

Respiratory Research

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

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Abstract:	<p>Background The increasing use of silver nanoparticles (AgNPs) in consumer products is concerning. We examined the potential toxic effects when inhaled in Brown-Norway (BN) rats with a pre-inflammatory state compared to Sprague-Dawley (SD) rats.</p> <p>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 both rat strains. Rats were sacrificed at day 1 and day 7 after exposure and measurement of lung function.</p> <p>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 at Day 1 and 7, while in BN rats, this was only seen at Day 1; surfactant protein D levels decreased at day 7 at the high dose in SD rats, but was increased at Day 1 at the 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.</p> <p>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.</p>	
Corresponding Author:	Kian Fan Chung Imperial College London UNITED KINGDOM	
Corresponding Author Secondary Information:		
Corresponding Author's Institution:	Imperial College London	
Corresponding Author's Secondary Institution:		
First Author:	Kian Fan Chung	
First Author Secondary Information:		
Order of Authors:	Kian Fan Chung	
	Joanna Seiffert	

Alison Buckley
Bey Leo
Jie Zhu
Farhana Hussain
Chang Guo
James Warren
Alan Hodgson
Jicheng Gong
Mary Ryan
Junfeng Zhang
Alexandra Porter
Terry Tetley
Andrew Gow
Rachel Smith
Ranran Dai
Nicholas G Martin

Order of Authors Secondary Information:

Response to Reviewers:	<p>RESPONSE TO REVIEWER: Reviewer reports:</p> <p>Reviewer #1: Seiffert and colleagues present a set of experiments designed to test the hypothesis that silver nanoparticles induce inflammation in rat models and that inflammation is exacerbated in rat models with preexisting inflammatory status. They utilize a well-designed nose-only system to deliver particles and exhaustively evaluate many common measures of pulmonary inflammation. They initially evaluate the efficacy of deposition and then consider cell-mediated inflammatory processes. The research questions are novel and important, especially in light of sparse data that demonstrate silver abundance and routes of exposure. The manuscript could be immediately strengthened by consideration of the following points:</p> <p>1.Despite initial questions about how much particulate mass was delivered through the apparatus (page 5 lines 18-27), the authors dispel most of the doubt by describing the controls undertaken (page 5, lines 37-50). Can this be done more effectively?</p> <p>Answer: We have re-arranged this part of Methods to address this issue. In addition this section is reduced in length with inclusion of references to various aspects of the methodology. We hope that this has been done more effectively.</p> <p>2.It was unclear in the methods section as to why the team measured silver in the two strains of rats differently. For example, why was the isolation done with wet lungs in the SD and dry in the NB? Would there be advantages to one over the other? And would deviations in the final assessment be expected in one method over the other?</p> <p>Answer: Two different methods of ICPMS assays were used for the 2 experiments done in the 2 different rat strains. This occurred from practical reasons. We originally had planned to do ICPMS only on the SD rats as we only had funding for the SD rats alone. We later decided that BN rats needed to be done and could only had it done in a lab that agreed to do the analyses free of charge for us. The reason why the analysis was done with wet lungs in SD and dry in BN rats is because of the 2 different methods used. We were not interested in whether one method was better than the other, but because of the internal controls for each method, we believe that the 2 methods are comparable. The data may be examined with greater certainty within the measurements made within each strain. However, we maintain that it is still valid to make comparisons across the two methods ie between the 2 strains, which shows that</p>
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there was more retention of silver in BN rat compared to SD rats.
We have corrected Fig 3A where the y axis should be per gram wet weight.

3.The manufacturer for the ELISA array should be provided, as well as the cat# for the product.

Answer: This has been added. It is Millipore Ltd, Watford, Hertfordshire, UK in the Milliplex Analyte Kit Finder.

4.Speaking of the methods section, can more be cited and less written? As it is, the methods section is quite expansive. This of course is necessary if methods are introduced, but some additional citations may allow for a slight paring down.

Answer: We have cut down on the Methods as suggested and added some references to the Methods.This has led to a reduction of 25% in length of Methods.Fig 1 has been removed as it is now in a recent publication referred to.

5.The results described on page 12, lines 14-18 appear problematic, are you sure you're stating what you wanted to here? Further, there is no mention of the panels for 5A or D...which should be listed in this area, even if very briefly.

Answer: We have now referred to Fig 5 A & C. On lines 14-18, we wanted to say that there were occasional aggregates of inflammatory cells consisting of eosinophils, neutrophils and mononuclear cells seen in BN rats but not in SD rats. This is corrected accordingly.

6.The histology images are too poor in many cases. The images would be immediately improved with white balancing as cloudiness, particularly along the edges, is distracting.

Answer: Thank you. We have replaced the images with better images with white balancing. We hope that this has increased the quality of these figures.

7.A citation would be very helpful at the end of the last sentence in the discussion's second paragraph (page 15, line 42).

Answer: This has been added in the form of 3 additional references.

8.In general, the discussion reads as though there may be too much recitation of results and not as much coordination with this work and the work of others. More attention as to how this work support or clarifies other research would be an improvement. Without being too speculative, is there support for a longer term study with the end points being lung remodeling and cell targeting?

Answer: Discussion has been altered as suggested by Reviewer. We have now discussed our findings in the light of what there is already in the literature. We did not do a long term study but we have now quoted the studies where observations have been made over a longer term. One study stated that there was no evidence of airway remodelling. But clearly longer term studies are needed, as now pointed out in the Discussion.

9.It would be helpful to include a designation to Fig 10 that shows the experiments only involved BN rats. All other figures show whether experiments involved both or only one of the 2 strains.

Answer: This has been added on the Figure. Fig 10 is now Fig 9.

[Click here to view linked References](#)

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

¹Joanna Seiffert, ² Alison Buckley, ³Bey Leo, ⁴Nicholas G Martin, ¹Jie Zhu, ¹Ranran Dai,
¹Farhana Hussain, ²Chang Guo, ²James Warren, ²Alan Hodgson, ⁵Jicheng Gong, ³Mary P Ryan,
⁵Junfeng (Jim) Zhang, ³Alexandra Porter, ¹Terry D Tetley, ⁶Andrew Gow, ²Rachel Smith and
¹Kian Fan Chung.

¹Airways Disease, National Heart & Lung Institute, Imperial College, London SW3, UK;

²Nanoparticle Inhalation Research Group, Public Health England, Oxfordshire, UK;

³Department of Material Science, Chemistry and the London Centre for Nanotechnology,
Imperial College, London SW3, UK; ⁴ Department of Clinical Biochemistry, Imperial College
Healthcare NHS Trust, Charing Cross Hospital, London, W6 8RF, ⁵Nicholas School of
Environment & Duke Global Health Institute, Duke University, Durham, USA; ⁶Department of
Pharmacology and Toxicology, Rutgers University, Piscataway, NJ, USA;.

Running title: Pulmonary toxicity of inhaled AgNPs

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Correspondence:

Professor K F Chung,

National Heart & Lung Institute

Imperial College London,

Dovehouse St,

London SW3 6LY, UK

f.chung@imperial.ac.uk

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Abstract: 349w

Background

The increasing use of silver nanoparticles (AgNPs) in consumer products is concerning. We examined the potential toxic effects when inhaled in Brown-Norway (BN) rats with a pre-inflammatory state compared to Sprague-Dawley (SD) rats.

Methods

We determined the effect of AgNPs generated from a spark generator (mass concentration: 600-800 $\mu\text{g}/\text{mm}^3$; mean diameter: 13-16 nm; total lung doses: 8 [Low] and 26-28 [High] μg) inhaled by the nasal route in both rat strains. 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-1 β , 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 at Day 1 and 7, while in BN rats, this was only seen at Day 1; surfactant protein D levels decreased at day 7 at the high dose in SD rats, but was increased at Day 1 at the 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 relatively higher 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.

Key words: lungs, silver nanospheres, inhalation, inflammation

Introduction

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2 Silver nanoparticles (AgNPs) are often suspended in a liquid which can be aerosolised or
3 used in a solid form and are widely used as anti-microbials in consumer products especially
4 for textiles, personal hygiene products, medical equipment, wound dressings, respiratory
5 devices, catheters and disinfectant sprays. Therefore, humans run the risk of inhaling AgNPs
6 [1]. What is known about the potential pulmonary toxicity of inhaled AgNPs has been
7 limited to studies in rodents. Subchronic inhalation of AgNPs induced mild, dose-dependent
8 pulmonary inflammation and alterations in pulmonary function [2-6], with evidence that
9 inhaled AgNPs may also enter the systemic circulation to become distributed to extra-
10 pulmonary organs such as the liver and brain [2, 7, 8]. Exposure of rats to AgNP aerosols for
11 90 days led to modest increased pulmonary function changes, with evidence of genotoxicity
12 and accumulation of tissue macrophages [9], with persistence of lung function and
13 inflammatory changes for up to 12 weeks after cessation of exposure [10]. On the other
14 hand, studies using lower inhaled doses have reported minimal or no toxicity [7, 11-13]. By
15 contrast, direct instillation of AgNPs produced higher levels of inflammation, oxidative stress
16 and cytotoxicity compared to AgNP inhaled at similar doses [14-16].

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31 In our previous study, the inflammatory response induced by intratracheal instillation of
32 AgNPs was dependent on particle size with a greater pulmonary inflammatory response
33 with a 20nm size than an 110 nm size but with little influence imposed by citrate or polyvinyl
34 phosphate-capping [15]. We also observed a pulmonary inflammatory response
35 characterised by an intense eosinophilia and neutrophilia in the Brown-Norway (BN) rat
36 compared to a predominantly neutrophilic response in Sprague-Dawley (SD) rats. The
37 response to AgNPs by direct inhalation into lungs, which would be the main route of
38 exposure if humans were exposed to AgNPs from consumer products, remains unclear
39 particularly in the potential differences in the response between the 2 rat strains. The BN
40 rat is known to readily develop features of allergic asthma, namely lung eosinophilia and
41 bronchial hyperresponsiveness (BHR) following sensitisation and exposure to allergens and
42 possess features of chronic lung inflammation in contrast to the SD rat [17-19].

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54 AgNPs from consumer products are more likely to be inhaled, and the potential toxic
55 effects of AgNPs will certainly depend on the route on entry of these NPs. Therefore, in
56 order to characterise further the bioreactivity of inhaled AgNPs in the lungs, we have studied
57 the effect of freshly-generated uncoated silver nanoparticles produced by a spark discharge

1 generator. We determined the threshold lung deposited dose on the lung inflammatory
2 response and cytokine levels. An important determining factor of the pulmonary response is
3 the interactions of these particles with the lung lining fluid constituents that include
4 dipalmitoylated phospholipid (DPPC) and surfactant-specific proteins A, B, C and D, which
5 influence the aggregation, dissolution and uptake of these nanoparticles by pulmonary
6 macrophages and alveolar cells and hence their cytotoxicity [20-22]. We therefore
7 measured the distribution of the silver particles in the lungs, assayed silver levels, and
8 related these to the surfactant composition and lung function changes. We determined
9 whether there would be differences in these parameters between the 2 rat strains.
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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 [23]. The rate of primary particle production and final size was dependent on the sparking frequency (90-300 Hz). The particles were passed through a krypton-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 chambers as previously described[23]. Animals were held in restraining tubes attached to the chamber ports, thus each had an individual aerosol supply directed to the nose area.

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 with continuous monitoring using a TEOM™ ambient particulate monitor (Model 1400a, Thermo Scientific, Franklin, MA, USA) [23]. The AgNP aerosol was immediately diluted to prevent coagulation using a Palas® ejector dilution system (Model VKL 100, Palas GmbH, Karlsruhe, Germany). 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 with aerosol particle size distribution determined using a scanning mobility particle sizer (SMPS; model 3936N76, TSI Inc., Shoreview, MN, USA) and aerosol particle shape using high resolution transmission electron microscopy (TEM) (JEOL 3000F, JEOL Inc., Tokyo, Japan)[23]. Projected area equivalent diameters were calculated for >1500 particles randomly selected using the image analysis software Image J (<http://imagej.nih.gov/ij/>).

Dose estimation

Estimates of the deposited dose, D (μg), in the lung and alveolar regions were determined using the formula, $D = C \times MV \times T \times DE \times 10^{-6}$, where C (μ/m^3) is the aerosol mass concentration, MV (ml/min) the rat minute ventilation, T (min) the exposure duration, and DE the deposition efficiency. The minute ventilation was derived from measurements of tidal volume (TV) and breathing frequency (f) of 6 rats of similar weight to those used in this study made using head-out plethysmograph (EMMS, Bordon, UK) for the duration of nose-only inhalation experiments similar to those described here. The average MV , TV and f were $190 \text{ mL}/\text{min}$, 1.6 ml and 130 min^{-1} respectively. The deposition efficiencies for the lung and alveolar regions, of 31% and 21%, respectively, were calculated using the multiple-path particle dosimetry (MPPD) model (version 2.11, Applied Research Associates, Inc.) [24] using the aerosol CMD and GSD (Table 1) and the breathing parameters indicated above.

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 in the rat species described here (Project Licence number: PPL 70/7581). These experiments were approved by the Imperial College BioSciences Animal Ethics Committee.

Male pathogen-free Sprague-Dawley (SD) (10-12 weeks, 250-320g) and Brown-Norway (BN) rats (10-12 weeks, 260-380g) were purchased from Harlan, UK and housed under filter tops. 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 either 24 hours or 7 days.

Respiratory Mechanics

Respiratory mechanics was measured by the forced oscillation technique at pulmonary end-expiratory pressures (PEEPs) of $3 \text{ cm H}_2\text{O}$. Rats were anaesthetised with ketamine ($80 \text{ mg}/\text{Kg}$) and Xylazine ($10 \text{ mg}/\text{Kg}$) 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,

1 PA]. Rats were ventilated through a tracheostomy (10 mL/Kg air at 90 breaths per minute)
2 using a computer-controlled ventilator (Spira, EMMS, UK). Three successive deep lung
3 inflations (30 cmH₂O) were performed to standardise volume history. Tidal breathing was
4 interrupted by an 8s broadband input signal, containing multiple frequencies between 0.5
5 and 20Hz. Respiratory impedance was calculated at each frequency using the Fast Fourier
6 transformation of the pressure and flow signals. As a function of frequency, the impedance
7 (Zrs) data can be separated into both resistance (R_L) and elastance (E_L) spectra which were
8 fitted to a constant phase model which partitions the respiratory mechanics into the central
9 airway (R_n) and coefficients of tissue damping (G) and tissue elastance (H) [25].
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18 *Bronchoalveolar lavage (BAL)*

19 BAL was performed as previously described [15]. Differential cell counts were performed on
20 cytology of BAL. BAL supernatants were stored for analysis of various analytes.
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27 *Tissue processing and staining*

28 Paraffin blocks were prepared from lungs and sections stained with hematoxylin and eosin
29 (H & E) and carbol-chromotrope for visualisation of eosinophils as previously described [15].
30 The lung inflammatory response was measured on a 0-3 scale, as previously defined [15].
31 Eosinophils in the lamina propria of largest airways in each lung section were counted
32 around 5 x 2nd-3rd generation large airways of ~10mm in length. Eosinophils up to 2mm
33 from each airway were counted and expressed as eosinophils per millimetre of basement
34 membrane length.
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43 We also used a Silver Enhancing Kit (Cat no: SE100, Sigma-Aldrich, Saint Louis, USA) to
44 visualise silver nanoparticles using light microscope with positive cells appearing black.
45 Uptake into cells in walls of bronchi or blood vessels, alveoli septa and alveolar space of the
46 left lung lobe were counted. Twenty fields covering the whole left section were counted and
47 data are expressed as number of positive silver cells per field.
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54 *Measurement of silver in lungs*

55 We used different inductively-coupled plasma mass spectrometry (ICP-MS) methods for
56 each rat species. For the Sprague-Dawley rats, the concentration of silver in lung lobes was
57 quantified by 7700 ICPMS machine (Agilent Technologies) following digestion of tissue
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1 samples. A standard reference material was analysed with samples (National Research
2 Council Canada (LUTS-1) non-defatted lobster hepatopancreas) and the detection limit for
3 the analysis was 5 ng per sample. For the Brown-Norway rats, we used 7900 ICPMS
4 machine (Agilent Technologies) on snap-frozen tissue. After freeze drying, tissues were
5 digested by microwave and ICPMS performed in no-gas mode using mass 107 as the
6 quantifier and mass 71 as the internal standard. The ICP-MS was calibrated using silver
7 standards diluted from a 1 ppm stock.
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13 *Measurement of malondialdehyde*

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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
KH₂PO₄ (pH=6.8). The detection limit, extraction recovery and analytical precision were 1.8
nM, 75.9%, and 2.2%, respectively.

66 *Total phospholipid and surfactant proteins in BAL*

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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].

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Cytokine and chemokine levels in BAL

Cytokines and chemokines including KC, CCL11 (eotaxin), Interferon (IFN)- γ , IL-1 β , IL-4, IL-6, IL-13, IL-17A, CCL2 (MCP-1) and CCL3 (MIP-1 α) were measured in BAL supernatants using a Milliplex MAP rat cytokine panel (Millipore Analyte Kit Finder, Millipore Ltd, Watford, UK) 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.

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 $3.68 \pm 0.48 \times 10^7$ and $4.55 \pm 0.70 \times 10^7/\text{cm}^3$ (**Table 1; Fig 1**). Mass concentrations measured gravimetrically ranged from 617 ± 25 to $801 \pm 33 \mu\text{g}/\text{m}^3$. **Fig 1** shows representative images of separate Ag nanospheres collected during exposures, illustrating their spherical form. In SD rats, low and high lung doses were estimated using deposition fractions from the MPPD model 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 an estimated deposited dose of 28 μg of AgNP particles were $9.97 \pm 2.79 \mu\text{g}/\text{g}$ (wet weight) at 24 hours. Assuming a typical rat lung weight of 1 g this suggests significant clearance within the first 24 hours, and lower levels of $4.99 \pm 2.21 \mu\text{g}/\text{g}$ wet weight at day 7 ($P < 0.05$), indicate further clearance had occurred from the lung by this time (**Fig 2 A**). The silver concentration in BN rat lung at 24 hours was 74.46 $\mu\text{g}/\text{g}$ dry weight). Typically lung dry weight is ~20% of wet weight so this corresponds to around 15 $\mu\text{g}/\text{g}$ wet weight, which is higher by 50% compared to that found in the SD rats. For the BN rats, there was a non-significant reduction measured at Day 7 (**Fig 2 B**), which suggests slower clearance from the BN rat lung.

In BN rats, there was a clear dose-dependent increase in the number of silver-positive staining cells in the lung at both 1 and 7 days post inhalation, this dose-dependent trend was less clear for the SD rats, particularly at 1 day where the number for both doses were very similar (**Fig 2 C & D**). The numbers of silver positive stained cells were between 2 and 3 times higher for the BN compared to the SD rats for the high dose exposure. For both SD and BN rats numbers of stained cells at 1 and 7 days were similar, indicating no consistent time-dependence.

Silver staining was not observed in non-exposed SD and BN lungs (**Fig 3 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

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cells consisted mainly of macrophages scattered in the alveolar space and lung interstitium of SD lungs (**Fig 3 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 3 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 3**).

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 in SD rat lungs (**Fig 4B & D**) compared to the control air-exposed rats (**Fig 4A & 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 5A**). 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 5C**). Areas of inflammation consisting of eosinophils, neutrophils, and mononuclear cells in BN rat lungs could be seen (**Fig 3E&F**). Eosinophil counts in the airway wall were increased in BN rats at day 1 after the 28 μg exposure dose but not at day 7 (**Fig 5D**). There was no eosinophil increase in the SD lung tissue (**Fig 5B**).

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 6**). 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 a small increase in lymphocyte numbers at the high dose exposure in SD rats ($P<0.03$; data not shown).

BAL total protein, malonaldehyde (MDA), phospholipid and surfactant proteins

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

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

24 25 26 27 *BAL cytokines/chemokines*

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29 Most of the changes in cytokine levels occurred at day 1. In the SD rat, levels of IL-1β,
30 KC, IL-17A, CCL2 (MCP-1) and CCL3 (MIP-1α) increased at the 28 µg dose, with increases also
31 seen at 8 µg for IL1β, KC and CCL3 (**Fig 8**). On the other hand, levels of IFNγ fell in a dose-
32 dependent manner. CCL2 and CCL3 remained elevated at the high dose at day 7. In the BN
33 rat, there were similar trends apart from an additional increase in IL-6 at day 1 at both 8 and
34 26 µg doses. Levels of KC, CCL2 and CCL3 were generally higher in BN rat compared to SD rat.
35 There were no changes in levels of IL-4, IL-13 and CCL-11 (eotaxin) (*data not shown*).

36 37 38 39 40 41 42 43 44 45 *Lung function*

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47 There were no changes in large airway resistance (Rn), tissue damping (G) or tissue
48 elastance (H) in the SD rats at either time-point compared with the air control at a
49 physiological PEEP of 3 cm H₂O, suggesting normal airway and parenchymal functioning of
50 the SD lung (*data not shown*). In BN rats, at PEEP 3, Rn increased at both AgNP doses,
51 although this was only statistically significant for the 8 µg dose (**Fig 9 A**). Increasing the PEEP
52 from 3 to 6 and 9 cm H₂O resolved the increase in Rn recorded at PEEP 3 (**Fig 9 B & C**). On
53 the other hand, a small non-significant decrease in Rn at 7 days at PEEP 3 for the 8 µg dose,
54 was not resolved by PEEP suggesting some ongoing effects on lung function. There was also
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1 a significant increase in H for BN rats at PEEP 3 at the highest dose (**Fig 9 D**), suggesting
2 effects on mechanical pulmonary function also originated in the parenchyma and that the
3 BN rats had stiffer lungs. Again, increasing the PEEP from 3 to 6 and 9 cm H₂O, resolved the
4 increases in H, suggesting that this may be due to a recruitment phenomenon as before (**Fig**
5 **9 E & F**). Similar to the SD rats, there were no changes in G in the BN rats suggesting that no
6 parenchymal distortion had occurred (*data not shown*).
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Discussion

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2 In this study, we report that inhalation of freshly-generated silver nanoparticles of
3 spherical shape of 15nm diameter induced an acute pulmonary neutrophilic inflammation
4 with the production of proinflammatory and pro-neutrophilic cytokines in SD and BN rats.
5 However, there were differences between the 2 strains in that the BN rat also showed an
6 eosinophilic inflammation and also was the only strain to respond with a deterioration of
7 lung function. In addition, our study further delineates the differential response of the lungs
8 of these 2 rat strains to inhalation of silver nanospheres in relation to the clearance of silver
9 from the lungs and the phospholipid and surfactant production.
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Silver tracing and clearance in the lungs

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21 The number of cells, particularly macrophages, containing silver increased with dose
22 exposure in both strains, with the number of silver-positive cells in the BN rat 2 to 3-fold
23 higher than in the SD rat at each time-point for the high dose. The uptake of nanoparticles
24 into macrophages is likely to result in oxidative stress, the release of pro-
25 inflammatory mediators and subsequent pulmonary inflammation [15, 29, 30].
26 Concomitantly, we found that the amount of silver in the lungs persisted to the seventh day,
27 despite a small reduction by that time, but the levels found in the lungs of Brown-Norway
28 rats were approximately 50% higher than in the lungs of Sprague-Dawley rats after the high
29 dose exposure. The levels of silver measured in our Sprague-Dawley rats were higher than
30 those previously reported in Sprague-Dawley or Fischer rats exposed to spark-generated
31 uncoated silver nanoparticles of a similar size, partly due to the higher levels of exposure we
32 delivered in our study [2, 6, 7, 31, 32]. The calculation of silver levels from the day 1 and day
33 7 measurements in the Sprague-Dawley rats indicate a clearance of 50%, which is in a similar
34 range to the clearance of 15 nm spark-produced AgNPs inhaled by Fischer rats at a lower
35 concentration of 179 $\mu\text{g}/\text{m}^3$ (compared to $\sim 700 \mu\text{g}/\text{m}^3$ for our study) for a similar exposure
36 pattern (6h/d for 4 days) of 62% between 1 and 7 days post exposure [6]. Clearance of such
37 uncoated AgNPs have been reported to be much faster, for example in the study of
38 Takenaka et al [2], it was 62% one day after exposure, increasing to 96% on day 7 (clearance
39 between 1 day and 7 days was 89%) in Fischer rats. In a recent study of nebulised AgNPs of
40 primary particle size 20 nm suspended in citrate buffer with a high mass concentration of 7.2
41 mg/m^3 delivered to Sprague-Dawley rats for 6 hours, a clearance of 34% between day 1 and
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1 day 7 was reported [33]. The comparison between different studies is difficult because of
2 differences in amount of exposure, dose of nanoparticles, method of generation of
3 nanoparticle aerosols, particle size and even animal gender, but most of these studies would
4 indicate that the silver persists for a period of at least 7 days after inhalation. In the study of
5 Anderson et al [33], one third of the initial silver inhaled persisted even at 56 days, and this
6 was associated with a greater persistence of silver-positive macrophages at 21 and 56 days.
7 In our study, the clearance between 1 day and 7 days for the Brown-Norway rat compared
8 to the Sprague-Dawley rat was lower at 33%, and was associated with a 2 to 3-fold higher
9 number of silver-containing lung macrophages compared to SD lungs. It is possible that the
10 pre-inflamed lung of the Brown-Norway rat may retain more nanoparticles with a slower
11 clearance rate leading to higher levels of silver found in the lungs when compared to the
12 Sprague-Dawley rat.
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25 **Phospholipid and surfactant levels and influence on lung function**

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27 In accord with previous reports of the inhalation of nanoparticles of titanium oxide, silica
28 or cadmium oxide or ultrafine diesel exhaust particles in rats or mice [34-37], we found that
29 inhalation of AgNPs dose-dependently induced an increase in the levels of phospholipids and
30 total protein in bronchoalveolar lavage fluid maximal at day 1 to a similar extent in both
31 strains. Coating of the AgNPs with phospholipid may have important implications on
32 pulmonary homeostasis by interfering with the biophysical surfactant function such as
33 decreased adsorption of pulmonary surfactant at the air-liquid interface as has been
34 observed for gold nanoparticles [22]. On the other hand, we found that the inhalation of
35 AgNPs resulted in diametrically-different effects on the measured levels of SP-D in BAL fluid.
36 While there was a decrease in SP-D levels in Sprague-Dawley rats at day 7 following the high
37 dose exposure, there was an increase in SP-D levels in BAL from Brown-Norway rats at the
38 low dose exposure at day 1, with a non-significant increase at the high dose at day 7.
39 Interestingly, the levels of SP-D at baseline in BAL fluid was nearly 3-fold higher in Sprague-
40 Dawley rats compared to Brown-Norway rats. The reduction in SP-D in Brown-Norway rats
41 may be due to increased SP-D turnover by alveolar macrophages, possibly as a result of
42 binding to AgNPs [21]. The acute increase in SP-D levels in day 1 seen in Brown-Norway rat
43 may represent an increased production from alveolar Type II cells. Increased amounts of SP-
44 D may lead to increased aggregation of nanoparticles [20], hence targeting them towards
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macrophage and lung clearance [38]. In a recent study, we have also shown that incubating AgNPs with Curosurf^R reduced the amount of IL-6 and IL-8 release from human alveolar Type 1 cells exposed to these AgNPs [39].

Lung function changes only occurred in Brown-Norway 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 [15]. 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 [40].

Inhalation versus direct instillation

The results of inhalation of AgNPs in the present study can be compared with our own work on the effect of direct instillation of these nanoparticles into the lungs [15]. In the previous study, we 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 by the inhaled route when a maximal dose of 26-28 µg was deposited in the lungs. The highest inhaled dose used 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 Sprague-Dawley 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 Brown-Norway rats at day 1 also, but not in Sprague-Dawley rats, changes that were similar as those observed in the current study. The inflammatory data we have observed are similar to those reported from a study of Silva et al [41] who reported that instillation of AgNPs into the lungs of rats induced an inflammatory response at day 7 with resolution by day 21, while at day 56, there was no inflammatory response and no evidence of airway wall remodelling. By contrast, Song et al [10] reported that in Sprague-Dawley rats exposed to spark-generated

1 AgNP aerosol, there was persistence of mild inflammation observed at 12 weeks. Longer
2 term exposure studies are needed.
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5 **Conclusions**

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7 The pre-existing inflammatory state of the Brown-Norway rat is likely to underlie the
8 increased amount of silver retained in the lungs with a reduced clearance rate that may
9 underlie the increased inflammatory response, induction of SP-D and phospholipid and
10 airway and parenchymal dysfunction observed in this rat strain but not in Sprague-Dawley
11 rats. Our findings would indicate that inhalation of AgNPs in people with pre-existing
12 inflammation in the lungs such as asthma patients or those with chronic obstructive
13 pulmonary disease would lead to a greater degree of inflammation with heightened
14 consequences on lung function.
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25 **Competing interests**

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27 The authors declare no competing interests.
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30 **Authors' contribution**

31 JS, BA, FH & CG carried out the in-vivo exposure studies; JW, AH & RS setting-up the spark
32 generation of nanoparticles and analysis of particles; CG, BS, BL & RS measurement of silver
33 in lungs by ICPMS; RD, JZ & JS the histological preparation and measurements; JG & JZ the
34 measurement of malonylaldehyde; JS & FH lung function measurements, counting of
35 inflammatory cells and cytokine assays; and AP, TDT, AG, RS & KFC supervised various
36 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

	Sprague-Dawley rats			Brown-Norway rats		
	Control	Low Dose	High Dose	Control	Low Dose	High Dose
Count Median Diameter (nm)	-	13.4 ± 1.0	14.1 ± 2.3	-	15.9 ± 0.8	15.7 ± 2.8
Geometric Standard Deviation	-	1.60 ± 0.03	1.58 ± 0.06	-	1.58 ± 0.02	1.56 ± 0.06
Concentration /cm³	<1	(4.50 ± 0.21) x 10 ⁷	(4.55 ± 0.70) x 10 ⁷	<1	(3.89 ± 0.18) x 10 ⁷	(3.68 ± 0.48) x 10 ⁷
Mass concentration (µg/m³)	-	801 ± 33	670 ± 49	-	791 ± 32	617 ± 25
Exposure duration (mins)	720	180	720	720	180	720
Lung burden (µg)	0	8	28	0	8	26
Alveolar dose (µg)	0	6	19	0	6	18

Legend to Figures:

Fig 1. *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 2. *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 3. 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 $8\mu\text{g}$; in Panel C, at lung dose of $28\mu\text{g}$ AgNPs and in Panel E, BN at lung dose $8\mu\text{g}$ and Panel F, $26\mu\text{g}$ 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 = $20\mu\text{m}$ for all).

Fig 4. 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 & C). In

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2 Panel B, a few black silver particles are deposited on the surface of epithelial cells (arrow) at
3 24 hours after 28µg AgNPs inhalation. In Panel D, more visible agglomerated black silver
4 positivity were observed on the luminal surface and on surface epithelial cells (arrows) and
5 subepithelial connective tissue (arrow heads) at 7 days post-8µg AgNPs inhalation (internal
6 scale bar = 20 µm for all).
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11 **Fig 5.** Lung tissue inflammatory scores (*Panels A & B*) and eosinophil counts per mm length
12 of airway wall (*Panels C & D*) in Sprague Dawley and Brown Norway rats exposed to silver
13 nanoparticles at 1 and 7 days post inhalation as a function of lung deposited doses of silver.
14 Individual data-points shown for 8-12 rats per group with horizontal bar showing mean.
15 *P<0.05, **P<0.01, ***P<0.001 versus the air only control (0) within each time-point.
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23 **Fig 6:** Differential cell counts in bronchoalveolar lavage fluid after inhalation of silver
24 nanoparticles at 1 and 7 days post-inhalation in Sprague Dawley rats (*Panels A-D*) and in
25 Brown Norway rats (*Panels E-H*) as a function of lung dose. Data shown as mean ± SD (n=8-
26 12 rats per group). *P<0.05, **P<0.01, ***P<0.001 versus the Air only control (0) within
27 each time-point.
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34 **Fig 7:** Levels of total protein (*Panels A & E*), malonaldehyde (MDA; *Panels B & F*) and
35 surfactant protein D (SP-D; *Panels D & H*) in bronchoalveolar lavage fluid in Sprague Dawley
36 and Brown Norway rats as a function of deposited lung dose of silver nanoparticles.
37 Phospholipid levels (*Panels C & D*) were measured in the large aggregate fraction. Data
38 shown as mean ± SD (n=8-12 rats per group). *P<0.05, **P<0.01, ***P<0.001 versus the Air
39 only control (0) within each time-point.
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48 **Fig 8.** Cytokine levels in bronchoalveolar lavage fluid in Sprague Dawley and Brown Norway
49 rats as a function of deposited lung dose of silver nanoparticles. Data shown as mean ± SD
50 (n=8-12 rats per group). *P<0.05, **P<0.01, ***P<0.001 versus the air only control (0)
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58 **Fig 9:** Large airway resistance (Rn) (*Panels A, B & C*) and tissue elastance (H) (*Panels D, E & F*)
59 in Brown Norway rats exposed to silver nanoparticles at PEEP 3, 6 and 9 cm H₂O, at 1 and 7
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1 days post inhalation as a function of lung deposited doses of silver. Data shown as mean \pm
2 SD, n = 6 rats per group. *P<0.05 and **P<0.01 versus the air only control (0) within each
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7 **Pulmonary effects of inhalation of spark-generated silver nanoparticles in**
8 **Brown-Norway and Sprague-Dawley rats**
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11 ¹Joanna Seiffert, ² Alison Buckley, ³Bey Leo, ⁴[Nicholas G Martin](#), ¹Jie Zhu, ¹[Ranran Dai](#),
12 ¹Farhana Hussain, ²Chang Guo, ²James Warren, ²Alan Hodgson, ⁴~~Jicheng~~⁵[Jicheng](#) Gong,
13 ³Mary P Ryan, ⁴~~Junfeng~~⁵[Junfeng](#) (Jim) Zhang, ³Alexandra Porter, ¹Terry D Tetley, ⁵~~Andrew~~
14 ⁶[Andrew](#) Gow, ²Rachel Smith and ¹Kian Fan Chung.
15
16
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18

19 ¹Airways Disease, National Heart & Lung Institute, Imperial College, London SW3, UK;
20 ²Nanoparticle Inhalation Research Group, Public Health England, Oxfordshire, UK;
21 ³Department of Material Science, Chemistry and the London Centre for Nanotechnology,
22 Imperial College, London SW3, UK; ⁴ [Department of Clinical Biochemistry, Imperial College](#)
23 [Healthcare NHS Trust, Charing Cross Hospital, London, W6 8RF](#), ⁵Nicholas School of
24 Environment & Duke Global Health Institute, Duke University, Durham, USA; ⁵~~Department~~
25 ⁶[Department](#) of Pharmacology and Toxicology, Rutgers University, Piscataway, NJ, USA;.
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33 **Running title:** Pulmonary toxicity of inhaled AgNPs
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38 Words: 5421; Table: 1; Figures: ~~109~~
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42 Correspondence:

43 Professor K F Chung,
44 National Heart & Lung Institute
45 Imperial College London,
46 Dovehouse St,
47 London SW3 6LY, UK
48 f.chung@imperial.ac.uk
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7 **Abstract: 349w**

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9 *Background*

10 The increasing use of silver nanoparticles (AgNPs) in consumer products is concerning. We
11 examined the potential toxic effects when inhaled in Brown-Norway (BN) rats with a pre-
12 inflammatory state compared to Sprague-Dawley (SD) rats.
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14 *Methods*

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16 We determined the effect of AgNPs generated from a spark generator (mass concentration:
17 600-800 $\mu\text{g}/\text{mm}^3$; mean diameter: 13-16 nm; total lung doses: 8 [Low] and 26-28 [High] μg)
18 inhaled by the nasal route in both rat strains. Rats were sacrificed at day 1 and day 7 after
19 exposure and measurement of lung function.
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22 *Results*

23 In both strains, there was an increase in neutrophils in bronchoalveolar lavage (BAL) fluid at
24 24 hours at the high dose, with concomitant eosinophilia in BN rats. While BAL inflammatory
25 cells were mostly normalised by Day 7, lung inflammation scores remained increased
26 although not the tissue eosinophil scores. Total protein levels were elevated at both lung
27 doses in both strains. There was an increase in BAL IL-1 β , KC, IL-17, CCL2 and CCL3 levels in
28 both strains at Day 1, mostly at high dose. Phospholipid levels were increased at the high
29 dose in SD rats at Day 1 and 7, while in BN rats, this was only seen at Day 1; surfactant
30 protein D levels decreased at day 7 at the high dose in SD rats, but was increased at Day 1 at
31 the low dose in BN rats. There was a transient increase in central airway resistance and in
32 tissue elastance in BN rats at Day 1 but not in SD rats. Positive silver-staining was seen
33 particularly in lung tissue macrophages in a dose and time-dependent response in both
34 strains, maximal by day 7. Lung silver levels were ~~highest~~ relatively higher in BN rat and
35 present at day 7 in both strains.
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42 *Conclusions*

43 Presence of cellular inflammation and increasing silver-positive macrophages in lungs at day
44 7, associated with significant levels of lung silver indicate that lung toxicity is persistent even
45 with the absence of airway luminal inflammation at that time-point. The higher levels and
46 persistence of lung silver in BN rats may be due to the pre-existing inflammatory state of the
47 lungs.
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51 **Key words:** lungs, silver nanospheres, inhalation, inflammation
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7 **Introduction**

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9 Silver nanoparticles (AgNPs) are often suspended in a liquid which can be aerosolised or
10 used in a solid form and are widely used as anti-microbials in consumer products especially
11 for textiles, personal hygiene products, medical equipment, wound dressings, respiratory
12 devices, catheters and disinfectant sprays. Therefore, humans run the risk of inhaling AgNPs
13 [1]. What is known about the potential pulmonary toxicity of inhaled AgNPs has been
14 limited to studies in rodents. Subchronic inhalation of AgNPs induced mild, dose-dependent
15 pulmonary inflammation and alterations in pulmonary function [2-6], with evidence that
16 inhaled AgNPs may also enter the systemic circulation to become distributed to extra-
17 pulmonary organs such as the liver and brain [2, 7, 8]. Exposure of rats to AgNP aerosols for
18 90 days led to modest increased pulmonary function changes, with evidence of genotoxicity
19 and accumulation of tissue macrophages [9], with persistence of lung function and
20 inflammatory changes for up to 12 weeks after cessation of exposure [10]. On the other
21 hand, studies using lower inhaled doses have reported minimal or no toxicity [7, 11-13]. By
22 contrast, direct instillation of AgNPs produced higher levels of inflammation, oxidative stress
23 and cytotoxicity compared to AgNP inhaled at similar doses [14-16].

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31 In our previous study, the inflammatory response induced by intratracheal instillation of
32 AgNPs was dependent on particle size with a greater pulmonary inflammatory response
33 with a 20nm size than an 110 nm size but with little influence imposed by citrate or polyvinyl
34 phosphate-capping [15]. We also observed a pulmonary inflammatory response
35 characterised by an intense eosinophilia and neutrophilia in the Brown-Norway (BN) rat
36 compared to a predominantly neutrophilic response in Sprague-Dawley (SD) rats. The
37 response to AgNPs by direct inhalation into lungs, which would be the main route of
38 exposure if humans were exposed to AgNPs from consumer products, remains unclear
39 particularly in the potential differences in the response between the 2 rat strains. The BN
40 rat is known to readily develop features of allergic asthma, namely lung eosinophilia and
41 bronchial hyperresponsiveness (BHR) following sensitisation and exposure to allergens and
42 possess features of chronic lung inflammation in contrast to the SD rat [17-19].

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48 AgNPs from consumer products are more likely to be inhaled, and the potential toxic
49 effects of AgNPs will certainly depend on the route on entry of these NPs. Therefore, in
50 order to characterise further the bioreactivity of inhaled AgNPs in the lungs, we have studied
51 the effect of freshly-generated uncoated silver nanoparticles produced by a spark discharge

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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 (DPPE) 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 [20-22]. We therefore measured the distribution of the silver particles in the lungs, assayed silver levels, and related these to the surfactant composition and lung function changes. We determined whether there would be differences in these parameters between the 2 rat strains.

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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 [23]. 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 as previously described ~~for sampling, by a flow-splitting cone with the rats are held in restraining tubes attached to the chamber~~ [23]. ~~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. Animals were held in restraining tubes attached to the chamber ports, thus each had an individual aerosol supply directed to the nose area.~~ Each animal has an individual aerosol supply directed to the nose area.

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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 with continuous monitoring using a-A TEOM™ ambient particulate monitor (Model 1400a, Thermo Scientific, Franklin, MA, USA) [23]. ~~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%).~~ The AgNP-AgNP 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

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7 numbers. ~~with~~The aerosol particle size distribution ~~was~~ determined ~~every 3 minutes~~ using a
8 scanning mobility particle sizer (SMPS; model 3936N76, TSI Inc., Shoreview, MN, USA) ~~and~~
9 ~~The shape of the aerosol particles particle shape delivered to the manifold was determined~~
10 ~~with using~~ high resolution transmission electron microscopy (TEM) (JEOL 3000F, JEOL Inc.,
11 Tokyo, Japan)[23]. ~~Samples for TEM were taken directly onto 400 mesh nickel TEM grids with~~
12 ~~lacey carbon film using an electrostatic precipitator (TSI 3089 nanometer aerosol sampler,~~
13 ~~TSI Inc., Shoreview, MN, USA).~~ Projected area equivalent diameters were calculated for
14 >1500 particles randomly selected ~~from each experiment~~ using the image analysis software
15 Image J (<http://imagej.nih.gov/ij/>).
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22 *Dose estimation*

23 Estimates of the deposited dose, D (μg), in the lung and alveolar regions were determined
24 using the ~~following~~ formula, $D = C \times MV \times T \times DE \times 10^{-6}$, where C (μm^3) is the aerosol mass
25 concentration, MV (ml/min) the rat minute ventilation, T (min) the exposure duration, and
26 DE the deposition efficiency. The minute ventilation was derived from measurements of tidal
27 volume (TV) and breathing frequency (f) of 6 rats of similar weight to those used in this study
28 made using head-out plethysmographs (EMMS, Bordon, UK) ~~in the same exposure system~~
29 for the duration of nose-only inhalation experiments similar to those described here. The
30 average MV, TV and f were 190 mL/min, 1.6 ml and 130 min^{-1} respectively, ~~in line with~~
31 ~~typical values for animals of similar weight found in the literature.~~ The deposition efficiencies
32 for the lung and alveolar regions, of 31% and 21%, respectively, were calculated using the
33 multiple-path particle dosimetry (MPPD) model (version 2.11, Applied Research Associates,
34 Inc.) [24] using the aerosol CMD and GSD (Table 1) and the breathing parameters indicated
35 above.
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44 *Study design*

45 The experiments were performed within the legal framework of the United Kingdom under a
46 Project License granted by the Home Office of Her Majesty's government. The researchers
47 hold Personal Licenses provided by the Home Office to perform the experiments in the rat
48 species described here (Project Licence number: PPL 70/7581). These experiments were
49 ~~reviewed and~~ approved by the Imperial College BioSciences Animal Ethics Committee.
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7 Male ~~specific~~-pathogen-free Sprague-Dawley (SD) ~~rats~~ (10-12 weeks, 250-320g) and Brown-
8 Norway (BN) rats (10-12 weeks, 260-380g) were purchased from Harlan, UK and housed
9 under filter tops. Rats were randomly assigned into groups and exposed for 3 hours on one
10 day (low dose) or for 3 hours on four consecutive days (high dose). Three exposure
11 conditions were defined: filtered air only controls for 3 hours on 4 consecutive days, low
12 dose AgNP exposure and high dose AgNP exposure (**Table 1**). Following exposure, the rats
13 were returned to their cages for ~~a period of~~ either 24 hours or 7 days.
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19 *Respiratory Mechanics*

20 Respiratory mechanics was measured by the forced oscillation technique at pulmonary end-
21 expiratory pressures (PEEPs) of 3 cm H₂O. Rats were anaesthetised with ketamine (80 mg/Kg)
22 and Xylazine (10mg/Kg) i.p. and the depth of anaesthesia was quantified by loss of pedal
23 reflex and by pulse oximetry, using the MouseOx Plus [STARR Life Sciences Corp., Oakmont,
24 PA]. ~~A tracheostomy was performed with a 15G cannula and the rats~~ Rats were ventilated
25 ~~through a tracheostomy with~~ (10 mL/Kg air at ~~a frequency of~~ 90 breaths per minute) using a
26 computer-~~controlled~~ ventilator (Spira, EMMS, UK). ~~Prior to measuring lung function, t~~ three
27 successive deep lung inflations (30 cmH₂O) were performed to standardise volume history.
28 ~~Normal t~~idal breathing was interrupted by an 8s broadband input signal, containing
29 multiple frequencies between 0.5 and 20Hz. Respiratory impedance was calculated at each
30 frequency using the Fast Fourier transformation of the pressure and flow signals. As a
31 function of frequency, the impedance (Zrs) data can be separated into both resistance (R_L)
32 and elastance (E_L) spectra ~~as calculated by the software. These spectra which~~ were fitted to a
33 constant phase model which partitions the respiratory mechanics into ~~contributions by~~ the
34 central airway (R_n) and coefficients of tissue damping (G) and tissue elastance (H) [25].
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44 *Bronchoalveolar lavage (BAL)*

45 ~~Following measurement of respiratory mechanics, rats were sacrificed with an overdose of~~
46 ~~sodium pentobarbital (200 mg/Kg). The pulmonary vasculature was perfused with 120 mL of~~
47 ~~saline and BAL was performed in situ via the tracheal cannula with 2 x 10.5 mL aliquots of~~
48 ~~PBS. BAL cells from both aliquots were pooled for analysis of total and differential cells. BAL~~
49 ~~was performed as previously described [15]. Differential cell counts were performed on~~
50 ~~cytology of BAL.~~ BAL supernatants were ~~retained separately stored~~ for analysis of total
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~~proteins, monaldehyde (MDA), cytokines, chemokines, phospholipid and surfactant proteinsvarious analytes. BAL cells were reconstituted to 1×10^6 cells/mL and 100 μ L 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

~~Paraffin blocks were prepared from lungs and sections stained with hematoxylin and eosin (H & E) and carbol-chromotrope for visualisation of eosinophils as previously described [15]. The lung inflammatory response was measured on a 0-3 scale, as previously defined [15]. Eosinophils in the lamina propria of largest airways in each lung section were counted around $5 \times 2^{\text{nd}}\text{-}3^{\text{rd}}$ generation large airways of ~ 10 mm in length. Eosinophils up to 2mm from each airway were counted and expressed as eosinophils per millimetre of basement membrane length. 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 μ m thick serial lung sections were cut and stained with: 1) hematoxylin and eosin (H & E) (BDH, Lutterworth, U.K.) 2)~~

~~We also used a 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. to visualise Silver-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

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~~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

~~We used two different inductively-coupled plasma mass spectrometry (ICP-MS) methods for each rat species. For the Sprague-Dawley rats, the concentration of silver in lung lobes was quantified by inductively-coupled plasma mass spectrometry (ICP-MS) (7700, ICPMS machine (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. Results were determined as silver concentrations per unit wet weight of the lung tissue.~~

~~— For the Brown-Norway rats, the concentration of Ag in the lung lobes was quantified we used using 7900 ICPMS machine (CP-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. ICP-MS was also used to measure silver concentrations in the kidney and liver. Results were are determined reported as silver concentrations per unit dry weight of the lung tissue.~~

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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 KH₂PO₄ (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].

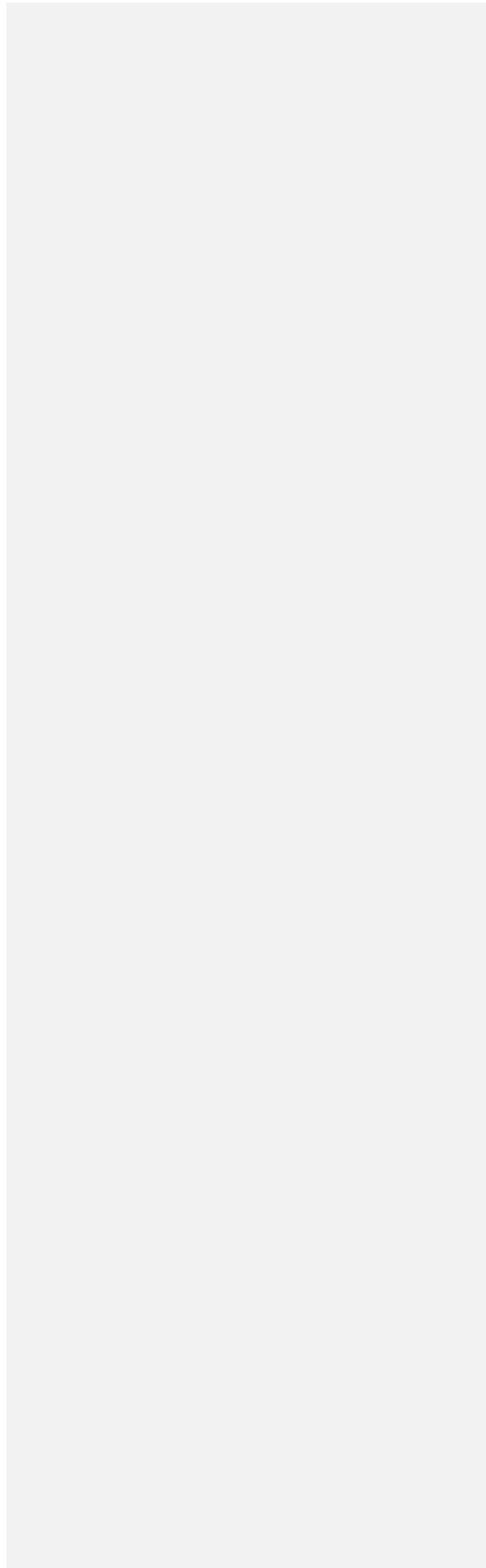
Cytokine and chemokine levels in BAL

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 ([Millipore Analyte Kit Finder, Millipore Ltd, Watford, UK](#)) according to the manufacturer's specifications.

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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.



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 $3.68 \pm 0.48 \times 10^7$ and $4.55 \pm 0.70 \times 10^7/\text{cm}^3$ (Table 1; Fig 21). Mass concentrations measured gravimetrically ranged from 617 ± 25 to $801 \pm 33 \mu\text{g}/\text{m}^3$. Fig 21 shows representative images of separate Ag nanospheres collected during exposures, illustrating their spherical form. In SD rats, low and high lung doses were estimated using deposition fractions from the MPPD model 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 an estimated deposited dose of 28 μg of AgNP particles were $9.97 \pm 2.79 \mu\text{g}/\text{g}$ (wet weight) at 24 hours. Assuming a typical rat lung weight of 1 g this suggests significant clearance within the first 24 hours, and lower levels of $4.99 \pm 2.21 \mu\text{g}/\text{g}$ (wet weight) at day 7 ($P < 0.05$), indicate further clearance had occurred from the lung by this time (Fig 3-2 A). The silver concentration in BN rat lung at 24 hours was $74.46 \mu\text{g}/\text{g}$ (dry weight). Typically lung dry weight is ~20% of wet weight so this corresponds to around $15 \mu\text{g}/\text{g}$ (wet weight), which is a bit higher but by 50% compared with very to that found in the SD rats similar to the SD values. For the BN rats, there was a non-significant reduction measured at Day 7 (Fig 3-2 B), which may interestingly suggest a slower clearance from the BN rat lung.

In both SD and BN rats, there was a clear dose-dependent increase in the number of silver-positive staining cells in the lung at both 1 and 7 days post inhalation, this dose-dependent trend was less clear for the SD rats, particularly at 1 day where the number for both doses were very similar with these being higher in BN rats compared with SD rats for the high dose exposure (Fig 3-2 C & D). The numbers of silver positive stained cells were between 2 and 3 times higher for the BN compared to the SD rats for the high dose exposure. For both SD and BN rats numbers of stained cells at 1 and 7 days were similar, indicating no consistent time-dependence, had similar numbers of silver positive cells at 1

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7 and 7 days, while BN rats had higher numbers of silver positive cells at 7 days for the highest
8
9 dose.

10 Silver staining was not observed in non-exposed SD and BN lungs (Fig 4-3 A & D).
11 There were black silver particles observed at 24 hours post-AgNP inhalation. Silver positivity
12 at 24 hours was as strong as at 7 days after AgNPs inhalation in both rat strains. Silver-
13 positive cells consisted mainly of macrophages scattered in the alveolar space and lung
14 interstitium of SD lungs (Fig 4-3 B & C). Silver-positive macrophages were embedded within
15 the inflammatory cells which infiltrate the alveolar septa and lamina propria of blood
16 vessels and airway mucosa and were deposited in granulomas of BN lungs (Fig 4-3 E & F).
17 Silver particles were also observed on the luminal surface and on surface epithelium and
18 subepithelial connective tissue of terminal bronchioles, scarcely at day 1 but more
19 prominent at day 7 (Fig 4-3).
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25 26 *Lung inflammatory changes*

27 Following inhalation of silver, there was a mild inflammation with a few inflammatory
28 cell infiltrations in the bronchial and vascular walls and alveolar septa in SD rat lungs (Fig
29 ~~5B4B & CD~~) compared to the control air-exposed rats (Fig 4A & C). Overall, tissue
30 inflammation scores were increased in lungs of SD rats at both day 1 and 7 and at both
31 exposure doses with the scores remaining unchanged during that period (Fig 6A5A). In BN
32 rats, there was a high level of baseline inflammation that remained unchanged after
33 inhalation of AgNPs at both levels of exposure and on both day 1 and day 7 (Fig 6E5C). Areas
34 of inflammation consisted consisting mainly of eosinophils, neutrophils, and
35 mononuclear cells monocytes/macrophage and also some of lymphocytes in BN rat lungs
36 could be seen (Fig 4E3E&F). Eosinophil counts in the airway wall were increased in BN rats at
37 day 1 after the 28 µg exposure dose but not at day 7 (Fig 6D5D). There was no eosinophil
38 increase in the SD rat lung tissues (Fig 6B5B).
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46 47 *BAL inflammatory cells*

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

10 11 12 *BAL total protein, malonaldehyde (MDA), phospholipid and surfactant proteins*

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

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

28 29 30 *BAL cytokines/chemokines*

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32 Most of the changes in cytokine levels occurred at day 1. In the SD rat, levels of IL-1 β ,
33 KC, IL-17A, CCL2 (MCP-1) and CCL3 (MIP-1 α) increased at the 28 μ g dose, with increases also
34 seen at 8 μ g for IL1 β , KC and CCL3 (**Fig 98**). On the other hand, levels of IFN γ fell in a dose-
35 dependent manner. CCL2 and CCL3 remained elevated at the high dose at day 7. In the BN
36 rat, there were similar trends apart from an additional increase in IL-6 at day 1 at both 8 and
37 26 μ g doses. Levels of KC, CCL2 and CCL3 were generally higher in BN rat compared to SD rat.
38 There were no changes in levels of IL-4, IL-13 and CCL-11 (eotaxin) (*data not shown*).

39 40 41 *Lung function*

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43 There were no changes in large airway resistance (Rn), tissue damping (G) or tissue
44 elastance (H) in the SD rats at either time-point compared with the air control at a
45 physiological PEEP of 3 cm H₂O, suggesting normal airway and parenchymal functioning of

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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 10-9 A**). Increasing the PEEP from 3 to 6 and 9 cm H₂O resolved the increase in Rn recorded at PEEP 3 (**Fig 10-9 B & C**). 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. There was also a significant increase in H for BN rats at PEEP 3 at the highest dose (**Fig 10-9 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 H₂O, resolved the increases in H, suggesting that this may be due to a recruitment phenomenon as before (**Fig 10-9 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*).

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Discussion

In this study, we ~~compared the pulmonary effects of inhaled silver nanospheres in 2 rat strains with different pre-existing pulmonary inflammatory profiles. report that i~~ 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 ~~in bronchoalveolar lavage fluid obtained from~~ SD and BN rats. However, there were differences between the 2 strains in that the BN rat also showed an eosinophilic inflammation and also was the only strain to respond with a deterioration of lung function. In addition, our study further delineates the differential response of the lungs of these 2 rat strains to inhalation of silver nanospheres in relation to the clearance of silver from the lungs and the phospholipid and surfactant production.

~~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. Therefore, the BN rat showed a greater response in terms of eosinophilia and lung function changes. There was 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 [28].~~

Silver tracing and clearance in the lungs

~~The persistence of tissue inflammation at day 7 was associated with the persistence of silver positive cells in the lungs and airway lumen of these rats. We identified the cells containing silver nanoparticles as being mostly macrophages found within the airway lumen and alveoli, also in inflammatory cells. T~~he number of cells, particularly macrophages, containing silver increased with dose exposure ~~and with time, reaching a maximal at 7 days in both strains but in both strains, with~~ the number of silver-positive cells in the BN rat 2 to ~~was~~ 3-fold higher than in the SD rat at ~~each lung dose and~~ each time-point for the high dose. The uptake of nanoparticles into macrophages is likely to result in oxidative stress, the release of pro-inflammatory mediators and subsequent pulmonary inflammation [15, 29, 30]. Concomitantly, we found that the amount of silver in the lungs persisted to by the seventh day, despite a small reduction by that time, but the levels found in the lungs of Brown-Norway rats were approximately 50% higher than in the lungs of Sprague-Dawley rats after

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7 ~~the high dose exposure.~~ Silver content of the lungs at Day 1 was measurable by ICPMS, with
8 ~~a reduction in silver by day 7 but with still significant amounts.~~ Interestingly, the levels of
9 ~~silver were up to 8 fold greater in BN lungs at day 1 and day 7 compared to SD lungs at the~~
10 ~~high dose exposure.~~ However, ~~T~~the levels of silver measured in our ~~SD-Sprague-Dawley~~ rats
11 were higher than those ~~previously~~ reported in ~~Sprague-Dawley~~ or Fischer rats exposed to
12 spark-generated uncoated silver nanoparticles of ~~a similar size as in our study~~, partly due to
13 the higher levels of exposure we delivered (~~for in our high dose~~)study [2, 6, 7, 31, 32]. The
14 calculation of silver levels from the day 1 and day 7 measurements in ~~our studies of the SD~~
15 ~~Sprague-Dawley~~ rats indicate a clearance ~~rate~~ of 50%, which is in a similar range to the
16 clearance of 15 nm spark-produced AgNPs inhaled by Fischer rats at a lower
17 ~~concentration~~dose of 179 µg/m³ (compared to ~700 µg/m³ for our study) ~~for a similar~~
18 ~~exposure pattern (6h/d for 4 days)~~ of 62% ~~between 1 and 7 days post exposure~~ [6].
19 Clearance of such uncoated AgNPs have been reported to be much faster, ~~with~~ for example
20 in the study of Takenaka et al [2], it was 62% one day after exposure, increasing to 96% on
21 day 7 (~~clearance between 1 day and 7 days was 89%~~) in Fischer rats. In a recent study of
22 nebulised AgNPs of ~~primary particle~~ size 20 nm suspended in citrate buffer with a high mass
23 concentration of 7.2 mg/m³ ~~delivered to SD-Sprague-Dawley rats for 6 hours~~, a clearance
24 ~~rate~~ of 34% between day 1 and day 7 was reported [33]. The comparison between different
25 studies is difficult because of differences in amount of exposure, dose of nanoparticles,
26 method of generation of nanoparticles aerosols, particle size and even animal gender, but
27 most of these ~~limited~~ studies would indicate that the silver persists for a period of at least 7
28 days after inhalation. ~~In t~~The study of Anderson et al [33], ~~showed that in their model~~ one
29 third of the initial silver inhaled persisted ~~even~~ at 56 days, ~~and this~~This was associated with
30 ~~a~~ greater persistence of silver-positive macrophages at 21 and 56 days. In our study, the
31 clearance ~~between 1 day and 7 days value~~ ~~rate~~ for the ~~BN-Brown-Norway~~ rat compared to
32 the ~~SD-Sprague-Dawley~~ rat was lower at 33%, ~~which started with an 8 fold higher silver~~
33 ~~concentration than in the SD rat.~~ This was also ~~and was~~ associated with a ~~2 to~~ 3-fold higher
34 number of silver-containing ~~lung~~ macrophages ~~in BN lungs~~ compared to SD lungs. ~~It is~~
35 ~~possible that~~ These data support the hypothesis that the pre-inflamed lung ~~in of~~ the ~~BN~~
36 ~~Brown-Norway~~ rat ~~may~~ retains more nanoparticles with a slower clearance rate leading to
37 higher levels of silver found in the lungs when compared to ~~the~~ ~~Sprague-Dawley~~ rat.
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7 **Phospholipid and surfactant levels and influence on lung function**

8 ~~Inhalation of AgNPs dose dependently induced an increase in the levels of phospholipids~~
9 ~~and total protein in BAL fluid maximal at day 1 to a similar extent in both strains. This is in~~
10 ~~accord with previous reports of similar observations in rats or mice following the inhalation~~
11 ~~of nanoparticles of titanium oxide, silica or cadmium oxide or ultrafine diesel exhaust~~
12 ~~particles in rats or mice [34-37], we found that inhalation of AgNPs dose-dependently~~
13 ~~induced an increase in the levels of phospholipids and total protein in bronchoalveolar~~
14 ~~lavage fluid maximal at day 1 to a similar extent in both strains.~~ Coating of the AgNPs with
15 phospholipid may have important implications on pulmonary homeostasis by ~~interference~~
16 ~~interfering with the~~ biophysical surfactant function such as decreased adsorption of
17 pulmonary surfactant at the air-liquid interface as has been observed for gold nanoparticles
18 [22]. On the other hand, we found that the inhalation of AgNPs resulted in diametrically-
19 different effects on the measured levels of SP-D in BAL fluid. While there was a decrease in
20 SP-D levels in ~~SD-Sprague-Dawley~~ rats at day 7 following the high dose exposure, there was
21 an increase in SP-D levels in BAL from ~~Brown-Norway BN~~ rats at the low dose exposure at
22 day 1, with a ~~suggestive non-significant~~ increase at the high dose at day 7. Interestingly, the
23 levels of SP-D at baseline in BAL fluid was nearly 3-fold higher in ~~SD-Sprague-Dawley~~ rats
24 compared to ~~BN-Brown-Norway~~ rats. The reduction in SP-D in ~~BN-Brown-Norway~~ rats may be
25 due to increased SP-D turnover by alveolar macrophages, possibly as a result of binding to
26 AgNPs [21]. The acute increase in SP-D levels in day 1 seen in ~~BN-Brown-Norway~~ rat may
27 represent an increased production from alveolar Type II cells. ~~The potential consequences of~~
28 ~~increased amounts of SP-D may lead to increased aggregation of nanoparticles [20], hence~~
29 ~~targeting them towards macrophage and lung clearance [38]. In a recent study, we have also~~
30 ~~shown that incubating AgNPs with Curosurf^R reduced the amount of IL-6 and IL-8 release~~
31 ~~from human alveolar Type 1 cells exposed to these AgNPs [39].~~

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44 Lung function changes only occurred in ~~BN-Brown-Norway~~ rats indicating a greater
45 sensitivity of the airways and lungs to the effect of inhaled AgNPs, similar to the lung
46 function responses observed with instillation of AgNPs [15]. Resolution of the increase in
47 lung resistance recorded at low positive end-expiratory pressure (PEEP) by increasing PEEP
48 suggests that this increase was a recruitment phenomenon which affected dynamic
49 breathing that may be related to surfactant dysfunction rather than inherent forces in the
50 lung which can occur following lung injury [40].

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Inhalation versus direct instillation

The results of inhalation of AgNPs in the present study can be compared with our own work on the effect of direct instillation of these nanoparticles into the lungs [15]. In the previous study, we 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-by~~ the inhaled route when a maximal dose of 26-28 µg was deposited in the lungs. The highest inhaled dose used 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-Sprague-Dawley~~ 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~~ Brown-Norway rats at day 1 also, but not in ~~SD-Sprague-Dawley~~ rats, changes that were similar as those observed in the current study. The inflammatory data we have observed are simialr to those reported from a study of -Silva et al [41] who reported that instillation of AgNPs into the lungs of rats induced an inflammatory response at day 7 with resolution by day 21, while at day 56, there was no inflammatory response and no evidence of airway wall remodelling. By contrast, Song et al [10]reported that in Sprague-Dawley rats exposed to spark-generated AgNP aerosol, there was persistence of mild inflammation observed at 12 weeks. Longer term exposure studies are needed.

Conclusions

~~Following the inhalation of freshly-generated uncoated AgNPs of 15 nm diameter, there was a transient increase in luminal inflammation with neutrophilia and eosinophilia, associated with the secretion of proinflammatory cytokines including the inflammasome marker, IL-1 β , and IL-17, oxidative stress and increased phospholipid levels. Lung cellular inflammation at day 7 was associated with persistent silver positive macrophages in the~~

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~~lungs and significant persistent levels of lung silver.~~ The pre-existing inflammatory state of the ~~BN-Brown-Norway~~ rat is likely 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 ~~and phospholipid, dysfunctional SP-D~~ and airway and parenchymal dysfunction ~~seen-observed~~ in this rat strain but not in ~~SD-Sprague-Dawley~~ rats. Our findings would indicate that inhalation of AgNPs in people with pre-existing inflammation in the lungs such as asthma patients or those with chronic obstructive pulmonary disease would lead to a greater degree of inflammation with heightened consequences on lung function.

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, ~~BS~~, BL & RS measurement of silver in lungs by ICPMS; ~~RD~~, 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

	Sprague-Dawley rats			Brown-Norway rats		
	Control	Low Dose	High Dose	Control	Low Dose	High Dose
Count Median Diameter (nm)	-	13.4 ± 1.0	14.1 ± 2.3	-	15.9 ± 0.8	15.7 ± 2.8
Geometric Standard Deviation	-	1.60 ± 0.03	1.58 ± 0.06	-	1.58 ± 0.02	1.56 ± 0.06
Concentration /cm³	<1	(4.50 ± 0.21) × 10 ⁷	(4.55 ± 0.70) × 10 ⁷	<1	(3.89 ± 0.18) × 10 ⁷	(3.68 ± 0.48) × 10 ⁷
Mass concentration (µg/m³)	-	801 ± 33	670 ± 49	-	791 ± 32	617 ± 25
Exposure duration (mins)	720	180	720	720	180	720
Lung burden (µg)	0	8	28	0	8	26
Alveolar dose (µg)	0	6	19	0	6	18

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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 21. *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 32. *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 43. 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

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7 brown positivity (red arrows). In Panel B, SD at lung dose of 8 μ g; in Panel C, at lung dose of
8 28 μ g AgNPs and in Panel E, BN at lung dose 8 μ g and Panel F, 26 μ g AgNPs. Positive silver-
9 stained cells are deposited in granuloma of BN lungs (arrow head pointing multi-nuclear
10 giant cells). There are high levels of eosinophilic inflammation (black arrows) in BN lungs
11 (internal scale bar = 20 μ m for all).
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16 **Fig 54.** Silver-enhancing and hematoxylin-eosin-stained terminal bronchioles of Sprague-
17 Dawley rat lungs showing no signals in air control at 24 hours and 7 days (Panels A & C). In
18 Panel B, a few black silver particles are deposited on the surface of epithelial cells (arrow) at
19 24 hours after 28 μ g AgNPs inhalation. In Panel D, more visible agglomerated black silver
20 positivity were observed on the luminal surface and on surface epithelial cells (arrows) and
21 subepithelial connective tissue (arrow heads) at 7 days post-8 μ g AgNPs inhalation (internal
22 scale bar = 20 μ m for all).
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28 **Fig 65.** Lung tissue inflammatory scores (Panels A & B) and eosinophil counts per mm length
29 of airway wall (Panels C & D) in Sprague Dawley and Brown Norway rats exposed to silver
30 nanoparticles at 1 and 7 days post inhalation as a function of lung deposited doses of silver.
31 Individual data-points shown for 8-12 rats per group with horizontal bar showing mean.
32 *P<0.05, **P<0.01, ***P<0.001 versus the air only control (0) within each time-point.
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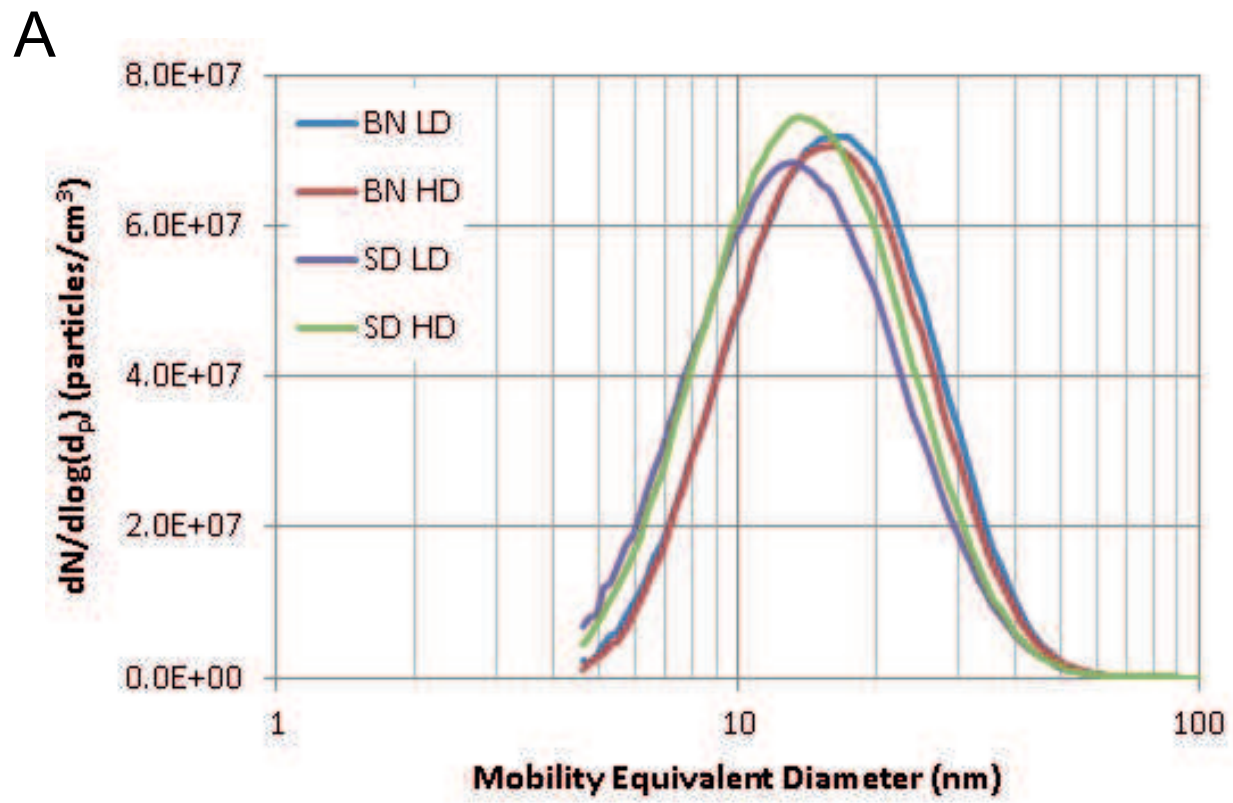
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37 **Fig 76:** Differential cell counts in bronchoalveolar lavage fluid after inhalation of silver
38 nanoparticles at 1 and 7 days post-inhalation in Sprague Dawley rats (Panels A-D) and in
39 Brown Norway rats (Panels E-H) as a function of lung dose. Data shown as mean \pm SD (n=8-
40 12 rats per group). *P<0.05, **P<0.01, ***P<0.001 versus the Air only control (0) within
41 each time-point.
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46 **Fig 87:** Levels of total protein (Panels A & E), malonaldehyde (MDA; Panels B & F) and
47 surfactant protein D (SP-D; Panels D & H) in bronchoalveolar lavage fluid in Sprague Dawley
48 and Brown Norway rats as a function of deposited lung dose of silver nanoparticles.
49 Phospholipid levels (Panels C & D) were measured in the large aggregate fraction. Data
50 shown as mean \pm SD (n=8-12 rats per group). *P<0.05, **P<0.01, ***P<0.001 versus the Air
51 only control (0) within each time-point.
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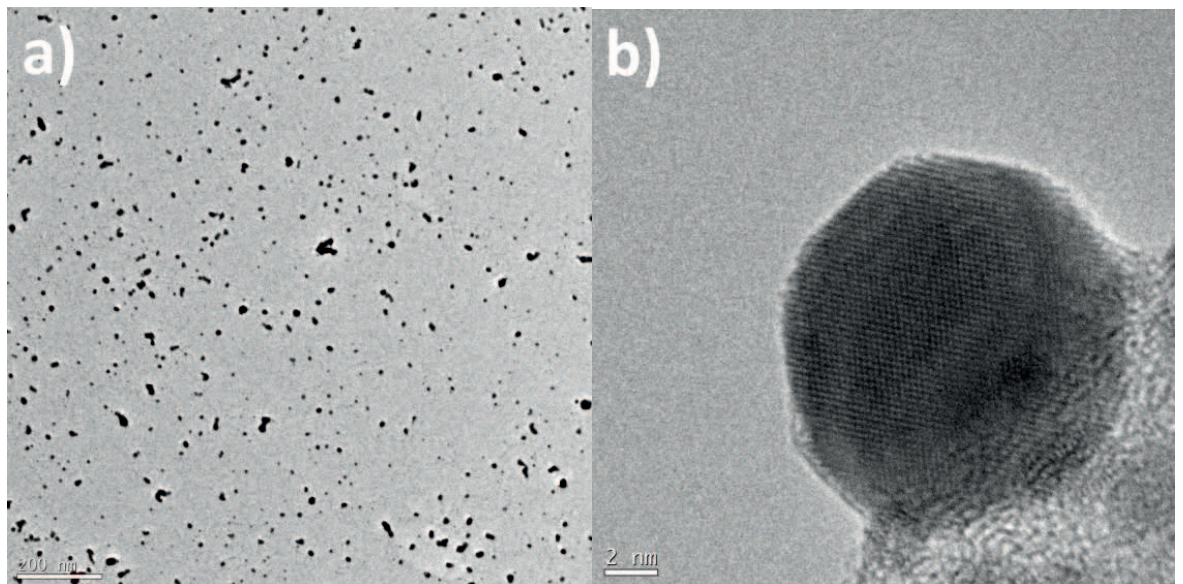
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Fig 98. Cytokine levels in bronchoalveolar lavage fluid in Sprague Dawley and Brown Norway rats as a function of deposited lung dose of silver nanoparticles. Data shown as mean \pm SD (n=8-12 rats per group). *P<0.05, **P<0.01, ***P<0.001 versus the air only control (0) within each time-point.

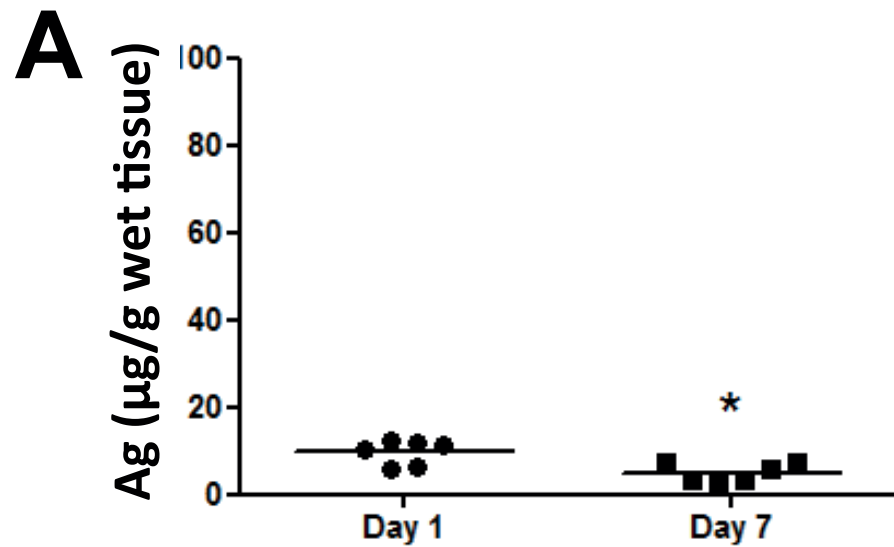
Fig 109: Large airway resistance (Rn) (*Panels A, B & C*) and tissue elastance (H) (*Panels D, E & F*) in Brown Norway rats exposed to silver nanoparticles at PEEP 3, 6 and 9 cm H₂O, at 1 and 7 days post inhalation as a function of lung deposited doses of silver. Data shown as mean \pm SD, n = 6 rats per group. *P<0.05 and **P<0.01 versus the air only control (0) within each time-point.



B



Sprague Dawley



Brown Norway

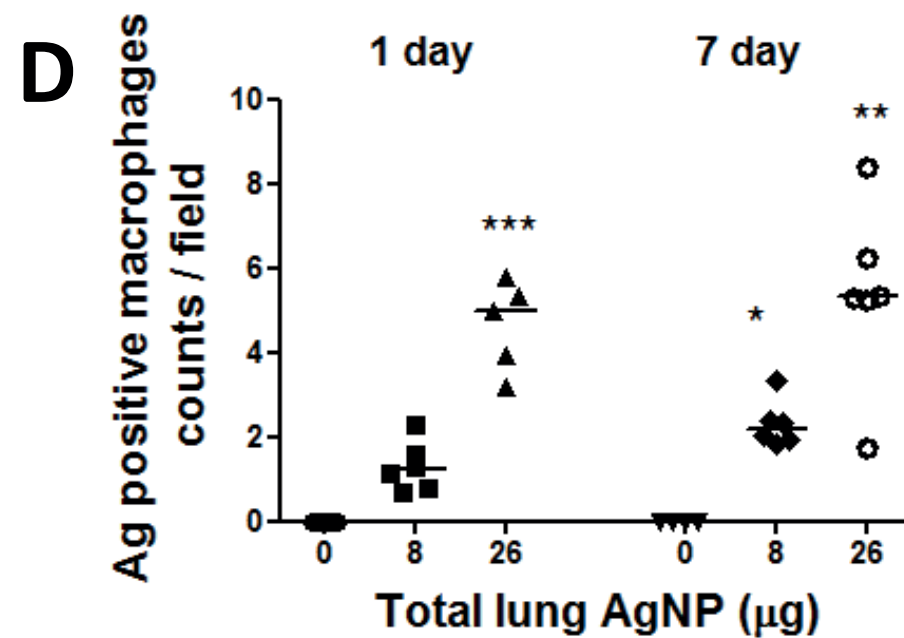
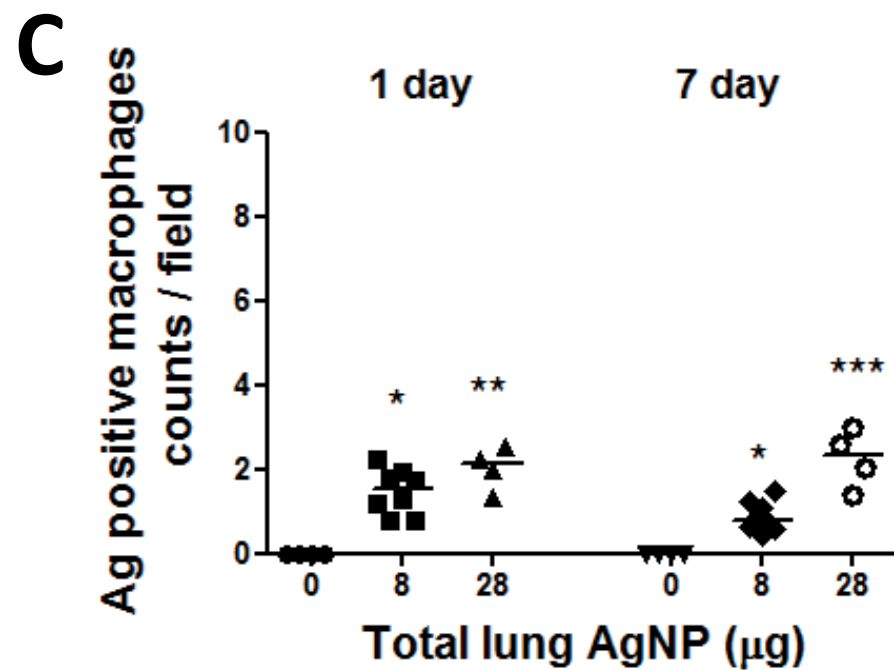
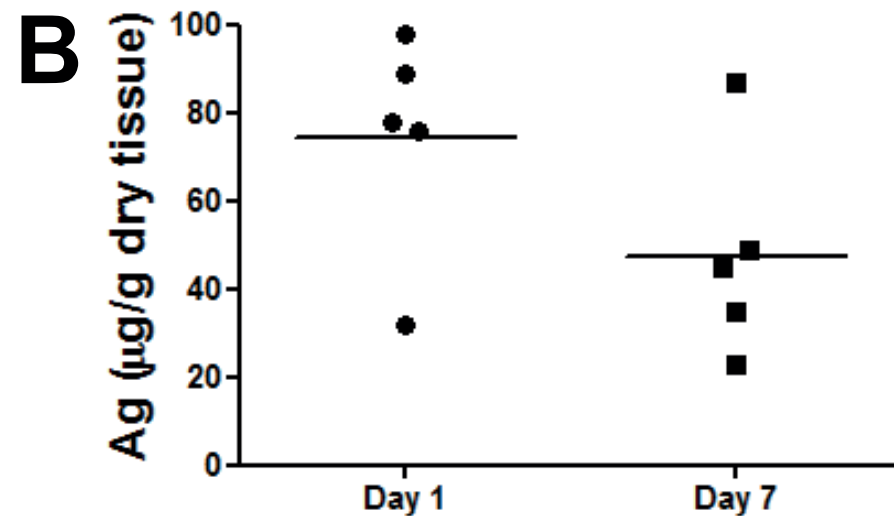


Fig 3

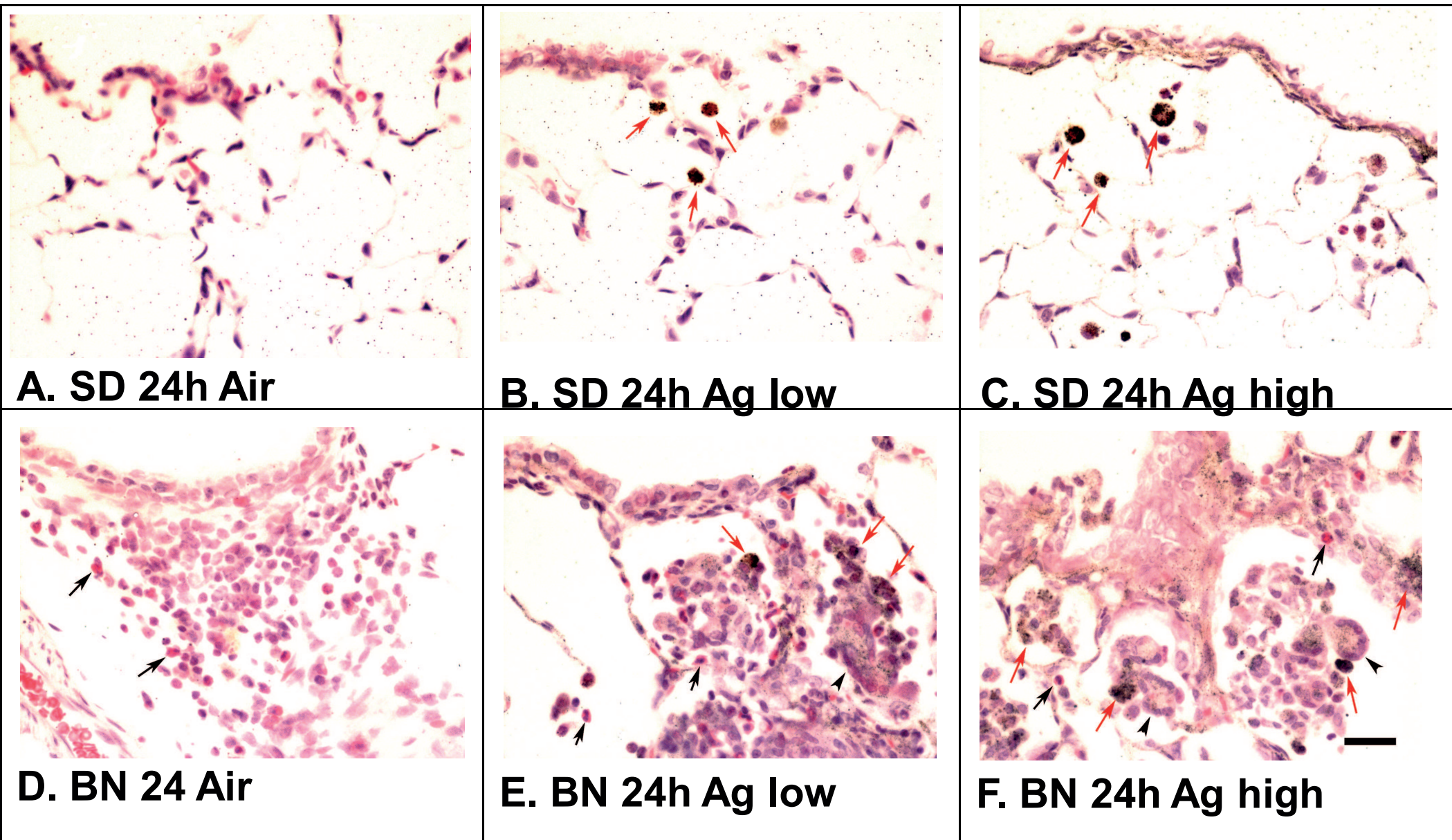


Fig 3

Figure 4

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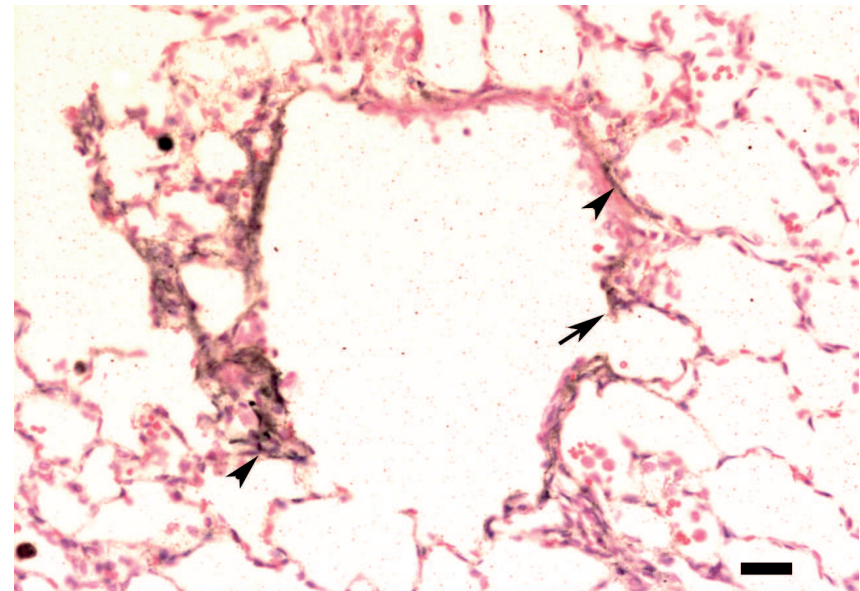
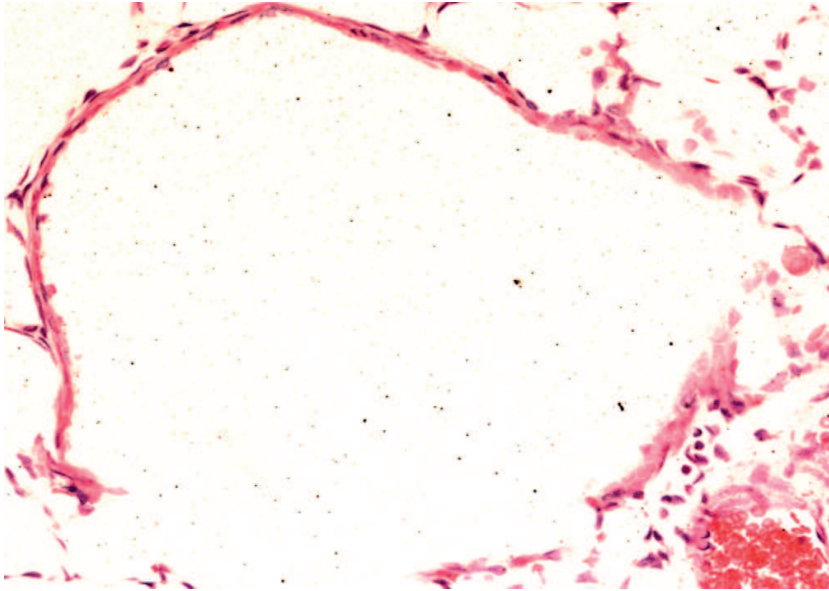
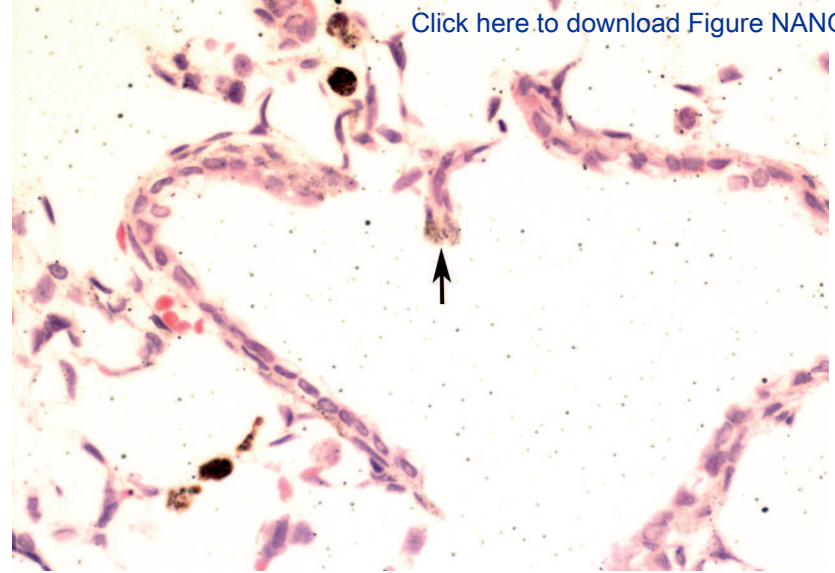
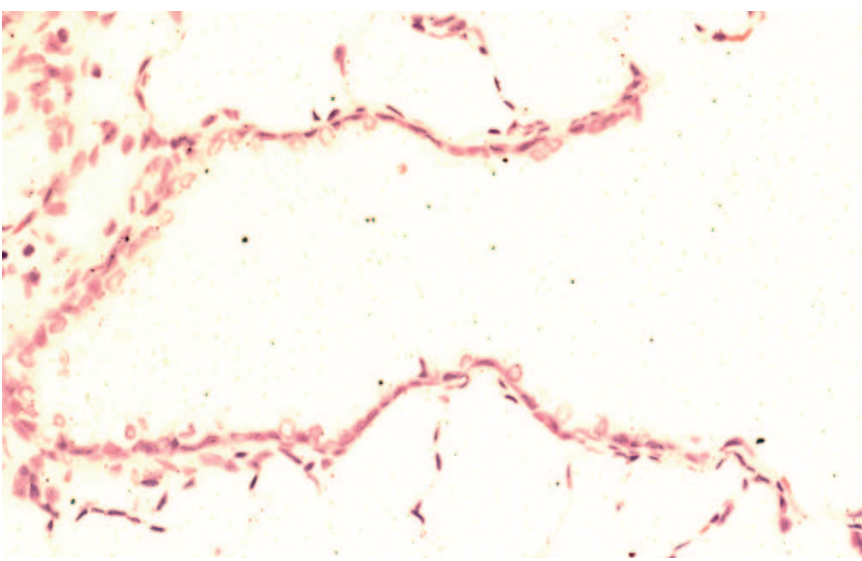
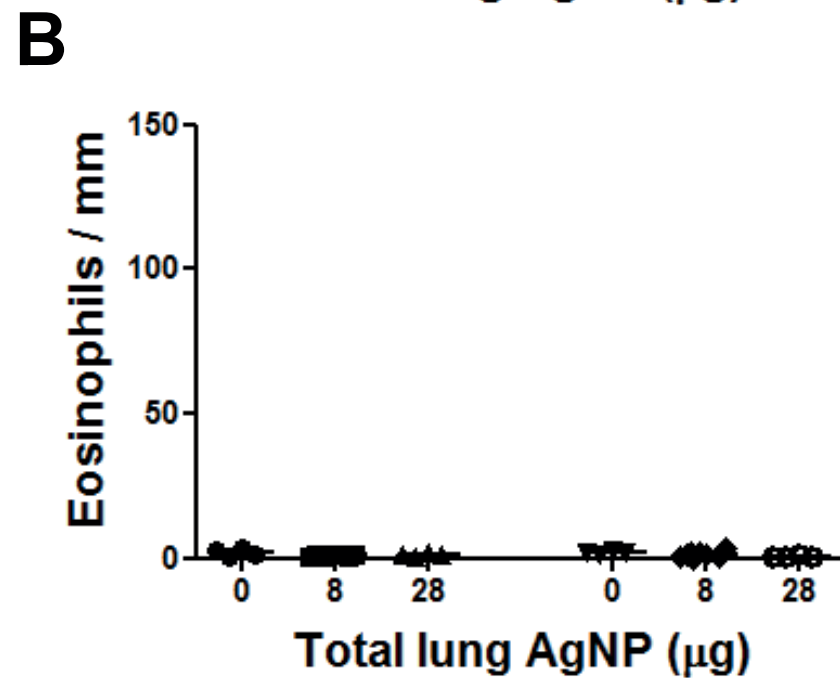
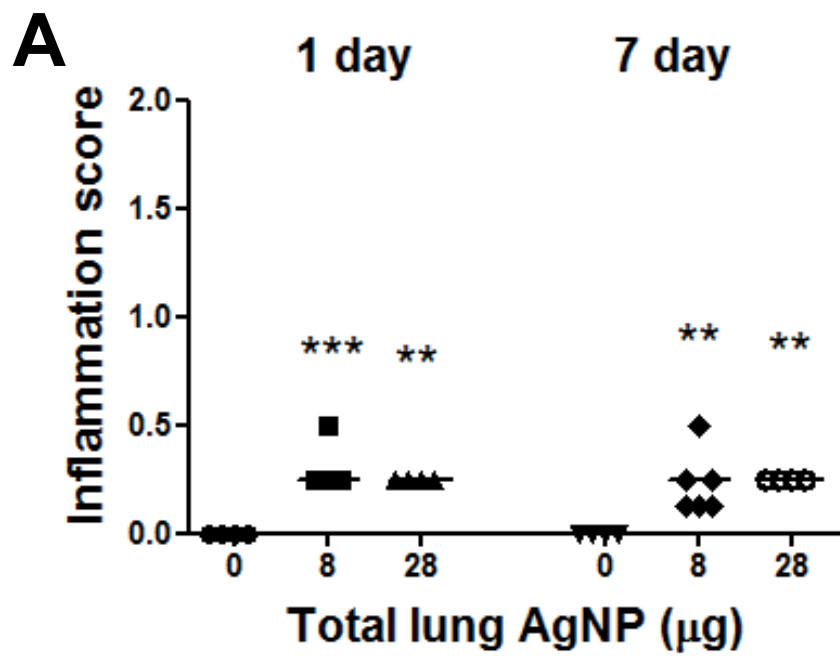
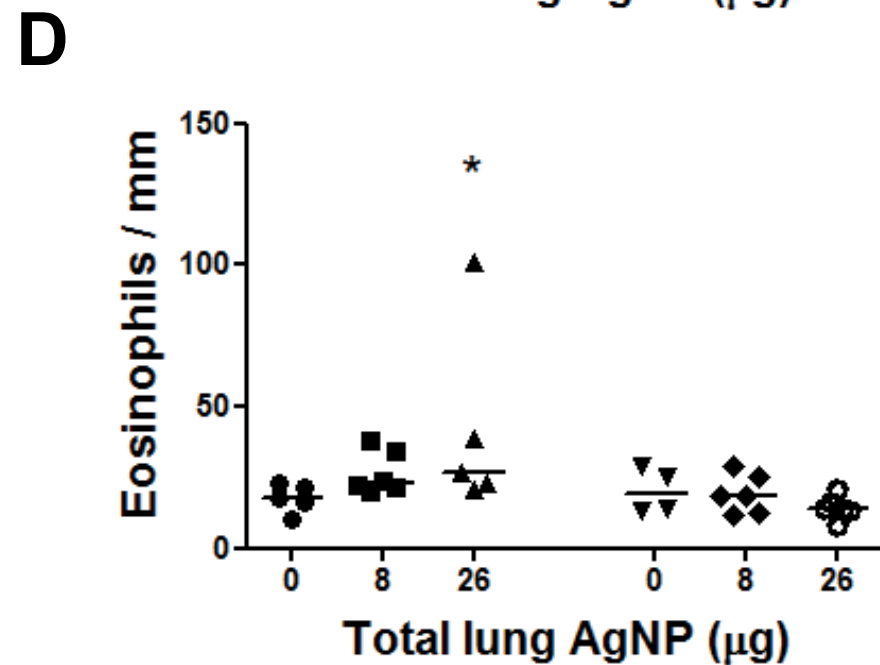
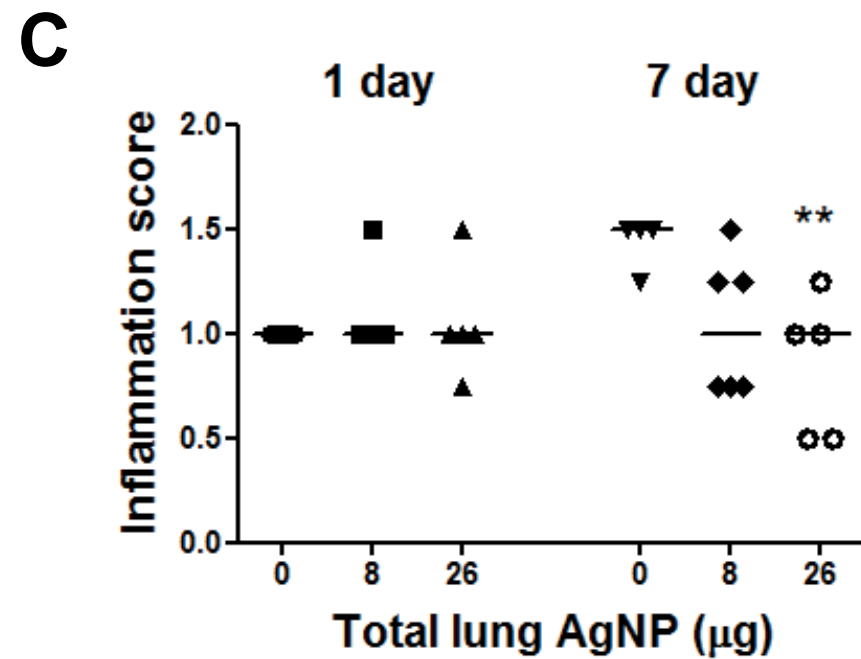


Fig 4

Sprague Dawley



Brown Norway



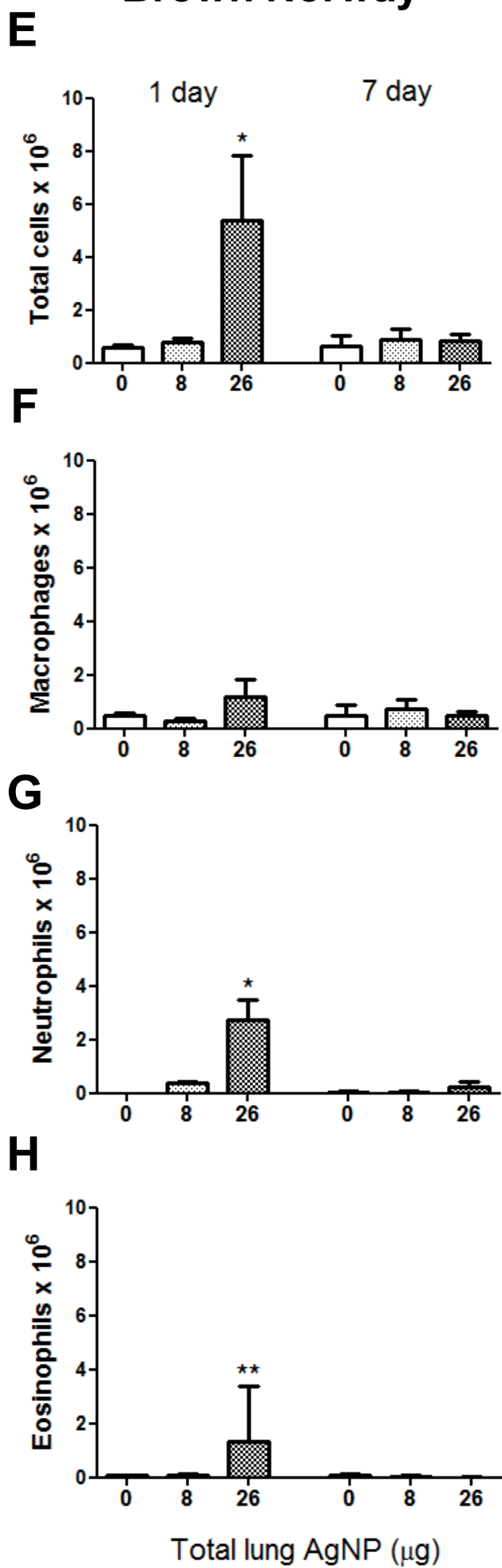
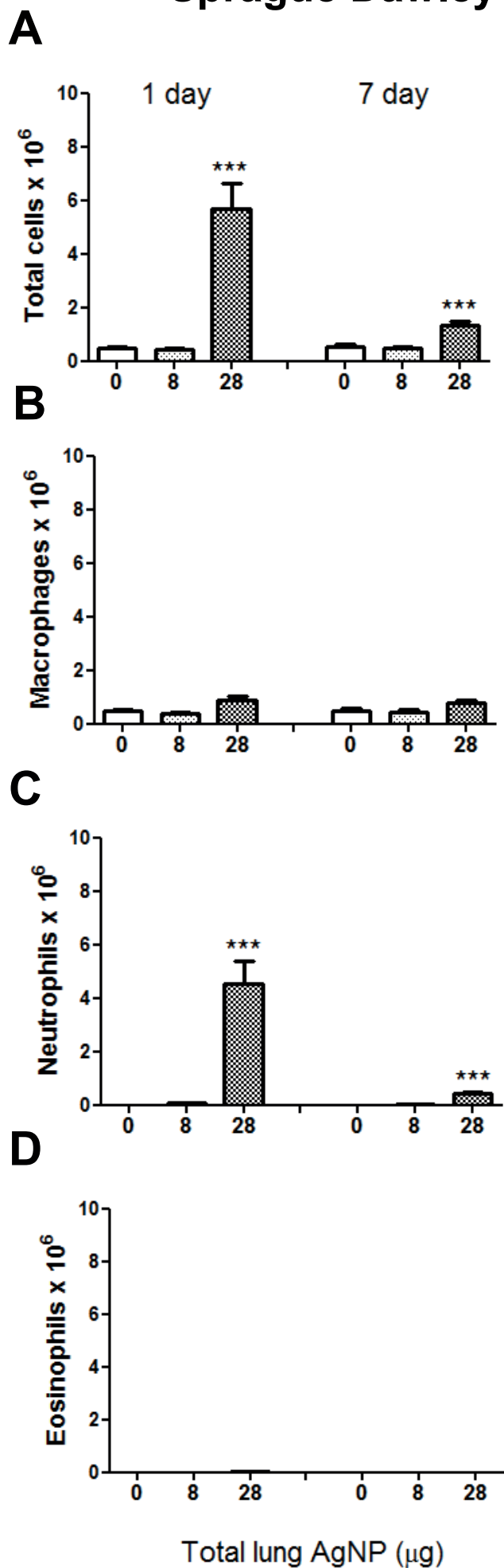


Fig 6

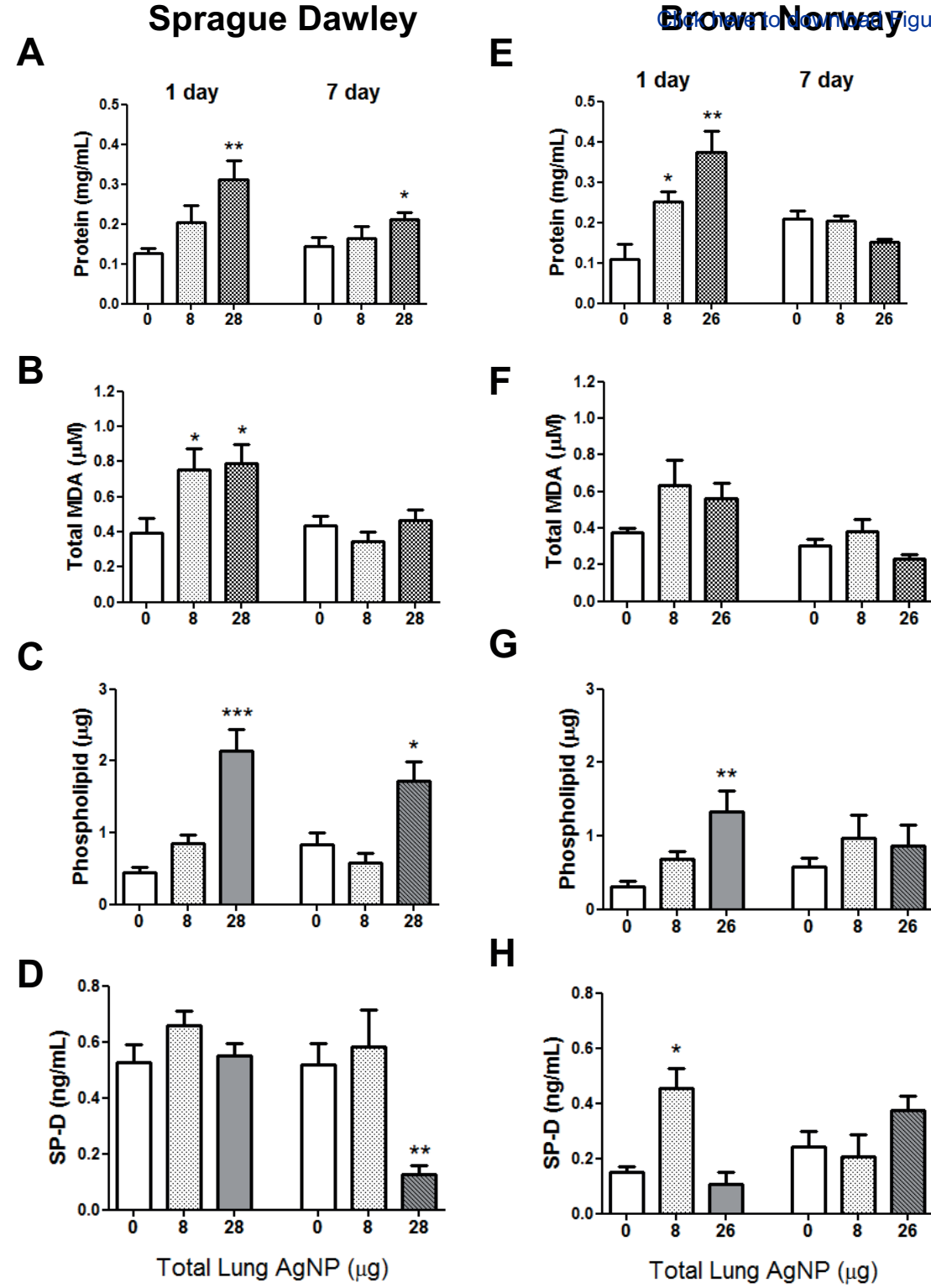


Fig 7

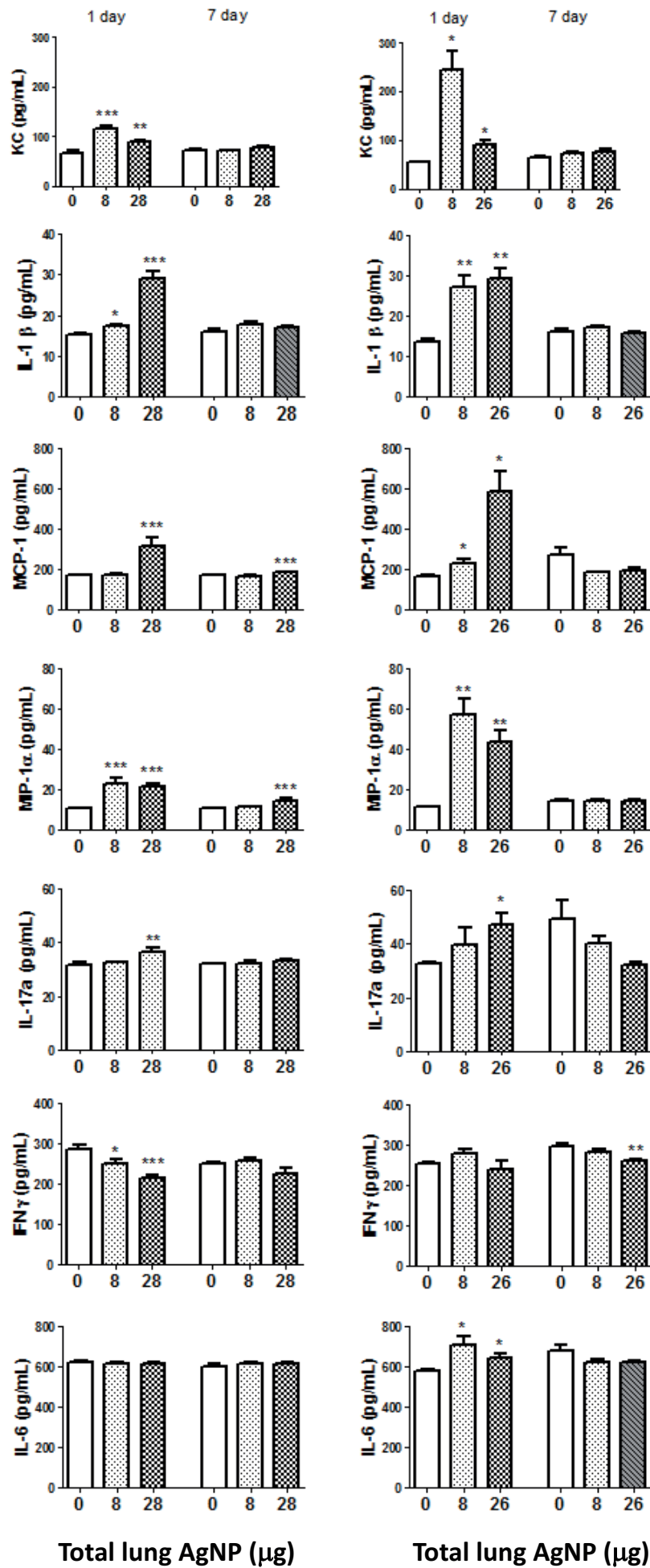


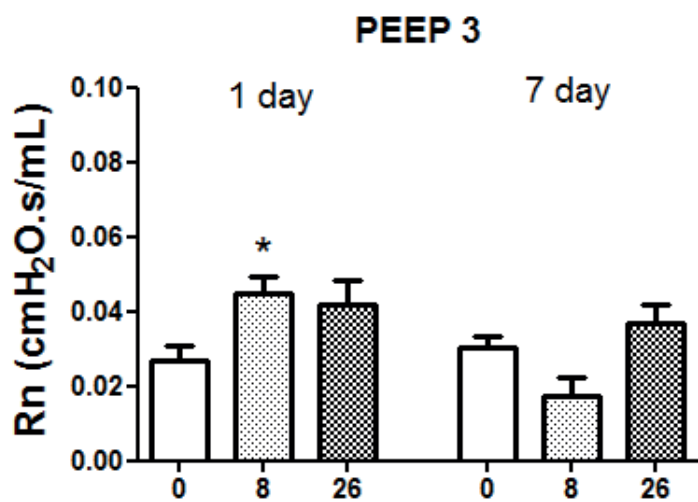
Fig 8

Brown-Norway rats

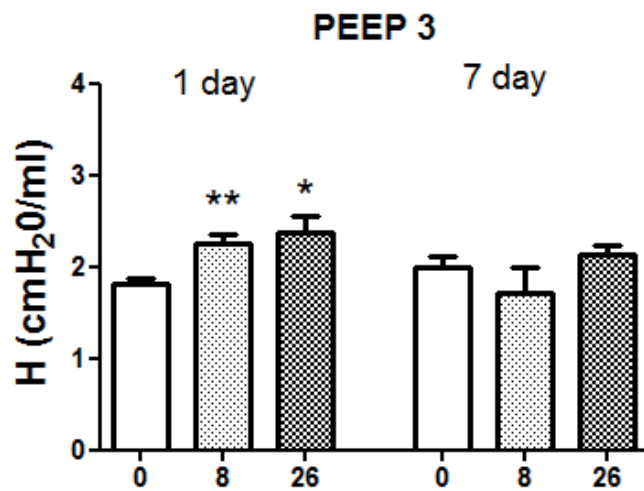
Large Airway Resistance

Tissue Elastance

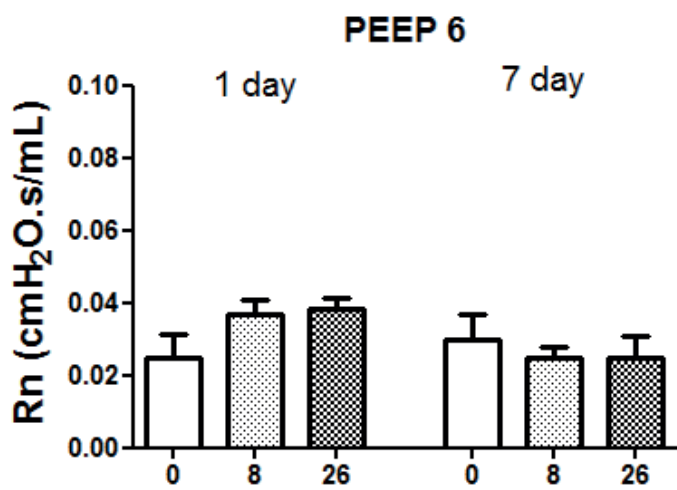
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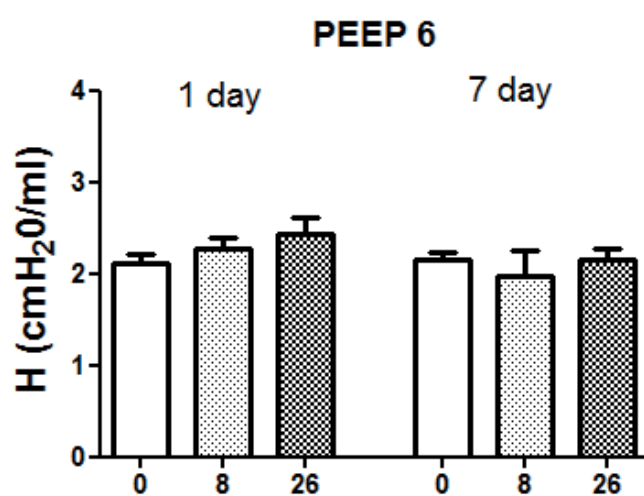
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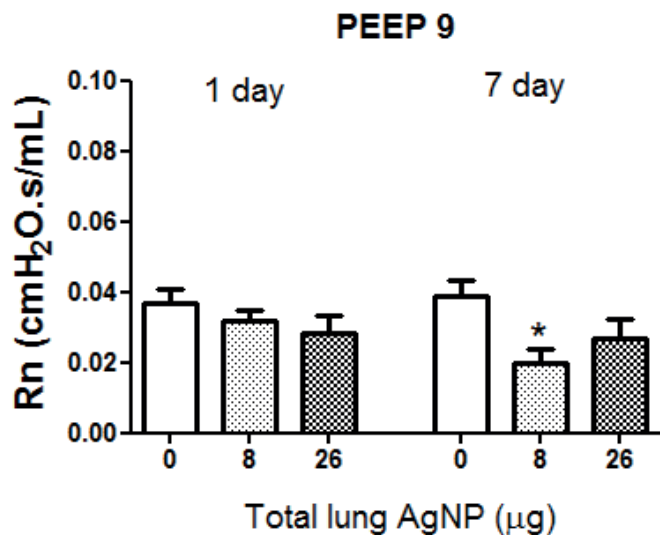
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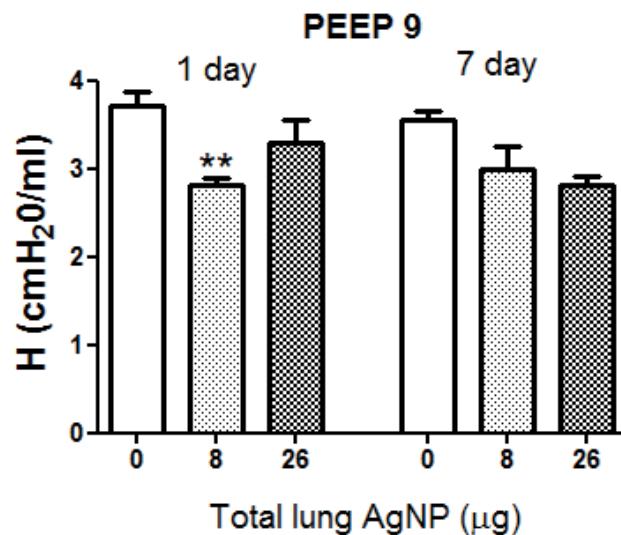


Fig 9