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Pulmonary effects of inhalation of spark-generated silver nanoparticles in Brown-Norway and Sprague-Dawley rats
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Abstract:
Background
The increasing use of silver nanoparticles (AgNPs) in consumer products is concerning over its potential toxic effects through inhalation. The pulmonary toxic response of AgNPs may be influenced by the lung pre-inflammatory state.

Methods
We determined the effect of AgNPs generated from a spark generator (mass concentration: 600-800 µg/mm³; mean diameter: 13-16 nm; total lung doses: 8 [Low] and 26-28 [high] µg) inhaled by the nasal route in Sprague-Dawley (SD) and Brown-Norway (BN) rats. Rats were sacrificed at day 1 and day 7 after exposure and measurement of lung function.

Results
In both strains, there was an increase in neutrophils in bronchoalveolar lavage (BAL) fluid at 24 hours at the high dose, with concomitant eosinophilia in BN rats. While BAL inflammatory cells were mostly normalised by Day 7, lung inflammation scores remained increased although not the tissue eosinophil scores. Total protein levels were elevated at both lung doses in both strains. There was an increase in BAL IL-1β, KC, IL-17, CCL2 and CCL3 levels in both strains at Day 1, mostly at high dose.
Phospholipid levels were increased at the high dose in SD rats, remaining elevated at Day 7, while in BN rats, the elevation was only seen at Day 1, while surfactant protein D levels decreased at day 7 at the high dose, but was increased at Day 1 low dose in BN rats. There was a transient increase in central airway resistance and in tissue elastance in BN rats at Day 1 but not in SD rats. Positive silver-staining was seen particularly in lung tissue macrophages in a dose and time-dependent response in both strains, maximal by day 7. Lung silver levels were highest in BN rat and present at day 7 in both strains.

Conclusions
Presence of cellular inflammation and increasing silver-positive macrophages in lungs at day 7, associated with significant levels of lung silver indicate that lung toxicity is persistent even with the absence of airway luminal inflammation at that time-point. The higher levels and persistence of lung silver in BN rats may be due to the pre-existing inflammatory state of the lungs.

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Pulmonary effects of inhalation of spark-generated silver nanoparticles in
Brown-Norway and Sprague-Dawley rats

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Running title: Pulmonary toxicity of inhaled AgNPs

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Key words: lungs, silver nanospheres, inhalation, inflammation
Introduction

Silver nanoparticles (AgNPs) are often suspended in a liquid which can be aerosolised or used in a solid form and are widely used as anti-microbials in consumer products especially for textiles, personal hygiene products, medical equipment, wound dressings, respiratory devices, catheters and disinfectant sprays. Therefore, humans run the risk of inhaling AgNPs (1). What is known about the potential pulmonary toxicity of inhaled AgNPs has been limited to studies in rodents. Subchronic inhalation of AgNPs induced mild, dose-dependent pulmonary inflammation and alterations in pulmonary function (2-6), with evidence that inhaled AgNPs may also enter the systemic circulation to become distributed to extra-pulmonary organs such as the liver and brain (2, 7, 8). Exposure of rats to AgNP aerosols for 90 days led to modest increased pulmonary function changes, with evidence of genotoxicity and accumulation of tissue macrophages (9), with persistence of lung function and inflammatory changes for up to 12 weeks after cessation of exposure (10). On the other hand, studies using lower inhaled doses have reported minimal or no toxicity (7, 11-13). By contrast, direct instillation of AgNPs produced higher levels of inflammation, oxidative stress and cytotoxicity compared to AgNP inhaled at similar doses (14-17).

In our previous study, the inflammatory response induced by intratracheal instillation of AgNPs was dependent on particle size with a greater pulmonary inflammatory response with a 20nm size than an 110 nm size but with little influence imposed by citrate or polyvinyl phosphate-capping (16). In addition, the pulmonary inflammatory response was characterised by an intense eosinophilia and neutrophilia in the Brown-Norway (BN) rat compared to a predominantly neutrophilic response in Sprague-Dawley (SD) rats. We elected to study these 2 rat strains because the BN rat is known to develop features of allergic asthma, namely lung eosinophilia and bronchial hyperresponsiveness (BHR) following sensitisation and exposure to allergens and possess features of a chronic lung inflammatory response in contrast to the SD rat (18-20).

In order to characterise further the bioreactivity of inhaled AgNPs in the lungs, we have studied the effect of freshly-generated uncoated silver nanoparticles produced by a spark discharge generator. We determined the threshold lung deposited dose on the lung inflammatory response and cytokine levels. An important determining factor of the pulmonary response is the interactions of these particles with the lung lining fluid constituents that include dipalmitoylated phospholipid (DPPC) and surfactant-specific
proteins A, B, C and D, which influence the aggregation, dissolution and uptake of these nanoparticles by pulmonary macrophages and alveolar cells and hence their cytotoxicity (21-23). We therefore measured the distribution of the silver particles in the lungs, assayed silver levels, and related these to surfactant composition and lung function changes. We determined whether there would be differences in these parameters between the 2 rat strains.
Results

AgNP dose in the lungs

The AgNPs had an average count median diameter (CMD) spanning between 13.4 ± 1.0 nm and 15.9 ± 0.8 nm on the different days of exposure with particle number concentrations between 3.68 ± 0.48 x 10^7 and 4.55 ± 0.70 x 10^7/cm^3 (Table 1; Fig 2). Mass concentrations measured by a TEOM™ ambient particulate monitor ranged from 617 ± 25 to 801 ± 33 µg/m^3. In SD rats, low and high lung doses were calculated to be 8 and 28 µg respectively, while in BN rats, they were 8 and 26 µg. Fig 2 shows representative images of separate Ag nanospheres collected during exposures, illustrating their spherical form. In SD rats, low and high lung doses were measured as 8 and 28 µg respectively, while in BN rats, these were 8 and 26 µg. The equivalent values for alveolar deposition were 6 and 19 µg for the SD rats and 6 and 18 µg for the BN rat.

Quantification and localisation of silver in lung tissue

Silver levels in the lungs of SD rats exposed to 28 µg of AgNP particles were 9.97 ± 2.79 µg/g at 24 hours with lower levels of 4.99 ± 2.21 µg/g at day 7 (P<0.05), suggesting some clearance had occurred from the lung by this time (Fig 3 A). The levels of silver measured in the kidney and liver were below the detection limit of the ICPMS standard curve at 1 and 7 days, suggesting that detectable silver had not translocated to these extrapulmonary regions (data not shown). Despite inhaling a similar dose as SD rats, BN rats had higher levels of silver in the lungs at 24 hours compared to the SD rats (74.46 µg/g), with a non-significant reduction measured at Day 7 (Fig 3 B).

In both SD and BN rats, there was a dose-dependent increase in the number of silver-positive staining cells in the lung at 1 day post inhalation, with these being higher in BN rats compared with SD rats for the high dose exposure (Fig 3 C & D). SD rats had similar numbers of silver-positive cells at 1 and 7 days, while BN rats had higher numbers of silver positive cells at 7 days for the highest dose.

Silver staining was not observed in non-exposed SD and BN lungs (Fig 4 A & D). There were black silver particles observed at 24 hours post-AgNP inhalation. Silver positivity at 24 hours was as strong as at 7 days after AgNPs inhalation in both rat strains. Silver-positive cells consisted mainly of macrophages scattered in the alveolar space and lung interstitium of SD lungs (Fig 4 B & C). Silver-positive macrophages were embedded within the
inflammatory cells which infiltrate the alveolar septa and lamina propria of blood vessels and airway mucosa and were deposited in granulomas of BN lungs (Fig 4 E & F). Silver particles were also observed on the luminal surface and on surface epithelium and subepithelial connective tissue of terminal bronchioles, scarcely at day 1 but more prominent at day 7 (Fig 4).

**Lung inflammatory changes**

Following inhalation of silver, there was a mild inflammation with a few inflammatory cell infiltrations in the bronchial and vascular walls and alveolar septa and also exudate in alveolar space in SD rat lungs (Fig 4B&C). Overall, tissue inflammation scores were increased in lungs of SD rats at both day 1 and 7 and at both exposure doses with the scores remaining unchanged during that period (Fig 6A). In BN rats, there was a high level of baseline inflammation that remained unchanged after inhalation of AgNPs at both levels of exposure and on both day 1 and day 7 (Fig 6C). Inflammatory cells consisted mainly of eosinophils, neutrophils, monocytes/macrophage and also some of lymphocytes with granuloma and giant cells in BN lungs (Fig 4E&F). Eosinophil counts in the airway wall were increased in BN rats at day 1 after the 28 μg exposure dose (Fig 6D). There was no increase at day 7. There was no eosinophil increase in the SD rat tissues (Fig 6B).

**BAL inflammatory cells**

In SD and BN rats, total cell numbers increased at 1 day post inhalation of the high 28 μg AgNP (P=0.0002 & p<0.02, respectively), and in SD rats, this remained elevated at day 7 (Fig 7). Low doses of AgNP (8 μg) did not elicit an increase in cells. The increase in total cells was reflected in an increase in neutrophils (P=0.0002) in SD rats, while there was both an increase in neutrophils (P<0.02) and eosinophils (P<0.01) in BN rats. Neutrophil numbers fell but remained elevated in SD rats at day 7 (P=0.009). There was also an increase in lymphocyte numbers at the high dose exposure in SD rats (P<0.03).

**BAL total protein, MDA, phospholipid and surfactant proteins**

Total BAL protein increased in the SD rats at 28μg dose only (P<0.001) at day 1, and in the BN rats at both 8 μg (P<0.02) and 26 μg (P<0.01). Total BAL protein levels returned to baseline levels by 7 day in both rat strains (Fig 8 A & E). In the SD rats, MDA increased at 1
day after inhalation of 8 (P<0.03) and 28 µg (P<0.01) of AgNP, with levels returning to baseline by day 7. There was a similar trend in the BN rats but the increases were not significant (Fig 8 B & F).

In the SD rat, SP-D levels in whole BAL did not change at 1 day while at 7 days, there was a decrease after the high dose. In the BN rat, SP-D levels increased after the low dose but not after the high dose at 1 day (Fig 8 D & H). There was no change in SP-D at the 26 µg dose at 7 days for the BN rats. Total phospholipid levels in the large aggregate fraction of BAL increased according to the dose of AgNPs exposed for both rat strains at day 1. By 7 day, phospholipid level remained elevated in the SD rat after the 26 µg dose, while in the BN rat, levels had returned to baseline (Fig 8 C & G).

**BAL cytokines/chemokines**

Most of the changes in cytokine levels occurred at day 1. In the SD rat, levels of IL-1β, KC, IL-17A, CCL2 (MCP-1) and CCL3 (MIP-1α) increased at the 28 µg dose, with increases also seen at 8 µg for IL1β, KC and CCL3 (Fig 9). On the other hand, levels of IFNγ fell in a dose-dependent manner. CCL2 and CCL3 remained elevated at the high dose at day 7. In the BN rat, there were similar trends apart from an additional increase in IL-6 at day 1 at both 8 and 26 µg doses. Levels of KC, CCL2 and CCL3 were generally higher in BN rat compared to SD rat. There were no changes in levels of IL-4, IL-13 and CCL-11 (eotaxin) (data not shown).

**Lung function**

There were no changes in large airway resistance (Rn), tissue damping (G) or tissue elastance (H) in the SD rats at either time-point compared with the air control at a physiological PEEP of 3 cm H₂O, suggesting normal airway and parenchymal functioning of the SD lung (data not shown). In BN rats, at PEEP 3, Rn increased at both AgNP doses, although this was only statistically significant for the 8 µg dose (Fig 10 A). Increasing the PEEP from 3 to 6 and 9 cm H₂O resolved the increase in Rn recorded at PEEP 3 (Fig 10 B & C), suggesting that this was a recruitment phenomenon which affected dynamic breathing. Changes in Rn in BN rats therefore may be related to surfactant dysfunction, rather than inherent forces in the lung which can occur following lung injury (28). On the other hand, a small non-significant decrease in Rn at 7 days at PEEP 3 for the 8 µg dose, was not resolved by PEEP suggesting some ongoing effects on lung function, which may be due to lung injury.
There was also a significant increase in H for BN rats at PEEP 3 at the highest dose (Fig 10 D), suggesting effects on mechanical pulmonary function also originated in the parenchyma and that the BN rats had stiffer lungs. Again, increasing the PEEP from 3 to 6 and 9 cm H$_2$O, resolved the increases in H, suggesting that this may be due to a recruitment phenomenon as before (Fig 10 E & F). Similar to the SD rats, there were no changes in G in the BN rats suggesting that no parenchymal distortion had occurred (data not shown).
Discussion

Lung inflammatory responses

This study was designed to compare and contrast the pulmonary effects of inhaled silver nanospheres in 2 rat strains with different pre-existing pulmonary inflammatory profiles. Inhalation of freshly-generated silver nanoparticles of spherical shape of 15nm diameter induced an acute pulmonary neutrophilic inflammation with the production of proinflammatory and pro-neutrophilic cytokines KC, CCL2, CCL3, IL-17A and IL-1β in the bronchoalveolar lavage fluid obtained from SD and BN rats. By contrast, there was both a neutrophilic and eosinophilic inflammation in the BN rat, associated with a similar profile of cytokines as found in SD rats, with only the addition of IL-6. BN rats but not SD rats also showed worsening of total lung resistance and tissue elastance. There was also an increase in oxidative stress marker, malonaldehyde, measured in BAL fluid which was only significant in SD rats. The effects shown were observed at the highest lung dose of 26-28 µg as calculated by the multiple-path particle dosimetry model, but not at the 8 µg lung dose, although cytokines IL-1β, KC and CCL3 were also induced at the lower dose. These measurements were transient being noticed at day 1 and were either reversed or significantly reduced by day 7. Therefore, the BN rat showed a greater response in terms of eosinophilia and lung function changes.

In contrast with the BAL measurements of inflammatory cells and cytokines, the inflammatory component seen in the lung tissue persisted at Day 7 at both lung doses, although this was not the case for the tissue eosinophils in BN rats. It is interesting to note that there is already a degree of inflammation in the lungs of BN rats at baseline characterised by inflammatory cell infiltration in the bronchial and vascular walls and alveolar septa with some exudates in the alveolar space, as previously reported (29), and that the increase in lung inflammatory response after inhalation of AgNPs is only small but significant. Interestingly, the pro-eosinophilic cytokines such as CCL11, IL-4 and IL-13 were not raised, indicating a lack of T-helper type 2 cytokine involvement.

Silver tracing in the lungs and clearance

This persistence of tissue inflammation at day 7 was associated with the persistence of silver-positive cells in the lungs and airway lumen. It is interesting to note that we identified the cells containing silver nanoparticles using the method of silver tracing, as being mostly
macrophages found within the airway lumen and alveoli, also in inflammatory cells in airway wall or in terminal bronchioles with also extracellular presence of silver. The number of cells expressing silver content increased with dose exposure and with time, reaching a maximal at 7 days in both strains but the number of silver-positive cells in the BN rat was 3-fold higher than in the SD rat at each lung dose and each time-point. The uptake of nanoparticles into macrophages is likely to result in oxidative stress, the release of pro-inflammatory mediators and subsequent pulmonary inflammation.

Silver content of the lungs at Day 1 was measurable by ICPMS, with a reduction in silver by day 7 but with still significant amounts. Interestingly, the levels of silver were up to 8-fold greater in BN lungs at day 1 and day 7 compared to SD lungs at the high dose exposure. However, the levels of silver measured in our SD rats were higher than those reported in SD or Fischer rats exposed to spark-generated uncoated silver nanoparticles of a similar size as in our study, partly due to the higher levels of exposure we delivered (for our high dose) (2, 6, 7, 30, 31). The calculation of silver levels from the day 1 and day 7 measurements in our studies of SD rats indicate a clearance rate of 50%, which is in a similar range to the clearance of 15 nm spark-produced AgNPs inhaled by Fischer rats at a lower dose of 179 µg/m³ (compared to ~700 µg/m³ for our study) of 62% (6). However, this study is similar to our delivery protocol of 4 consecutive exposures for 4 hours per day. Clearance of such uncoated AgNPs have been reported to be much faster with for example in the study of Takenata et al (2), it was 62% one day after exposure, increasing to 96% on day 7 in Fischer rats. In a recent study of nebulised AgNPs of size 20 nm suspended in citrate buffer with a high mass concentration of 7.2 mg/m³ to SD rats, a clearance rate of 34% between day 1 and day 7 was reported (32). The comparison between different studies is difficult because of differences in amount of exposure, dose of nanoparticles, method of generation of nanoparticles aerosol, particle size and even animal gender but most of these limited studies would indicate that the silver persists for a period of at least 7 days after inhalation. The study of Anderson et al (32) showed that in their model one third of the initial silver inhaled persisted at 56 days. This was associated with greater persistence of silver-positive macrophages at 21 and 56 days. In our study, the clearance value for the BN rat compared to the SD rat was lower at 33%, which started with an 8-fold higher silver concentration than in the SD rat. This was also associated with a 3-fold higher number of silver-containing macrophages in BN lungs compared to SD lungs. These data support the
hypothesis that the pre-inflamed lung in the BN rat retains more nanoparticles with a slower clearance rate leading to much higher levels of silver in the lungs compared to SD rat.

**Phospholipid and surfactant levels and influence on lung function**

Inhalation of AgNPs dose-dependently induced an increase in the levels of phospholipids and total protein in BAL fluid maximal at day 1 to a similar extent in both strains. This is in accord with previous reports of similar observations in rats or mice following the inhalation of nanoparticles of titanium oxide, silica or cadmium oxide or ultrafine diesel exhaust particles (33-36). Coating of the AgNPs with phospholipid may have important implications on pulmonary homeostasis by interference with biophysical surfactant function such as decreased adsorption of pulmonary surfactant to gold nanoparticles at the air-liquid interface (23). On the other hand, we found that the inhalation of AgNPs resulted in diametrically different effects on the measured levels of SP-D in BAL fluid. While there was a decrease in SP-D levels in SD rats at day 7 following the high dose exposure, there was an increase in SP-D levels in BAL from BN rats at the low dose exposure at day 1, with a suggestive increase at the high dose at day 7. Interestingly, the levels of SP-D at baseline in BAL fluid was nearly 3-fold higher in SD rats compared to BN rats. The reduction in SP-D in BN rats may be due to increased SP-D turnover by alveolar macrophages, possibly as a result of binding to AgNPs (22). The acute increase in SPD levels in day 1 seen in BN rat may represent an increased production from alveolar Type II cells. The potential consequences of increased amounts of SP-D may lead to increased aggregation of nanoparticles (21), hence targeting them towards macrophage and lung clearance (37).

Lung function changes only occurred in BN rats indicating a greater sensitivity of the airways and lungs to the effect of inhaled AgNPs, similar to the lung function responses observed with instillation of AgNPs (16). Resolution of the increase in lung resistance recorded at low positive end-expiratory pressure (PEEP) by increasing PEEP suggests that this increase was a recruitment phenomenon which affected dynamic breathing that may be related to surfactant dysfunction rather than inherent forces in the lung which can occur following lung injury.

**Inhalation versus direct instillation**
The results of inhalation of AgNPs can be compared with the effect of direct instillation of these nanoparticles into the lungs. In a previous study, we have instilled AgNPs at 20 nm diameter capped with citrate or polyvinylpyrrolidone (PVP), the diameter nearest to the spark-generated particles at an intra-tracheal dose of 30 µg in both strains. Indeed, the pattern of responses in these 2 strains in terms of the inflammatory response and of lung function changes were similar for both strains, albeit a lesser response in the inhaled route when a maximal dose of 26-28 µg was deposited in the lungs. The other difference is that the inhaled dose at which we saw an effect was acquired over a period of 12 hours of inhalation, while for the instillation, a larger bolus dose was administered over seconds. Thus, with the instilled dose of both citrate and pvp-capped 20nm silver nanoparticles, we saw a neutrophilic response at day 1 in both strains but only eosinophilic response significant at day 7 in SD rats. Similarly, there was an increase in protein concentration and levels of MDA in bronchoalveolar lavage fluid, with increased levels of KC at day 1 in both strains. Changes in lung function were only seen in BN rats at day 1 also, but not in SD rats. These changes were exactly similar as those observed here.

Conclusions

In conclusion, following the inhalation of freshly-generated uncoated AgNPs of 15 nm diameter, there is a transient increase in luminal inflammation with neutrophilia and eosinophilia, associated with the secretion of proinflammatory cytokines including the inflammasome marker, IL-1β, and IL-17, oxidative stress and increased phospholipid levels. Lung cellular inflammation at day 7 was associated with persistent silver-positive macrophages in the lungs and significant persistent levels of lung silver. The pre-existing inflammatory state of the BN rat is postulated to underlie the increased amount of silver retained in the lungs with a reduced clearance rate that may underlie the increased inflammatory response, induction of SP-D, dysfunctional SP-D and airway and parenchymal dysfunction seen in this rat strain but not in SD rats. The clinical implications for our finding is that people suffering from asthma may experience more serious toxic effects with AgNPs inhalation.
**Methods**

*Generation of silver nanoparticle aerosol and exposure system*

Aerosols of AgNPs were generated using a spark generator (DNP 4000, Palas, Karlsruhe, Germany) by the homogeneous nucleation of vapour produced between two electrodes (5 mm length and 1 mm diameter silver wire; Premion™ 99.999% purity, Alfa Aesar™, Heysham, UK) in an inert argon atmosphere at a flow rate of 5 L min⁻¹, as previously described. The rate of primary particle production and final size was dependent on the sparking frequency (90-300 Hz). The particles were passed through a krypon-85 charge neutraliser (Model 3077A, TSI Incorporated, Shoreview, MN, USA) and were diluted with oxygen and nitrogen to give a total aerosol flow-rate of 9 L min⁻¹. This was led into a custom-built nose-only exposure manifold consisting of 4 nose-only exposure stainless steel and anodised aluminium chambers, each with 9 ports for sampling, by a flow-splitting cone with the rats are held in restraining tubes attached to the chamber (Fig 1). Each animal has an individual aerosol supply directed to the nose area and exhaust flow (exhaled and excess aerosol flow) are separated from the inlet flow to avoid re-circulation.

*Characterisation and monitoring of AgNPs*

Aerosol mass concentrations were determined gravimetrically using Pallflex® emfab™ filters (Pall Life Sciences, Ann Arbor, MI, USA) with the aerosol drawn at 2 L min⁻¹. A TEOM™ ambient particulate monitor (Model 1400a, Thermo Scientific, Franklin, MA, USA) was also used to continuously monitored the aerosol mass concentration delivered to the exposure manifold at a sampling flow rate of 1 L min⁻¹. The TEOM data agreed well with the gravimetric mass concentration (maximum variation 10%). Particle size distribution and number concentration were continuously measured at the point-of-administration from one of the exposure chamber ports using a stainless steel sampling probe. The aerosol was immediately diluted to prevent coagulation using a Palas® ejector dilution system (Model VKL 100, Palas GmbH, Karlsruhe, Germany). To supply an aerosol flow rate of 0.6 L min⁻¹ to the instruments, HEPA-filtered compressed air was supplied to the diluter, drawing a sample flow rate of 0.12 L min⁻¹ from the exposure chamber and giving a dilution ratio of 150 ± 3 %. A condensation particle counter (CPC model 3775, TSI Inc., Shoreview, MN, USA) continuously monitored the concentration of particle numbers. The aerosol particle size distribution was determined every 3 minutes using a scanning mobility particle sizer (SMPS;
model 3936N76, TSI Inc., Shoreview, MN, USA). The shape of the aerosol particles delivered to the manifold was determined with high resolution transmission electron microscopy (TEM) (JEOL 3000F, JEOL Inc., Tokyo, Japan). Samples for TEM were taken directly onto 400 mesh nickel TEM grids with lacey carbon film using an electrostatic precipitator (TSI 3089 nanometer aerosol sampler, TSI Inc., Shoreview, MN, USA). Projected area equivalent diameters were calculated for >1500 particles randomly selected from each experiment using the image analysis software Image J (http://imagej.nih.gov/ij/).

**Dose estimation**

The estimated lung burden and dose to the alveolar region (µg) were calculated using the following equation: \( \text{Dose to lung/alveoli} = \text{MV} \times \text{T} \times (\text{C}/1000000) \times \text{DF} \) where MV is the minute ventilation of the exposed animal (ml.min\(^{-1}\)), T is the total exposure time (min), C is the average gravimetrically determined exposure mass concentration (µg.m\(^{-3}\)) and DF is the lung or alveolar deposition fraction. The mean MV, tidal volume and frequency of breathing for 6 BN rats were 208 ml/min, 1.6 ml and 130 min\(^{-1}\) respectively. The deposition fractions for the lung and alveolar region were calculated using the multiple-path particle dosimetry (MPPD) model (version 2.11, Applied Research Associates, Inc.) (24) with estimated total lung and alveolar deposition fractions of 31% and 21%, respectively. As the BN and SD rats were of similar size the same values were assumed applicable to each.

**Study design**

The experiments were performed within the legal framework of the United Kingdom under a Project License granted by the Home Office of Her Majesty’s government. The researchers hold Personal Licenses provided by the Home Office to perform the experiments described here. Male Sprague-Dawley (SD) rats (10-12 weeks, 250-320g) and male Brown-Norway (BN) rats (10-12 weeks, 260-380g) were purchased from Harlan, UK. Rats were randomly assigned into groups and exposed for 3 hours on one day (low dose) or for 3 hours on four consecutive days (high dose). Three exposure conditions were defined: filtered air only controls for 3 hours on 4 consecutive days, low dose AgNP exposure and high dose AgNP exposure (Table 1). Following exposure, the rats were returned to their cages for a period of either 24 hours or 7 days.
**Respiratory Mechanics**

Respiratory mechanics was measured by the forced oscillation technique at pulmonary end-expiratory pressures (PEEPs) of 3 cm H\(_2\)O. Rats were anesthetised with ketamine (80 mg/Kg) and Xylazine (10mg/Kg) i.p. and the depth of anaesthesia was quantified by loss of pedal reflex and by pulse oximetry, using the MouseOx Plus [STARR Life Sciences Corp., Oakmont, PA]. A tracheostomy was performed with a 15G cannula and the rats were ventilated with 10 mL/Kg air at a frequency of 90 breaths per minute using a computer controlled ventilator (Spira, EMMS, UK). Prior to measuring lung function, three successive deep lung inflations (30 cmH\(_2\)O) were performed to standardise volume history. Normal tidal breathing was interrupted by an 8s broadband input signal, containing multiple frequencies between 0.5 and 20Hz. Respiratory impedance was calculated at each frequency using the Fast Fourier transformation of the pressure and flow signals. As a function of frequency, the impedance (Z_{rs}) data can be separated into both resistance (R\(_L\)) and elastance (E\(_L\)) spectra as calculated by the software. These spectra were fitted to a constant phase model which partitions the respiratory mechanics into contributions by the central airway (Rn) and coefficients of tissue damping (G) and tissue elastance (H) (25).

**Bronchoalveolar lavage (BAL)**

Following measurement of respiratory mechanics, rats were sacrificed with an overdose of sodium pentobarbital (200 mg/Kg). The pulmonary vasculature was perfused with 120 mL of saline and BAL was performed in situ via the tracheal cannula with 2 x 10.5 mL aliquots of PBS. BAL cells from both aliquots were pooled for analysis of total and differential cells. BAL supernatants were retained separately for analysis of total proteins, monaldehyde (MDA), cytokines, chemokines, phospholipid and surfactant proteins. BAL cells were reconstituted to 1 x 10\(^6\) cells/mL and 100 µL was spun onto slides using a cyto-centrifuge. Staining for differential cell count was performed using the DIFF quick staining kit (Polysciences Inc. Warrington, PA, USA). At least 300 cells on the slides were counted and identified as macrophages, monocytes, neutrophils, eosinophils and lymphocytes. Total protein levels were measured by the Biorad protein assay (Biorad Laboratories, Hemel Hempstead, UK).

**Tissue processing and staining**
Following vascular perfusion, the lungs were removed en bloc and the left lung was inflated and fixed with freshly made 4% paraformaldehyde, via the tracheal cannula, at a pressure of 30 cm of water and then processed into paraffin blocks. 5 µm thick serial lung sections were cut and stained with: 1) hematoxylin and eosin (H & E) (BDH, Lutterworth, U.K.) 2) Silver Enhancing Kit (Cat no: SE100, Sigma-Aldrich, Saint Louis, USA) following manufacture’s instructions. Briefly, after dewaxing and hydrating lung sections to water, the sections were incubated with mixed equal parts of initiator and enhancer and then were counterstained with H & E. Silver nanoparticles were visualized using light microscope with positive cells appearing black. 3) Carbol-chromotrope for visualisation of eosinophils, which was made from Chromotrope 2R (BDH, Poole, UK). Sections were counterstained with haematoxylin and mounted with DPX mounting medium under glass coverslips. Eosinophils were visualized using light microscope with positive cells appearing red.

Lung inflammation score, positive-silver cell counts and eosinophil counts

The severity of inflammatory response was scored on a 0–3 scale defined as: 0 no inflammatory response; 1 mild inflammation with foci of inflammatory cells in bronchial or vascular wall and in alveolar septa; 2 moderate inflammation with patchy inflammation or localized inflammation in walls of bronchi or blood vessels and alveolar septa, and less than one-third of lung cross-sectional area is involved; and 3 severe inflammation with diffuse inflammatory cells in walls of bronchi or blood vessels and alveoli septa; between one-third and two-thirds of the lung area is involved.

Silver staining was counted as positive cells appearing black. Uptake into cells in walls of bronchi or blood vessels, alveoli septa and alveolar space of the left lung lobe were counted. Twenty fields covering the whole left section were counted and data are expressed as number of positive silver cells per field.

Eosinophil influx in the lamina propria of largest airways in each lung section was assessed. Eosinophils were counted around 5 x 2nd-3rd generation large airways which are ~10mm in length each. Eosinophils up to 2mm from each airway were counted. Data was expressed as eosinophils per millimetre of basement membrane length.

Measurement of silver in lungs
For the Sprague-Dawley rats, the concentration of silver in lung lobes was quantified by inductively coupled plasma mass spectrometry (ICP-MS) (7700x, Agilent Technologies) following digestion of tissue samples. A standard reference material was analysed with samples (National Research Council Canada (LUTS-1) non-defatted lobster hepatopancreas) and the detection limit for the analysis was 5 ng per sample.

For the Brown-Norway rats, the concentration of Ag in the lung lobes was quantified using ICP-MS (7900, Agilent Technologies) on the snap frozen tissue. After freeze drying, tissues were digested by microwave and ICPMS performed in no-gas mode using mass 107 as the quantifier and mass 71 as the internal standard. The ICP-MS was calibrated using silver standards diluted from a 1 ppm stock.

**Measurement of malondialdehyde**

BAL malondialdehyde (MDA) was measured using a HPLC system with fluorescent detection (Waters, Milford, MA, USA) set at 532 nm for the excitation wavelength and 553 nm for the emission wavelength. A Nova-Pak C18 column (Waters, Milford, MA, USA) was used with a mobile phase that was composed of 40% methanol and 60% water containing 50mM KH$_2$PO$_4$ (pH=6.8). The detection limit, extraction recovery and analytical precision were 1.8 nM, 75.9%, and 2.2%, respectively.

**Total phospholipid and surfactant proteins in BAL**

BAL supernatant was separated into two fractions using differential centrifugation. 1 mL of BAL from the first wash was centrifuged at 18,000 rpm for 30 minutes at 4°C to obtain large and small aggregate fractions. The large aggregate (LA) fraction (pellet) contains phospholipids, tubular myelin, lamellar bodies, large vesicles, SP-A, B and C. The supernatant contains the small aggregate fraction (SA) consisting of small vesicles, SP-D and little surface functional surfactant (26). The LA was re-suspended in 40 µL of physiological saline and 5 µL was separated into organic and aqueous fractions by a chloroform and methanol extraction. The lower layer containing the organic fraction was dried under nitrogen. Total organic phosphorus was extracted using a perchloric acid digestion (70%) for one hour at 200°C, with potassium phosphate standards treated in the same way as the samples and assayed using the method of Bartlett (27). Organic phospholipid was expressed as µg of total organic phosphate in 5 µL LA, equivalent to 1/5 mL of BAL. Protein concentrations in the LA and SA
were determined by the Bio-rad assay. SP-D was measured in whole BAL supernatants by ELISA [Cusabio Biotech Co., Suffolk, UK] and SP-B in the LA fraction by ELISA [Cusabio Biotech Co., Newmarket, Suffolk, UK.]

**Cytokine and chemokine levels in BAL**

Cytokines and chemokines including KC, CCL11 (eotaxin), Interferon (IFN)-γ, IL-1β, IL-4, IL-6, IL-13, IL-17A, CCL2 (MCP-1) and CCL3 (MIP-1α) were measured in BAL supernatants using a Milliplex MAP rat cytokine panel according to the manufacturer’s specifications.

**Data analysis**

Data analysis was performed using Prism 5 software. Data were treated non-parametrically as data was generally not normally distributed when tested using the Shapiro-Wilk normality test. A non-parametric ANOVA (Kruskal-Wallis test) was performed at each time point and comparison of the means of the multiple groups was assessed by Dunn’s post-hoc test. P values <0.05 were considered significant.

**Competing interests**

The authors declare no competing interests.

**Authors’ contribution**

JS, BA, FH & CG carried out the in-vivo exposure studies; JW, AH & RS setting-up the spark generation of nanoparticles and analysis of particles; CG, BL & RS measurement of silver in lungs by ICPMS; JZ & JS the histological preparation and measurements; JG & JZ the measurement of malonylaldehyde; JS & FH lung function measurements, counting of inflammatory cells and cytokine assays; and AP, TDT, AG, RS & KFC supervised various aspects of measurements. All authors read and approved the final manuscript.
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### Table 1

Characterisation of spark generated silver nanoparticles and lung burden on each of the exposure conditions for the two rat strains

<table>
<thead>
<tr>
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<th><strong>Sprague-Dawley rats</strong></th>
<th></th>
<th><strong>Brown-Norway rats</strong></th>
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<td></td>
<td>Control</td>
<td>Low Dose</td>
<td>High Dose</td>
<td>Control</td>
</tr>
<tr>
<td><strong>Count Median Diameter (nm)</strong></td>
<td>-</td>
<td>13.4 ± 1.0</td>
<td>14.1 ± 2.3</td>
<td>-</td>
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<tr>
<td><strong>Geometric Standard Deviation</strong></td>
<td>-</td>
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<td>1.58 ± 0.06</td>
<td>-</td>
</tr>
<tr>
<td><strong>Concentration /cm³</strong></td>
<td>&lt;1</td>
<td>(4.50 ± 0.21) x 10⁷</td>
<td>(4.55 ± 0.70) x 10⁷</td>
<td>&lt;1</td>
</tr>
<tr>
<td><strong>Mass concentration (µg/m³)</strong></td>
<td>-</td>
<td>801 ± 33</td>
<td>670 ± 49</td>
<td>-</td>
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<tr>
<td><strong>Exposure duration (mins)</strong></td>
<td>720</td>
<td>180</td>
<td>720</td>
<td>720</td>
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<tr>
<td><strong>Lung burden (µg)</strong></td>
<td>0</td>
<td>8</td>
<td>28</td>
<td>0</td>
</tr>
<tr>
<td><strong>Alveolar dose (µg)</strong></td>
<td>0</td>
<td>6</td>
<td>19</td>
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**Legend to Figures:**

**Fig 1.** Diagram of nose-only inhalation exposure system to silver nanoparticles generated from a spark generator. After passing through a krypton-85 charge neutraliser, the particles were led into a custom-built nose-only exposure manifold consisting of 4 nose-only exposure stainless steel and anodised aluminium chambers by a flow-splitting cone with the rats held in restraining tubes. A TEOM™ ambient particulate monitor continuously monitored the aerosol mass concentration delivered to the exposure manifold at a sampling flow rate of 1 L min\(^{-1}\). The aerosol particle size distribution was determined every 3 minutes using a scanning mobility particle sizer (SMPS).

**Fig 2.** Panel A: Density of particles as a function of the diameter of silver nanoparticles measured during each of the experimental conditions for each rat strain. BN-LD: Brown Norway rats exposed to low dose; BN-HD: Brown Norway rats exposed to high dose; SD-LD: Sprague-Dawley rats exposed to low dose; SD-HD: Sprague-Dawley rats exposed to high-dose. Panel B: Representative high resolution transmission electron microscopy (TEM) images of aerosol particles delivered to the exposure manifold at 12,000X magnification (scale bar 200 nm) (Panel B a) and 800,000X magnification (scale bar 2 nm) (Panel B b).

**Fig 3.** Panels A & B: Silver quantification using ICP-MS in lung tissue following inhalation of the high dose of freshly-generated silver nanoparticles. In both rat strains, there was a reduction in lung silver content at Day 7 compared to Day 1, although this only reached statistical significance in the Sprague-Dawley rats. The silver lung content was higher in Brown-Norway rats compared to Sprague-Dawley rats. Panels C & D: Counts of macrophages staining positive for silver in the lung tissue of Sprague Dawley and Brown Norway rats exposed to silver nanoparticles at 1 and 7 days post inhalation at each lung dose deposition. Data for individual rats are shown with the median for each group denoted as a horizontal bar. *P<0.05, **P<0.01, ***P<0.001 versus the air only control within each time-point.

**Fig 4.** Silver-enhancing and hematoxylin-eosin-stained Sprague-Dawley (SD) and Brown-Norway (BN) rat lung sections at 24 hours post-treatment. Panels A & D: SD and BN air control shows an absence of signal; silver-stained positive cells are seen as black or black
brown positivity (red arrows). In Panel B, SD at lung dose of 8μg; in Panel C, at lung dose of 28μg AgNPs and in Panel E, BN at lung dose 8μg and Panel F, 26μg AgNPs. Positive silver-stained cells are deposited in granuloma of BN lungs (arrow head pointing multi-nuclear giant cells). There are high levels of eosinophilic inflammation (black arrows) in BN lungs (internal scale bar = 20 μm for all).

**Fig 5.** Silver-enhancing and hematoxylin-eosin-stained terminal bronchioles of Sprague-Dawley rat lungs showing no signals in air control at 24 hours and 7 days (Panels A &D). In Panel B, a few black silver particles are deposited on the surface of epithelial cells (arrow) at 24 hours after 28μg AgNPs inhalation. In Panel C, more visible agglomerated black silver positivity were observed on the luminal surface and on surface epithelial cells (arrows) and subepithelial connective tissue (arrow heads) at 7 days post-8μg AgNPs inhalation (internal scale bar = 20 μm for all).

**Fig 6.** Lung tissue inflammatory scores (Panels A & B) and eosinophil counts per mm length of airway wall (Panels C & D) in Sprague Dawley and Brown Norway rats exposed to silver nanoparticles at 1 and 7 days post inhalation as a function of lung deposited doses of silver. Individual data-points shown for 8-12 rats per group with horizontal bar showing mean. *P<0.05, **P<0.01, ***P<0.001 versus the air only control (0) within each time-point.

**Fig 7:** Differential cell counts in bronchoalveolar lavage fluid after inhalation of silver nanoparticles at 1 and 7 days post-inhalation in Sprague Dawley rats (Panels A-D) and in Brown Norway rats (Panels E-H) as a function of lung dose. Data shown as mean ± SD (n=8-12 rats per group). *P<0.05, **P<0.01, ***P<0.001 versus the Air only control (0) within each time-point.

**Fig 8:** Levels of total protein (Panels A & E), malonaldehyde (MDA; Panels B & F) and surfactant protein D (SP-D; Panels D & H) in bronchoalveolar lavage fluid in Sprague Dawley and Brown Norway rats as a function of deposited lung dose of silver nanoparticles. Phospholipid levels (Panels C & D) were measured in the large aggregate fraction. Data shown as mean ± SD (n=8-12 rats per group). *P<0.05, **P<0.01, ***P<0.001 versus the Air only control (0) within each time-point.
Fig 9. Cytokine levels in bronchoalveolar lavage fluid in Sprague Dawley and Brown Norway rats as a function of deposited lung dose of silver nanoparticles. Data shown as mean ± SD (n=8-12 rats per group). *P<0.05, **P<0.01, ***P<0.001 versus the air only control (0) within each time-point.

Fig 10: Large airway resistance (Rn) (Panels A, B & C) and tissue elastance (H) (Panels D, E & F) in Brown Norway rats exposed to silver nanoparticles at PEEP 3, 6 and 9 cm H₂O, at 1 and 7 days post inhalation as a function of lung deposited doses of silver. Data shown as mean ± SD, n = 6 rats per group. *P<0.05 and **P<0.01 versus the air only control (0) within each time-point.
Figure 1

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Gas lines
Data connections
Lab extract system

MFC: Mass flow controller
HEPA: High efficiency particulate air filter
Δp: Differential pressure meter
NAS: Electrostatic nanometer aerosol sampler
TEOM: Tapered element oscillating microbalance
SMPS: Scanning mobility particle sizer
CPC: Condensation particle counter

Fig 1
Figure 2
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Fig 2
Figure 3

Sprague Dawley

Brown Norway

A

B

C

D

Ag (µg/g dry tissue)

Day 1

Day 7

Ag positive macrophages counts / field

Total lung AgNP (µg)

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<td>A.</td>
<td>SD 24h Air</td>
<td>B.</td>
<td>SD 24h Ag low</td>
<td>C.</td>
<td>SD 24h Ag high</td>
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<tr>
<td>D.</td>
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<td>E.</td>
<td>BN 24h Ag low</td>
<td>F.</td>
<td>BN 24h Ag high</td>
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Fig 4
Figure 6
Click here to download Figure: Fig6 INH.png

Sprague Dawley

A

1 day 7 day

Inflammation score

Total lung AgNP (μg)

B

Eosinophils / mm

Total lung AgNP (μg)

C

1 day 7 day

Inflammation score

Total lung AgNP (μg)

D

Eosinophils / mm

Total lung AgNP (μg)
Sprague Dawley  
Brown Norway  

1 day  
7 day  

**Fig 9**

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Figure 10

Click here to download Figure: Fig 10 INH.eps

Large Airway Resistance

A

PEEP 3

1 day 7 day

Rn (cm H2O·s/mL)

0 8 26 0 8 26

B

PEEP 6

1 day 7 day

Rn (cm H2O·s/mL)

0 8 26 0 8 26

C

PEEP 9

1 day 7 day

Rn (cm H2O·s/mL)

0 8 26 0 8 26

Tissue Elastance

D

PEEP 3

1 day 7 day

H (cm H2O/ml)

0 1.5 1 0 1.5 1

E

PEEP 6

1 day 7 day

H (cm H2O/ml)

0 1.5 1 0 1.5 1

F

PEEP 9

1 day 7 day

H (cm H2O/ml)

0 1.5 1 0 1.5 1

Total lung AgNP (μg)

Total lung AgNP (μg)