et al., 2000. Identifying subgroups of the general population that may be susceptible to short-term increases in particulate air pollution: a time-series study in Montreal, Quebec. *Res. Rep. Health Eff. Inst.* 97, 7–113 (Oct, discussion 115–120).

Good, R.A., 1989. A note on zinc and immunocompetence. *Zinc in Human Biology*. Springer, London:pp. 221–223 https://doi.org/10.1007/978-1-4471-3879-2_13 (Internet, cited 2017 Aug 17, ILSI Human Nutrition Reviews).

Gouveia, N., Fletcher, T., 2000. Respiratory diseases in children and outdoor air pollution in São Paulo, Brazil: a time series analysis. *Occup. Environ. Med.* 57 (7), 477–483 (Jul).

Guan, X., Hoffman, B., Dwivedi, C., Mathees, D.P., 2003. A simultaneous liquid chromatography/mass spectrometric assay of glutathione, cysteine, homocysteine and their disulfides in biological samples. *J. Pharm. Biomed. Anal.* 31 (2), 251–261.

Nanotechnology – project on emerging nanotechnologies. Available from: <http://www.nanotechproject.org/> (Internet, cited 2017 Aug 25).

Ibs, K.-H., Rink, L., 2003. Zinc-altered immune function. *J. Nutr.* 133 (5 Suppl 1), 1452S–1456S (May).

Jacobs, L., Emmerts, J., Mathieu, C., Hoyleaerts, M.F., Fierens, F., Hoet, P.H., et al., 2010. Air pollution-related prothrombotic changes in persons with diabetes. *Environ. Health Perspect.* 118 (2), 191–196 (Feb).

Jacobs, L., Buczyńska, A., Walgraeve, C., Delcloo, A., Potgieter-Vermaak, S., Van Grieken, R., et al., 2012. Acute changes in pulse pressure in relation to constituents of particulate air pollution in elderly persons. *Environ. Res.* 117, 60–67 (Aug).

Jain, S., Rachamalla, M., Kulkarni, A., Kaur, J., Tikoo, K., 2013. Pulmonary fibrotic response to inhalation of ZnO nanoparticles and toluene co-exposure through directed flow nose only exposure chamber. *Inhal. Toxicol.* 25 (13), 703–713 (Nov).

Kagan, V.E., Kapralov, A.A., St. Croix, C.M., Watkins, S.C., Kisin, E.R., Kotchey, G.P., et al., 2014. Lung macrophages “digest” carbon nanotubes using a superoxide/peroxynitrite oxidative pathway. *ACS Nano* 8 (6), 5610–5621 (Jun 24).

Kermanizadeh, A., Pojana, G., Gaiser, B.K., Birkedal, R., Bilanovic, D., Wallin, H., et al., 2013. In vitro assessment of engineered nanomaterials using a hepatocyte cell line: cytotoxicity, pro-inflammatory cytokines and functional markers. *Nanotoxicology* 7 (3), 301–313 (May).

Kiehl, S., Willeit, J., 1999. The natural course of atherosclerosis. Part II: vascular remodeling. *Bruneck Study Group. Arterioscler. Thromb. Vasc. Biol.* 19 (6), 1491–1498 (Jun).

Koster, T., Rosendaal, F.R., Reitsma, P.H., van der Velden, P.A., Briët, E., Vandenbroucke, J.P., 1994. Factor VII and fibrinogen levels as risk factors for venous thrombosis. A case-control study of plasma levels and DNA polymorphisms—the Leiden Thrombophilia Study (LETS). *Thromb. Haemost.* 71 (6), 719–722 (Jun).

Kotchey, G.P., Hasan, S.A., Kapralov, A.A., Ha, S.H., Kim, K., Shvedova, A.A., et al., 2012. A natural vanishing act: the enzyme-catalyzed degradation of carbon nanomaterials. *Acc. Chem. Res.* 45 (10), 1770–1781 (Oct 16).

LeBlanc, A.J., Moseley, A.M., Chen, B.T., Frazer, D., Castranova, V., Nurkiewicz, T.R., 2010. Nanoparticle inhalation impairs coronary microvascular reactivity via a local reactive oxygen species-dependent mechanism. *Cardiovasc. Toxicol.* 10, 27–36 (Mar, 1559–0259 (Electronic)).

Li, Y., Zhang, Y., Yan, B., 2014. Nanotoxicity overview: nano-threat to susceptible populations. *Int. J. Mol. Sci.* 15 (3), 3671–3697.

Luyts, K., Smulders, S., Napierska, D., Van, K.S., Poels, K., Scheers, H., et al., 2014. Pulmonary and hemostatic toxicity of multi-walled carbon nanotubes and zinc oxide nanoparticles after pulmonary exposure in Bmal1 knockout mice. *Part. Fibre Toxicol.* 11, 61 (Nov 14, 1743–8977 (Electronic)).

Marano, F., Hussain, S., Rodrigues-Lima, F., Baeza-Squibin, A., Boland, S., 2010. Nanoparticles: molecular targets and cell signalling. *Arch. Toxicol.* 85 (7), 733–741 (May 26).

Mercer, R.R., Scabilloni, J.F., Hubbs, A.F., Wang, L., Battelli, L.A., McKinney, W., et al., 2013. Extrapulmonary transport of MWCNT following inhalation exposure. *Part. Fibre Toxicol.* 10, 38.

Miller, M.R., Raftis, J.B., Langrish, J.P., McLean, S.G., Samutrtai, P., Connell, S.P., et al., 2017. Inhaled nanoparticles accumulate at sites of vascular disease. *ACS Nano* 11 (5), 4542–4552 (May 23).

Mutlu, G.M., Green, D., Bellmeyer, A., Baker, C.M., Burgess, Z., Rajamannan, N., et al., 2007. Ambient particulate matter accelerates coagulation via an IL-6-dependent pathway. *J. Clin. Invest.* 117 (10), 2952–2961 (Oct).

Nemmar, A., Nemery, B., Hoet, P.H., Van, R.N., Hoyleaerts, M.F., 2005. Silica particles enhance peripheral thrombosis: key role of lung macrophage-neutrophil cross-talk. *Am. J. Respir. Crit. Care Med.* 171, 872–879 (Apr 15, 1073–449X (Print)).

Niwa, Y., Hiura, Y., Sawamura, H., Iwai, N., 2008. Inhalation exposure to carbon black induces inflammatory response in rats. *Circ. J.* 72 (1), 144–149 (Jan).

- Nurkiewicz, T.R., Porter, D.W., Hubbs, A.F., Cumpston, J.L., Chen, B.T., Frazer, D.G., et al., 2008. Nanoparticle inhalation augments particle-dependent systemic microvascular dysfunction. *Part. Fibre Toxicol.* 5 (1).
- Oberdorster, G., Oberdorster, E., Oberdorster, J., 2005. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. *Environ. Health Perspect.* 113 (7), 823–839 (Jul).
- Park, E.-J., Roh, J., Kim, S.N., Kang, M.-S., Lee, B.-S., Kim, Y., et al., 2011. Biological Toxicity and Inflammatory Response of Semi-single-walled Carbon Nanotubes.
- Pope III, C.A., Dockery, D.W., 2006. Health effects of fine particulate air pollution: lines that connect. *J. Air Waste Manage.* 56, 709–742 (Jun, 1096–2247).
- Qian, X., Han, X., Chen, Y., 2017. Insights into the unique functionality of inorganic micro/nanoparticles for versatile ultrasound theranostics. *Biomaterials* 142, 13–30 (Oct).
- Sagripanti, A., Carpi, A., 1998. Natural anticoagulants, aging, and thromboembolism. *Exp. Gerontol.* 33 (7–8), 891–896 (Dec).
- Saptarshi, S., Duschl, A., Lopata, A., 2015. Biological reactivity of zinc oxide nanoparticles with mammalian test systems: an overview. *Nanomedicine* 10 (13), 15.
- Shannahan, J.H., Kodavanti, U.P., Brown, J.M., 2012. Manufactured and airborne nanoparticle cardiopulmonary interactions: a review of mechanisms and the possible contribution of mast cells. *Inhal. Toxicol.* 24 (5), 320–339 (Apr).
- Shvedova, A.A., Kisin, E., Murray, A.R., Johnson, V.J., Gorelik, O., Arepalli, S., et al., 2008. Inhalation vs. aspiration of single-walled carbon nanotubes in C57BL/6 mice: inflammation, fibrosis, oxidative stress, and mutagenesis. *Am. J. Phys. Lung Cell. Mol. Phys.* 295 (4), L552–L565 (Oct).
- Teeguarden, J.G., Webb-Robertson, B.J., Waters, K.M., Murray, A.R., Kisin, E.R., Varnum, S.M., et al., 2011. Comparative proteomics and pulmonary toxicity of instilled single-walled carbon nanotubes, crocidolite asbestos, and ultrafine carbon black in mice. *Toxicol. Sci.* 120, 123–135 (Mar, 1096–0929 (Electronic)).
- Tracy, R.P., Bovill, E.G., 1992. Thrombosis and cardiovascular risk in the elderly. *Arch. Pathol. Lab. Med.* 116 (12), 1307–1312 (Dec).
- Upadhyayula, V.K.K., Meyer, D.E., Curran, M.A., Gonzalez, M.A., 2014. Evaluating the environmental impacts of a nano-enhanced field emission display using life cycle assessment: a screening-level study. *Environ. Sci. Technol.* 48 (2), 1194–1205 (Jan 21).
- Vogelmeier, C., König, G., Bencze, K., Fruhmman, G., 1987. Pulmonary involvement in zinc fume fever. *Chest* 92 (5), 946–948 (Nov).
- Wei, Z., Wang, H., Chen, X., Wang, B., Rong, Z., Wang, B., et al., 2009. Time- and dose-dependent effect of psyllium on serum lipids in mild-to-moderate hypercholesterolemia: a meta-analysis of controlled clinical trials. *Eur. J. Clin. Nutr.* 63 (7), 821–827 (Jul).