GRAPHISTRENGTH© C100 MULTIWALLED CARBON NANOTUBES: THIRTEEN-WEEK INHALATION TOXICITY STUDY IN RATS WITH 13- AND 52-WEEK RECOVERY PERIODS COMBINED WITH COMET AND MICRONUCLEUS ASSAYS

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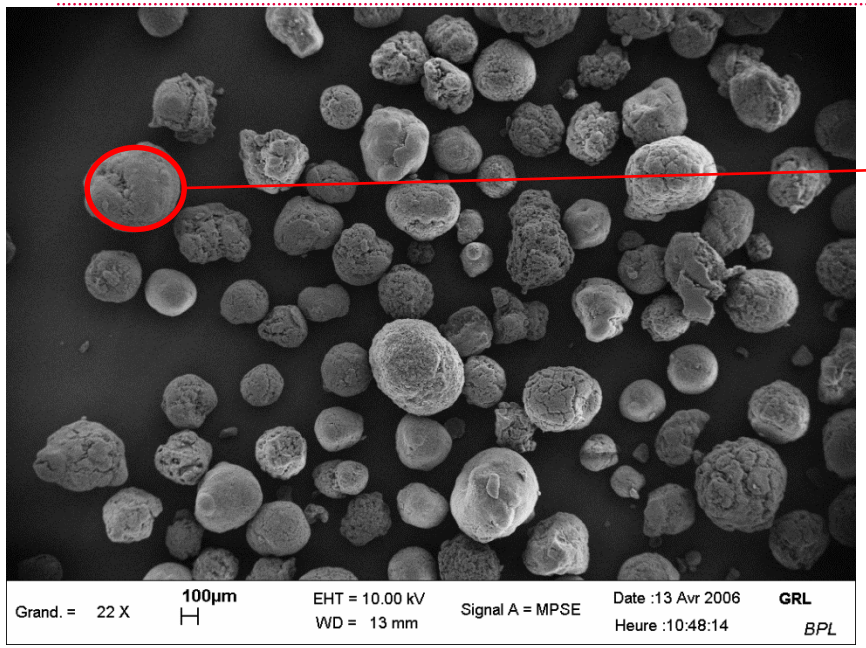
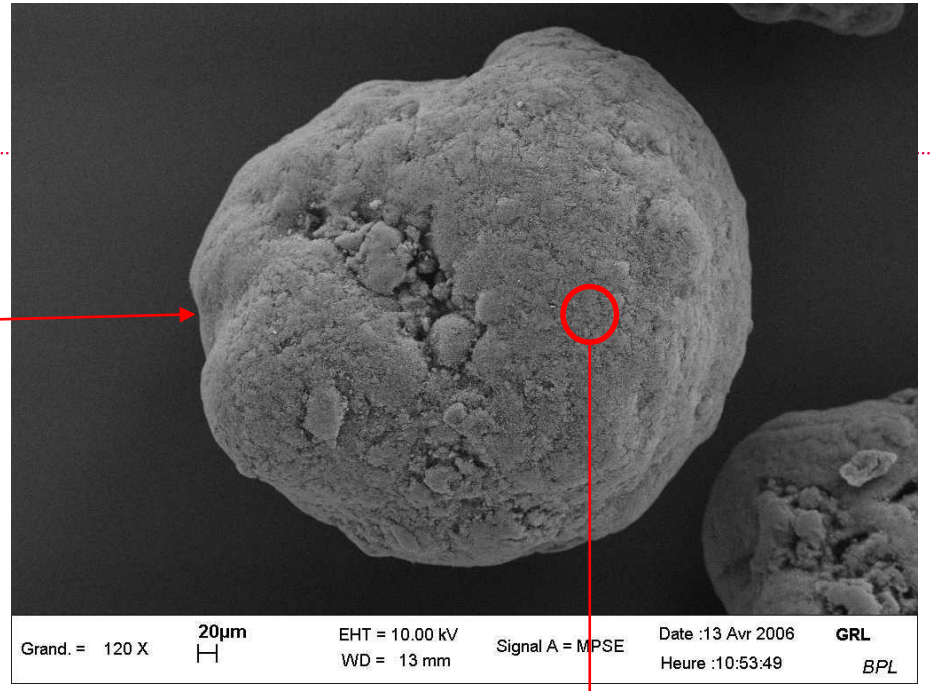
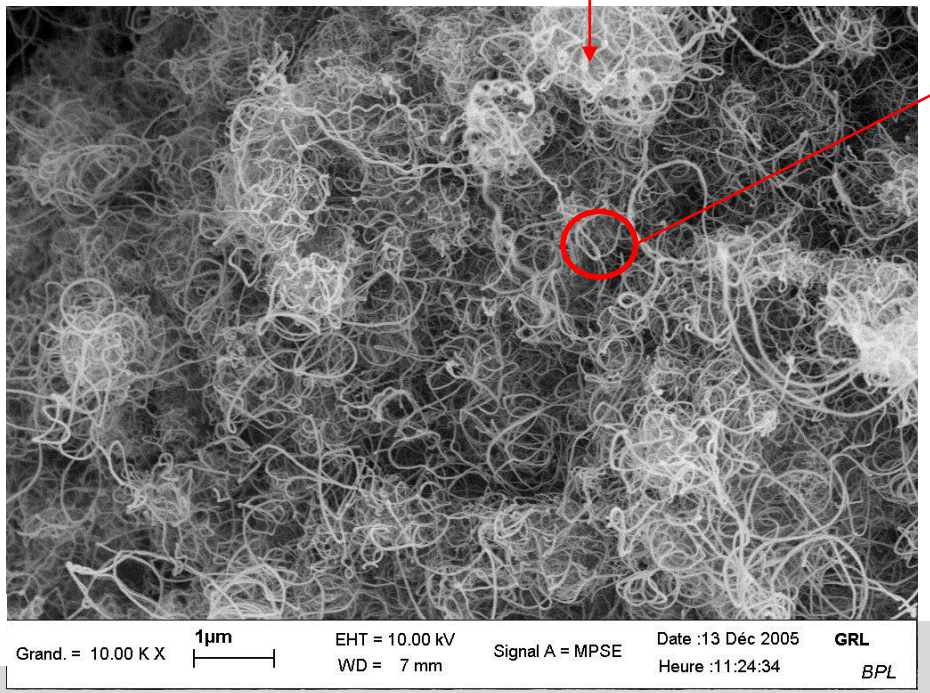
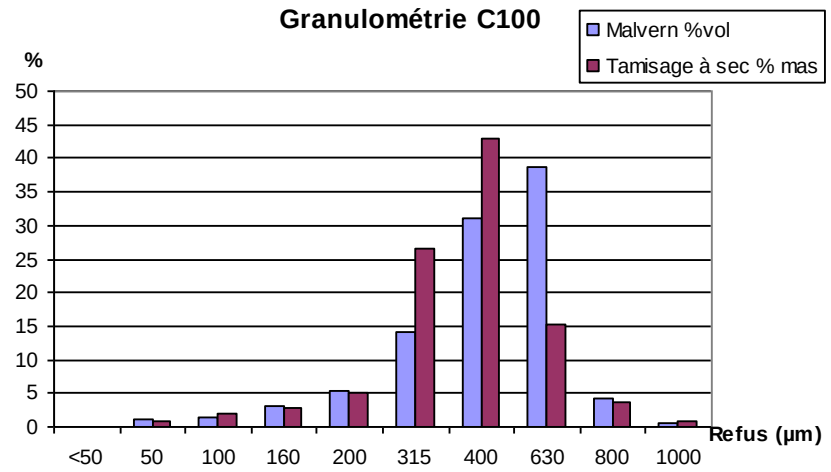
GRAPHISTRENGTH© C100



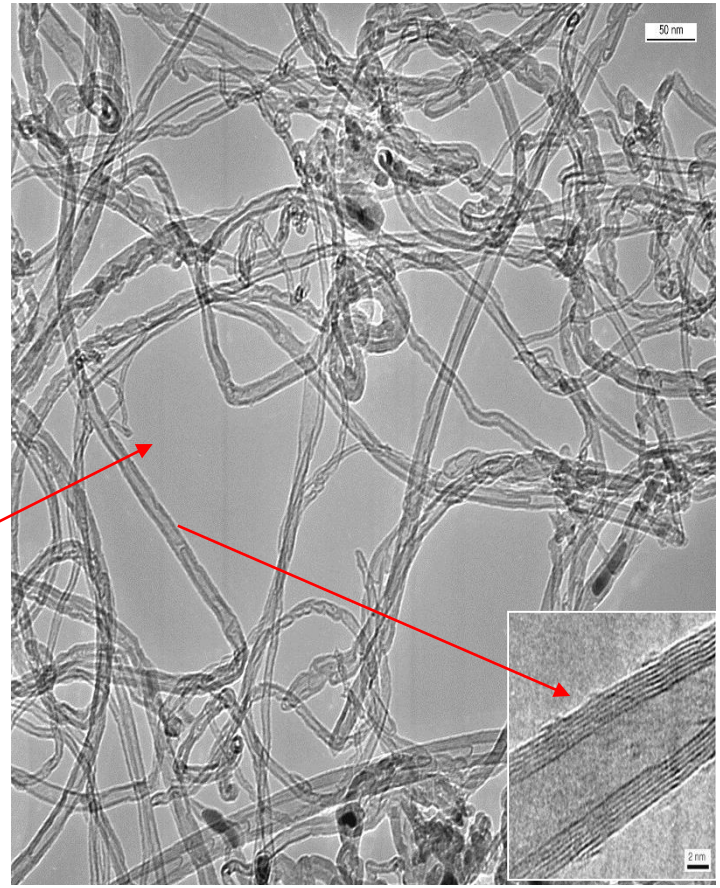






< 0.2 % of fragments of pellets < 15 µm

Entangled MWCNT (≈ 12 walls, outer diameter ≈ 12 nm, length ≈ 1 µm)

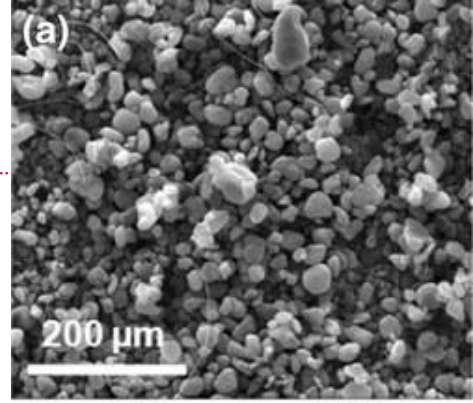


Régnier JF et al.
Nanosafe 2016, Grenoble

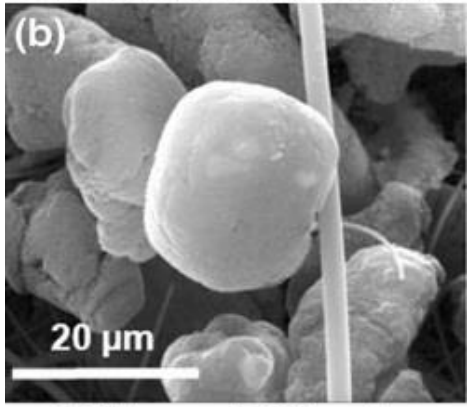
ARKEMA

OBJECTIVES OF THE STUDY

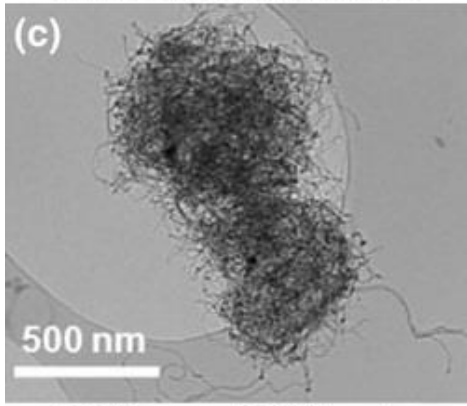
- **To develop an aerosol generation procedure**
  - the generated aerosol should have physico-chemical properties similar to the original material
  - The particles should have a respirable size (<3 µm)
- **To conduct an inhalation subchronic toxicity study for the safety assessment of Graphistrength© C100**
  - According to the OECD test guideline no. 413
  - Specific evaluation of the pulmonary inflammation parameters
  - Long recorery periods (3 and 12 months)
- **To assess the genotoxic potential in the cells at the site of contact and those distant from it**
  - Comet assay (OECD test guideline no. 489) in lung, liver and kidney cells
  - Micronucleus assay (OECD test guideline no. 474) in bone marrow cells



Quartz filter surface SEM image



SEM image of CNT bundles



TEM image of CNT bundle

R'mili et al. 2011 Journal of Nanoparticle Research **13(2)**:563–77

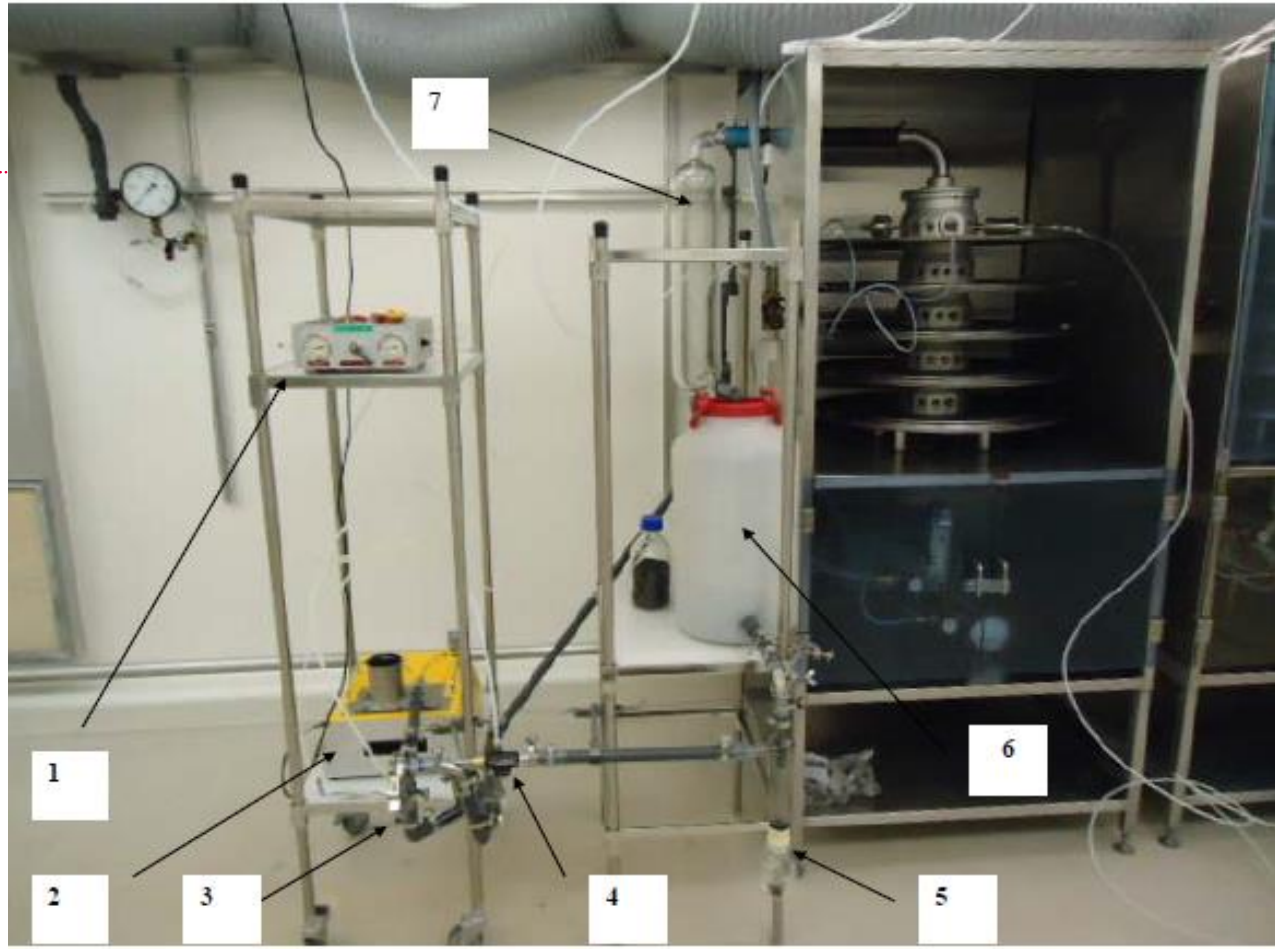
METHODS

Aerosol generation and monitoring

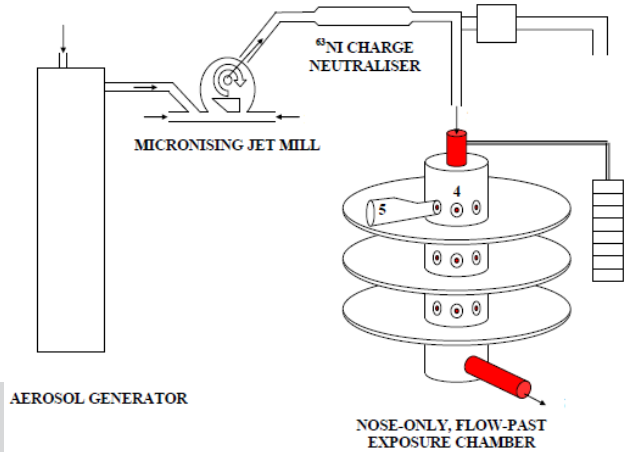
- **Graphistrength© C100 was ground in a ceramic ball mill for 12 hours under argon to reduce oxidation and was sieved**
- **Aerosol Generator (SAG 410) connected to a micronizing jet mill and a cyclone and two elutriators thereafter**
- **Gravimetric determinations of the aerosol concentrations using Millipore® durapore filters**
- **Cumulative particle size distribution of the test aerosol**
  - Mercer cascade impactors
  - Wide Range Particle Spectrometer©

Physico-chemical characterisations

- **Original Graphistrength© C100 and samples taken at different steps of the aerosol generation process**
  - SEM for the morphology of the particles
  - TEM for the walls number, diameters, length size and ends of the nanotubes
  - Laser method for the particle size
  - Porosimetry with mercury intrusion for the apparent density
  - BET method for specific area
  - Calcination for ash content and the elementary organic analysis
  - XPS for the chemical surface analysis
  - ICP for metal content



1: Air supply
2: Topas powder generator SAG 400
3: Jet-Mill
4: Air dilution system
5: Cyclone
6: Elutriator 1
7: Elutriator 2



METHODS

→ Animal exposure

- Four groups of 35 8-week old male and female Wistar rats
- Nose-only inhalation exposure, 6 h/day, 5 d/week for 4 or 13 weeks
- Target concentrations: 0, 0.05, 0.25 and 5.0 mg/m^3 air

→ Study design

Examinations	4-week interim animals	Main animals	13-week recovery animals	52-week recovery animals
Clinical signs, body weight, food consumption				
Ophthalmology				
Oestrus cycle				
Functional observation battery				
Blood pressure				
Hematology, blood chemistry and urinalysis				
Bronchoalveolar lavage fluid (cytology, biochemistry, cytokines)				
Full histopathology				
Respiratory tract histopathology				
Sperm analysis				
Genotoxicity assays (comet and micronucleus assays)				

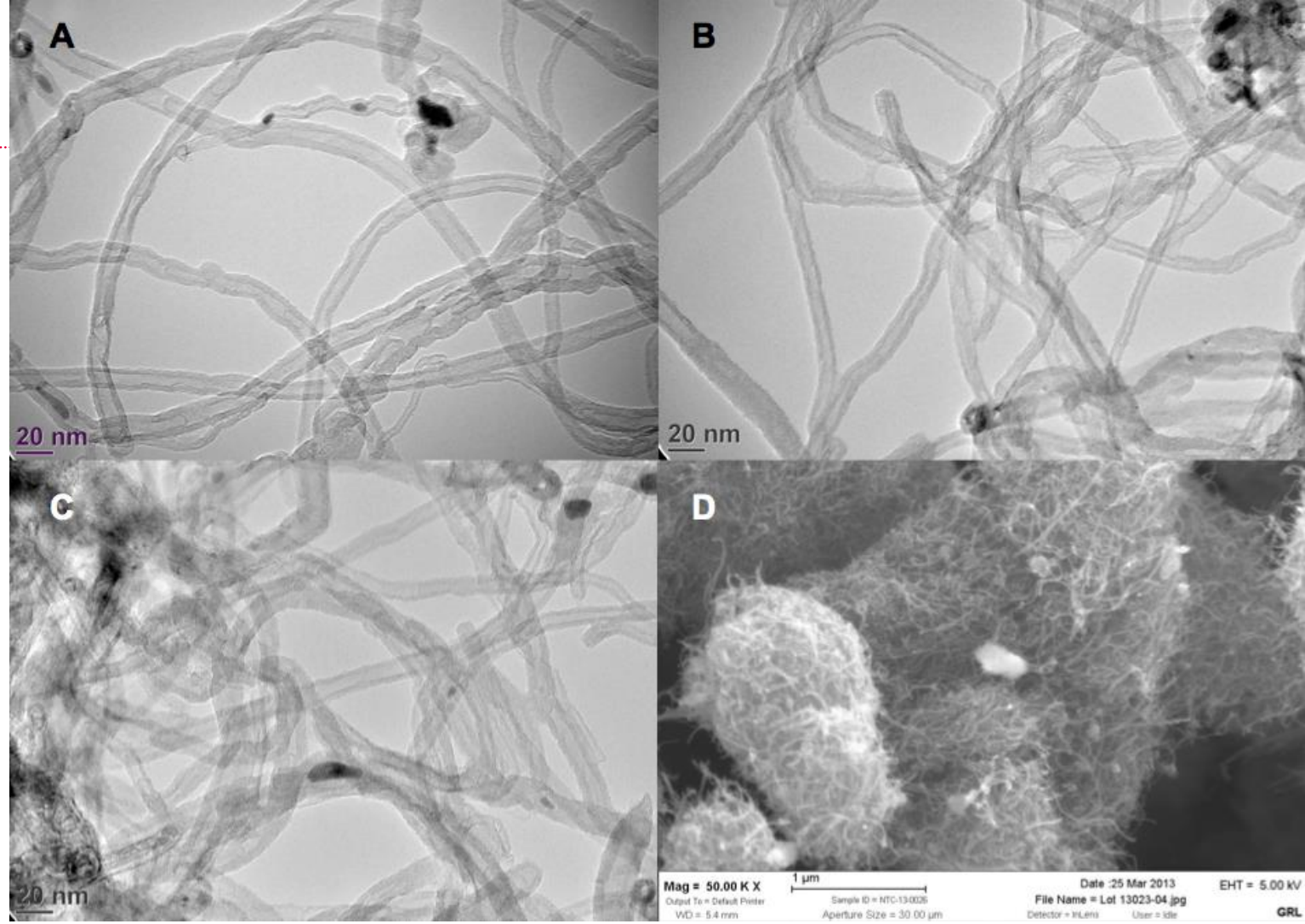
RESULTS

Physico-chemical analysis

- **Minor changes between the starting material and the ball milled or aerosol samples.**
  - For apparent density, surface to volume ratio, and MWCNT length, the changes observed are inherent to the process to generate the aerosol form.
- **No apparent alteration by TEM of the MWCNT structure between the original, milled and aerosolized Graphistrength© C100**

Atmosphere monitoring

- **Achieved aerosol concentrations: 0.06, 0.28 and 4.84 mg/m³ air**
- **Mean mass median aerodynamic diameter: 1.62-2.3 µm**
- **Mean GSD: 2.47-4.67**
- **% < 3µm: 63-74**



(A) TEM of original Graphistrength© C100 (B) TEM of milled Graphistrength© C100 under argon for 12 h and sieved (63 µm) (C) TEM of aerosol sample (D) SEM of aerosol sample.
(A, B, and C: 350'000x, D: 50'000x)

RESULTS

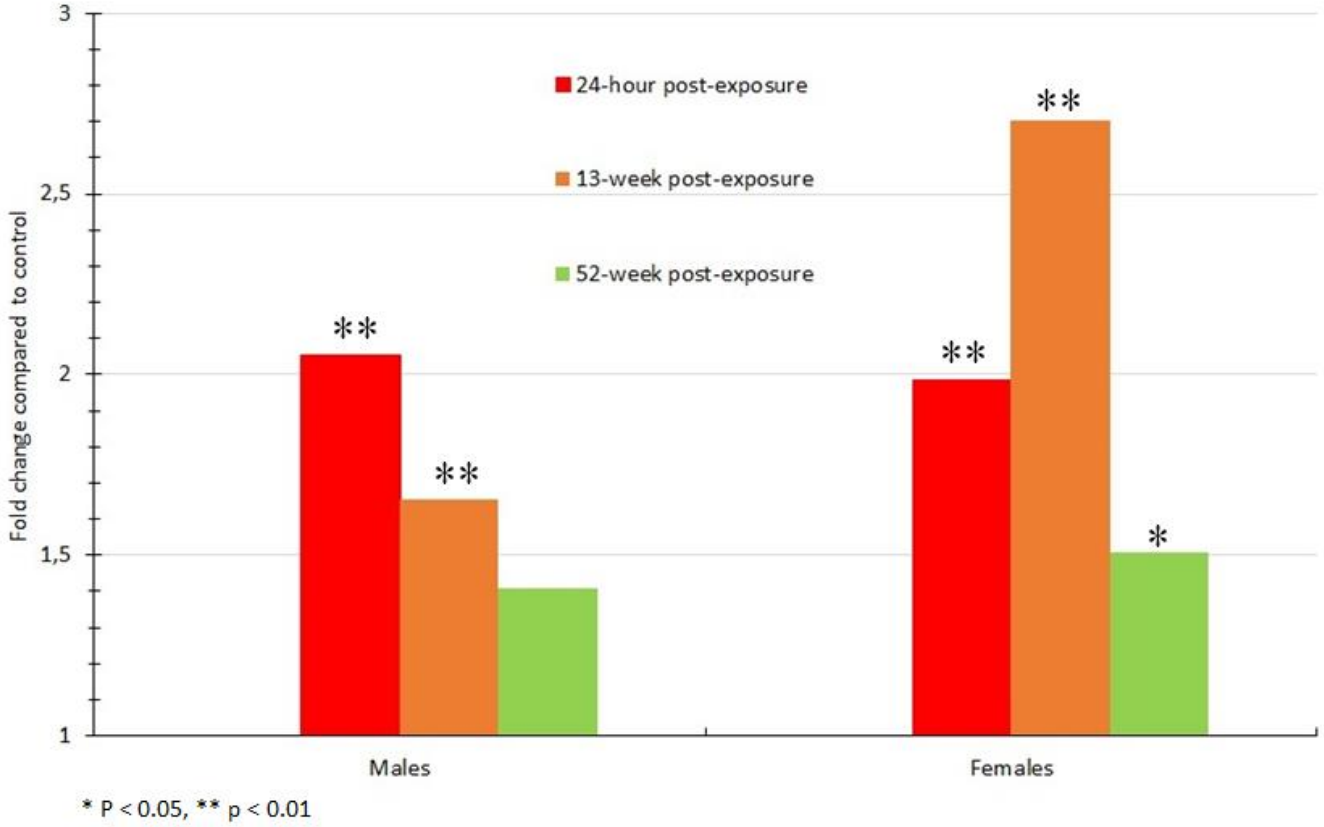
→ **No mortality and no specific clinical signs**

→ **No exposure-related adverse effects on:**

- body weight gain
- food consumption
- FOB parameters
- blood pressure
- ophthalmoscopic examinations
- blood chemistry and urinalysis parameters
- estrus cycle
- sperm parameters

→ **Haematology:**

- **Only in rats exposed to 5.0 mg/m³**
  - increase in relative and absolute neutrophil counts
  - slight decrease of the relative (but not absolute) lymphocyte counts

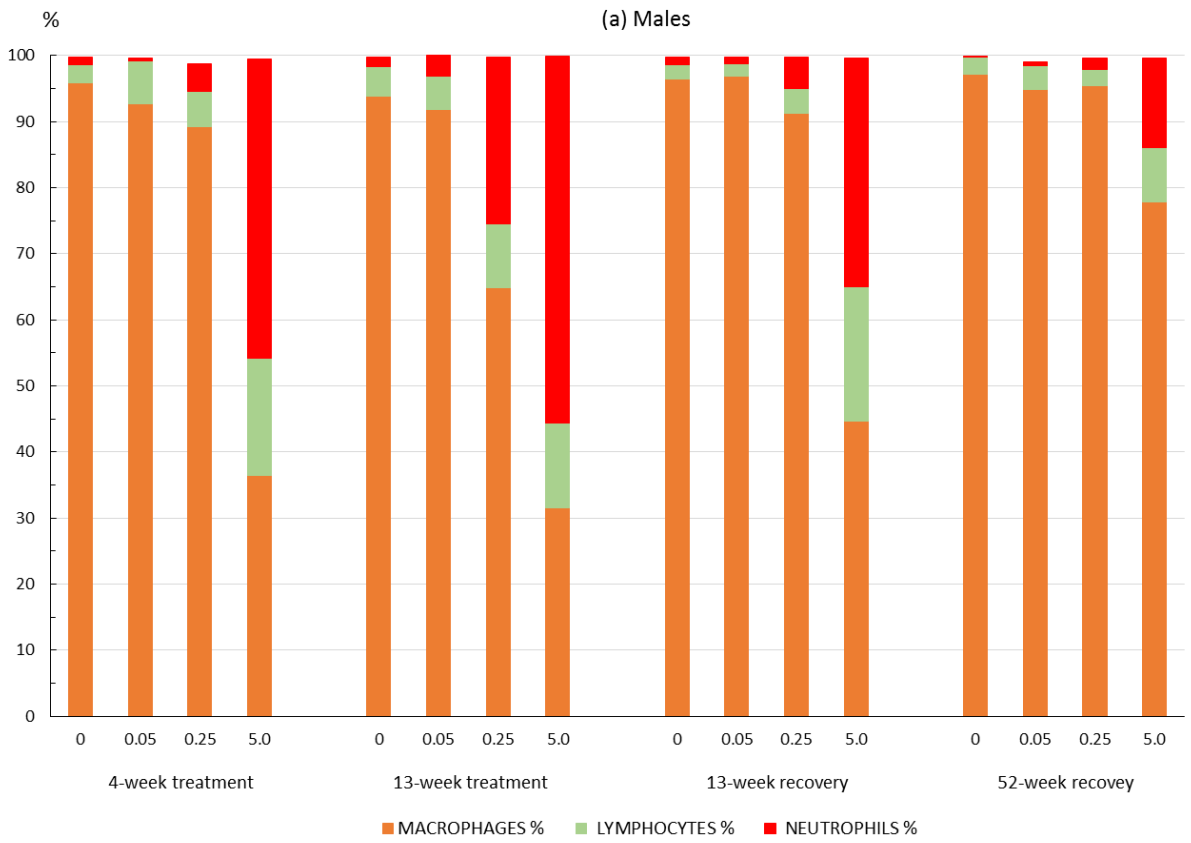


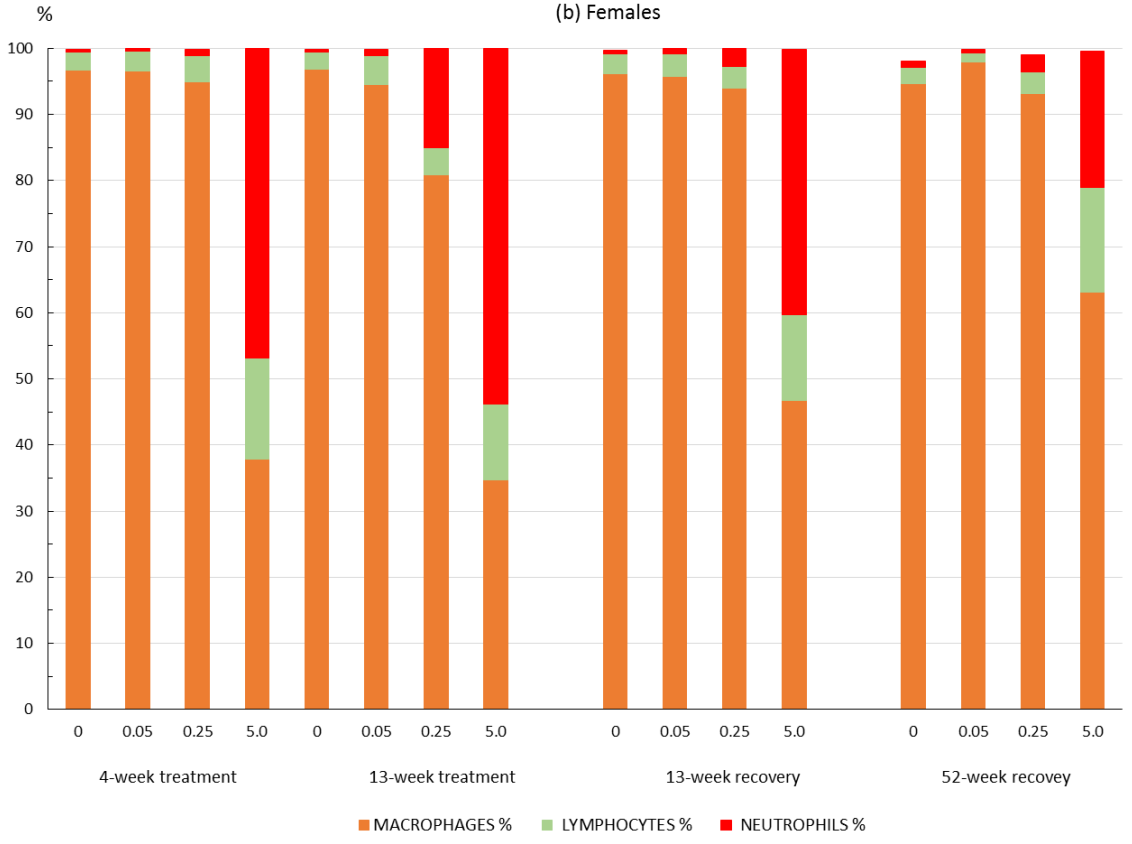
Absolute neutrophils counts in circulating blood of rats exposed to 5.0 mg/m³

RESULTS

BALF examinations

- Presence of black particles in the BALF, from minimal at 0.05 mg/m^3 to severe at 5.0 mg/m^3
- increase in neutrophils and lymphocytes with a concomitant decrease in macrophages at 5.0 mg/m^3. Slight effect at 0.25 mg/m^3 after 13 weeks of exposure, reversible after 13 and 52 weeks of recovery

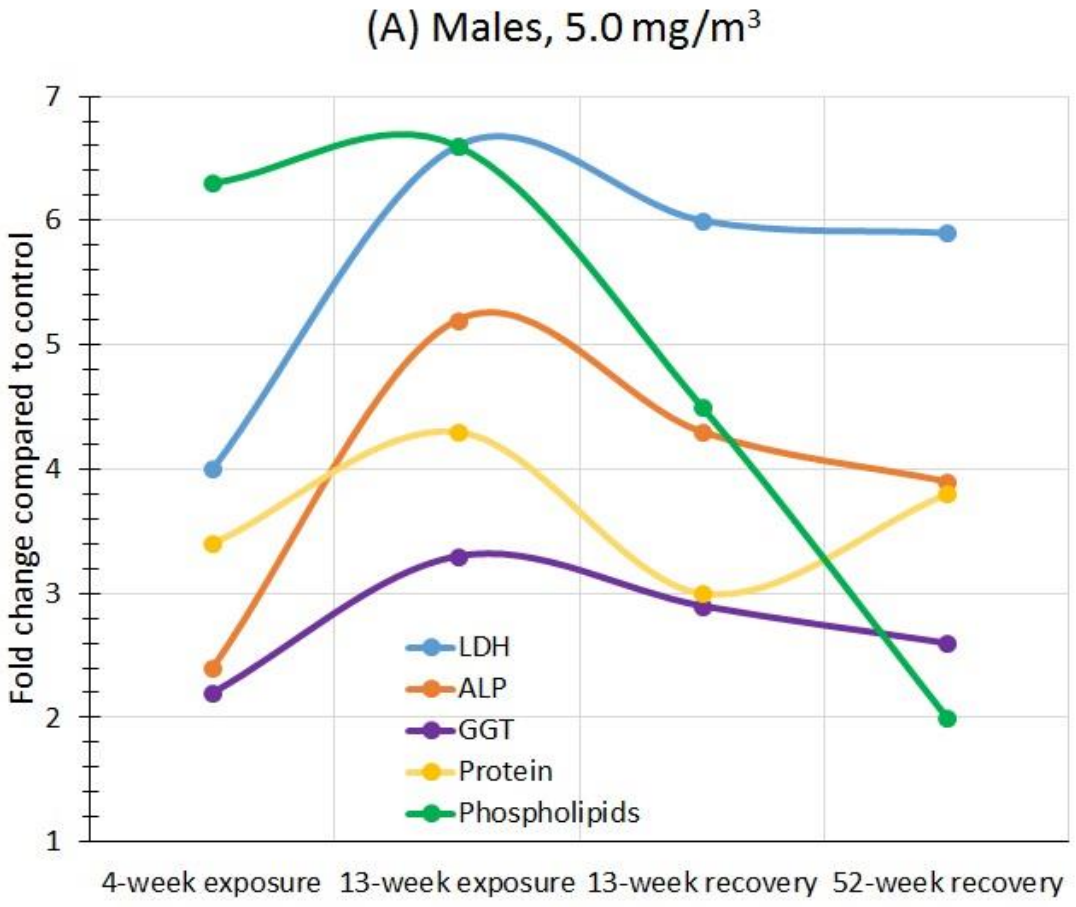


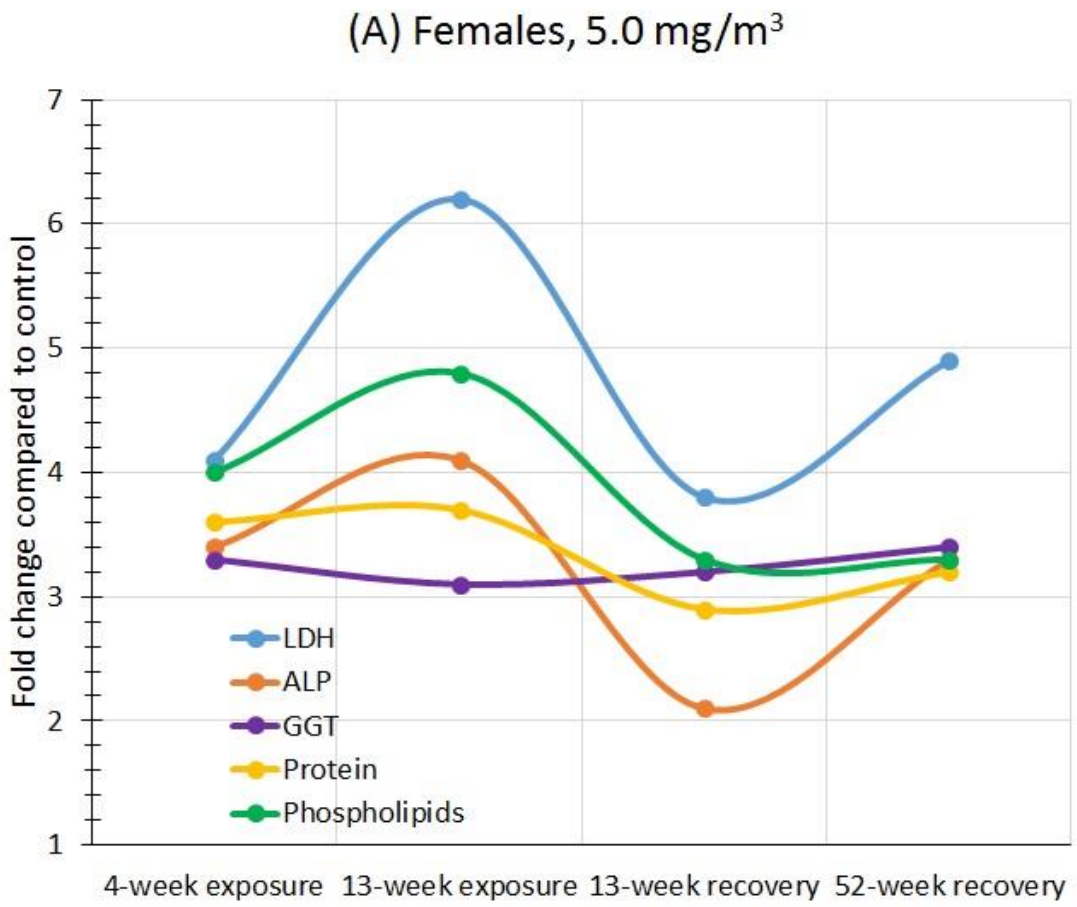


RESULTS

→ BALF examinations

- **5.0 mg/m³**: changes in all biochemical parameters, maximal after 13 weeks of exposure and slightly improved during the recovery periods.
- **0.25 mg/m³**: slight increase of GGT after 13 weeks of exposure, fully recovered in males, partially in females

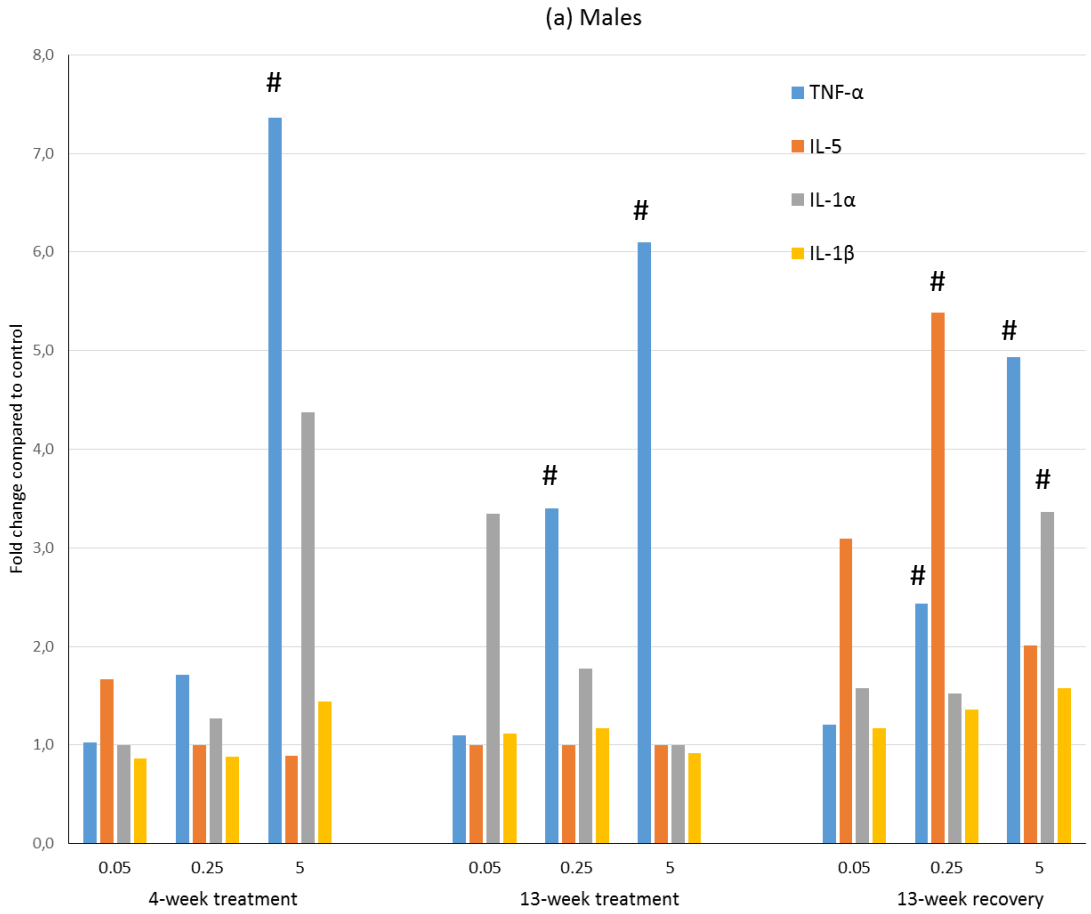


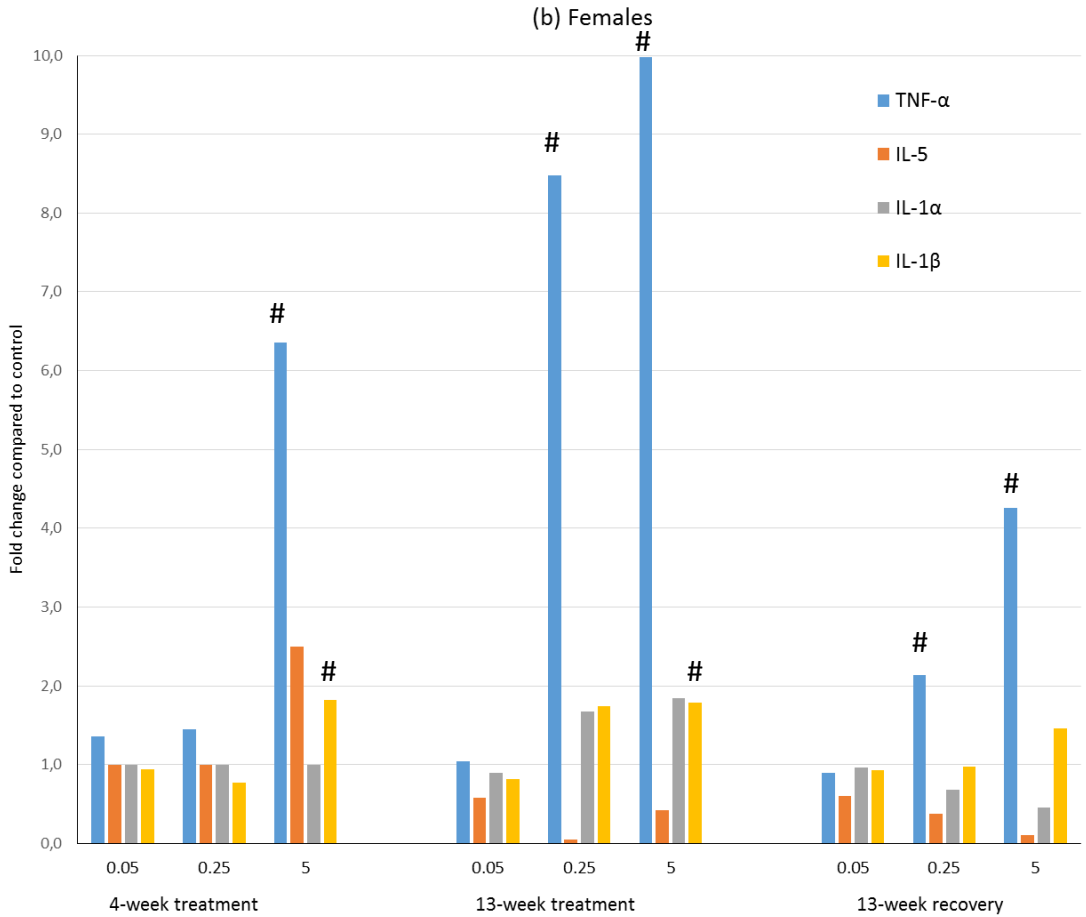


RESULTS

BALF examinations

- increases in TNF-a at 0.25 and 5.0 mg/m^3. Levels decreased after 13 weeks of recovery





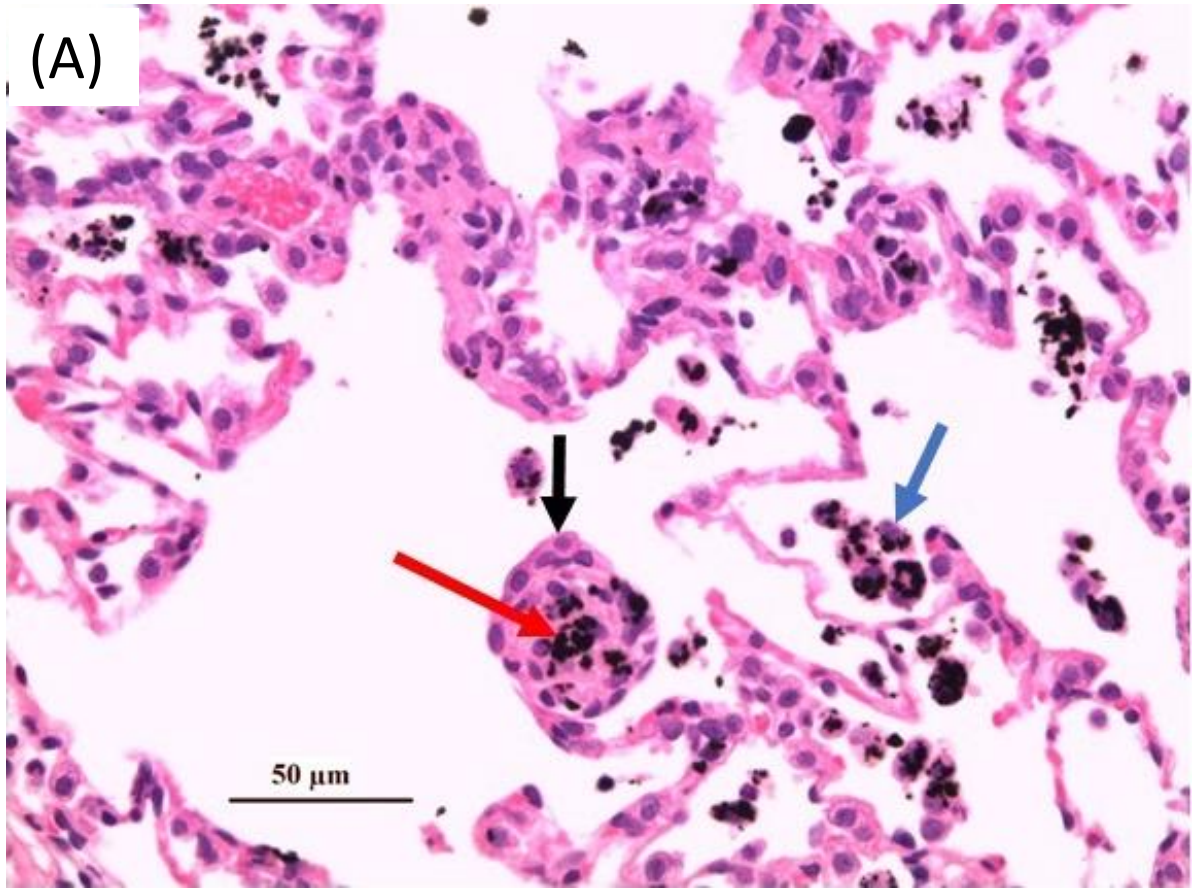
Mean value higher than “mean+2sd” of the corresponding control group

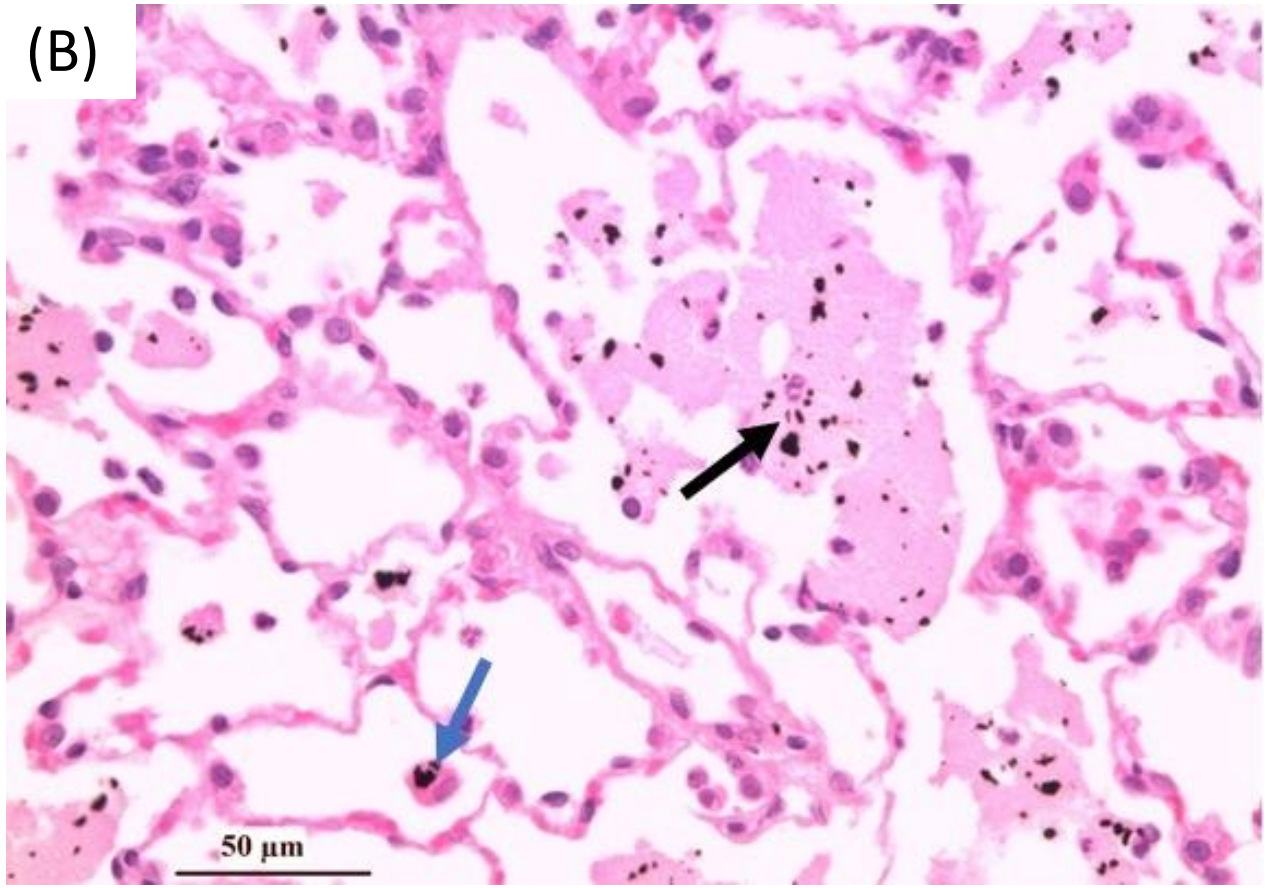
Histopathology

- **Excepted in the respiratory tract, no microscopic changes were observed**
  - No translocation of the MWCNT in liver, kidney, spleen, brain, olfactory bulb, et c…
  - No effect on the cardiovascular system
  - No effect on the reproductive organs
- **Respiratory Tract**
  - at all sacrifice periods, concentration-related deposition of black particles
    - 0.05 and 0.25 mg/m^3: within the alveolar macrophages
    - 5.0 mg/m^3: within tissue macrophages or free within the alveolar lumen
  - **5 mg/m^3**: significant microscopic changes

RESULTS

Lung, after 13 weeks of exposure to 5.0 mg/m^3

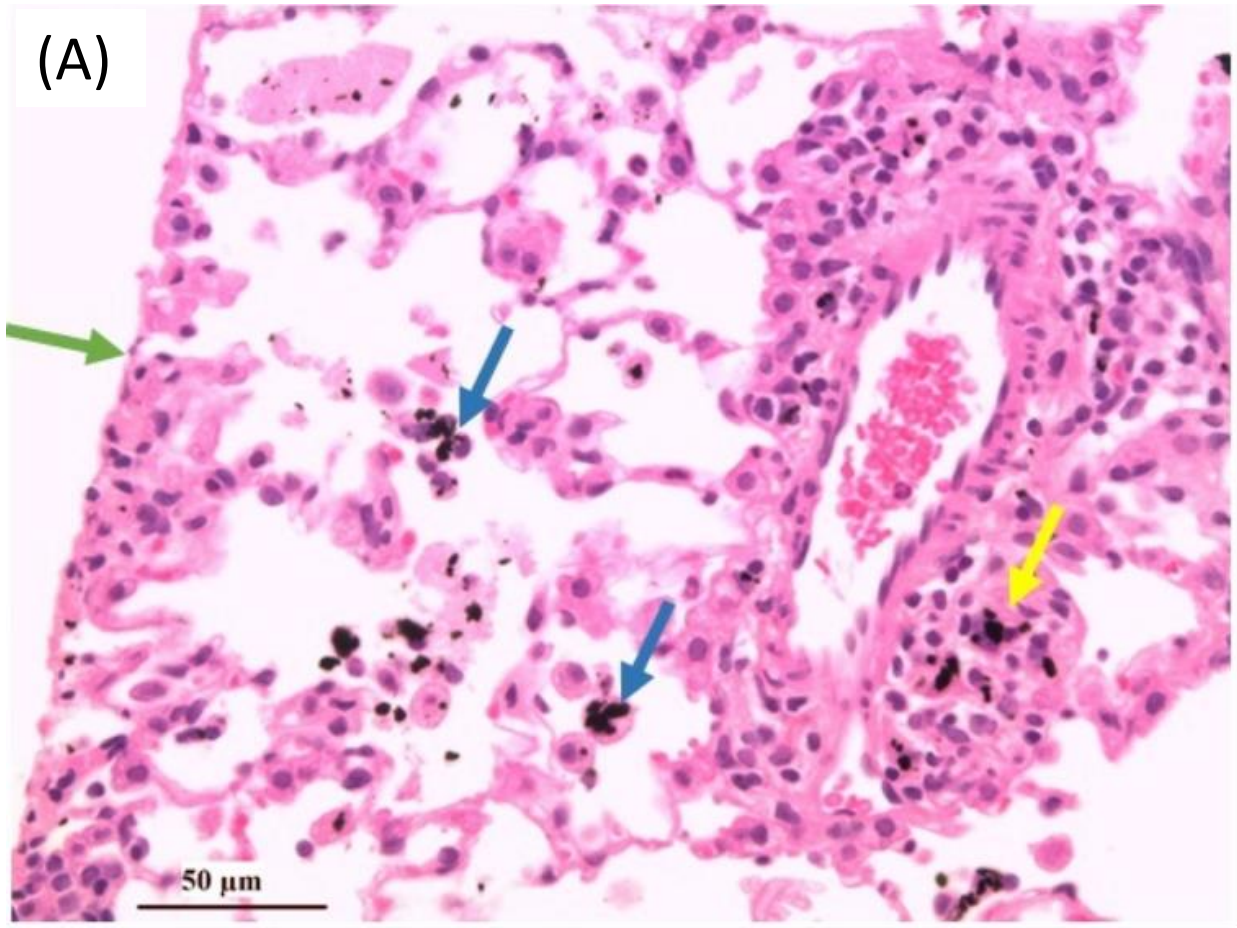


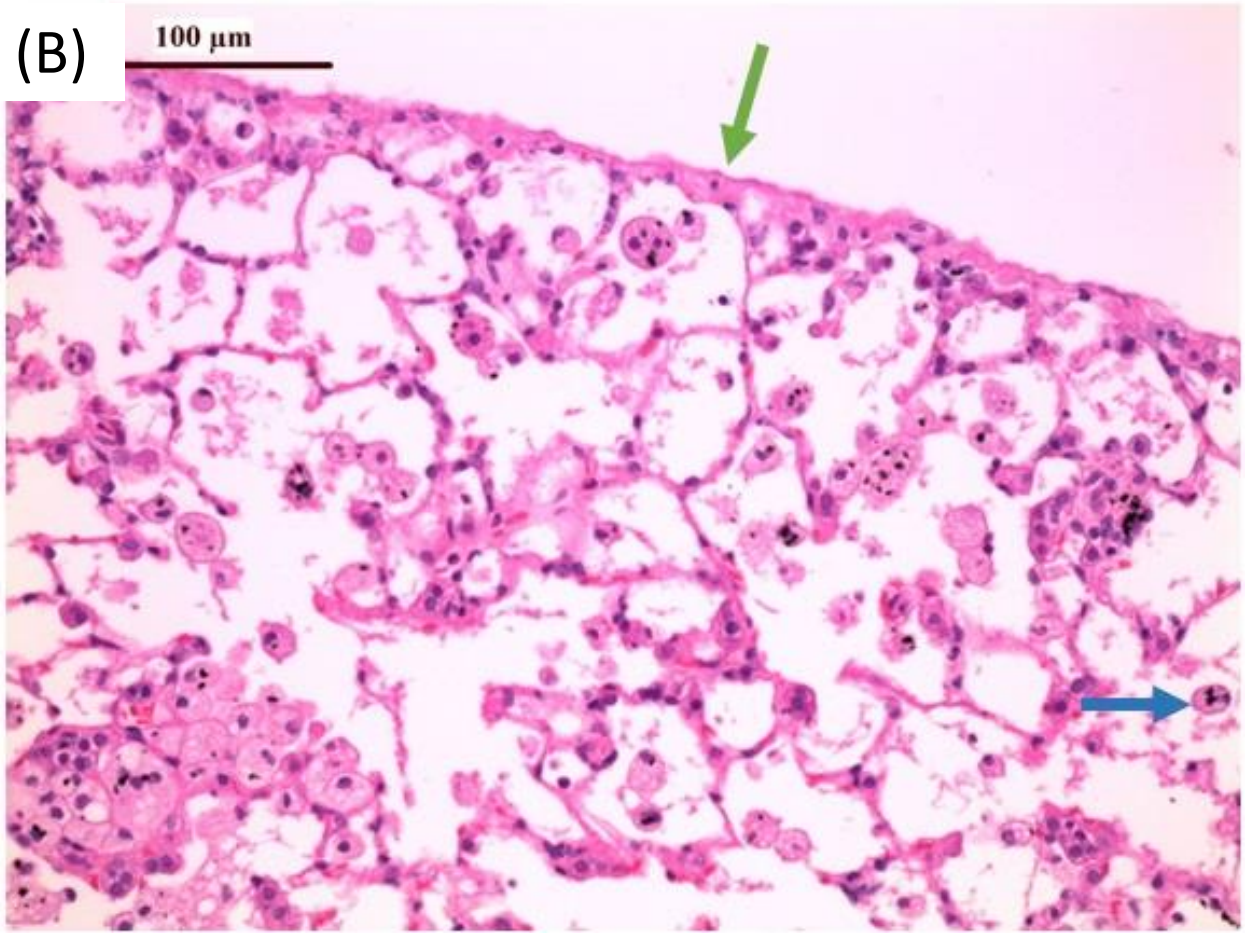


RESULTS

(A) 5.0 mg/m^3, after 13 weeks of exposure

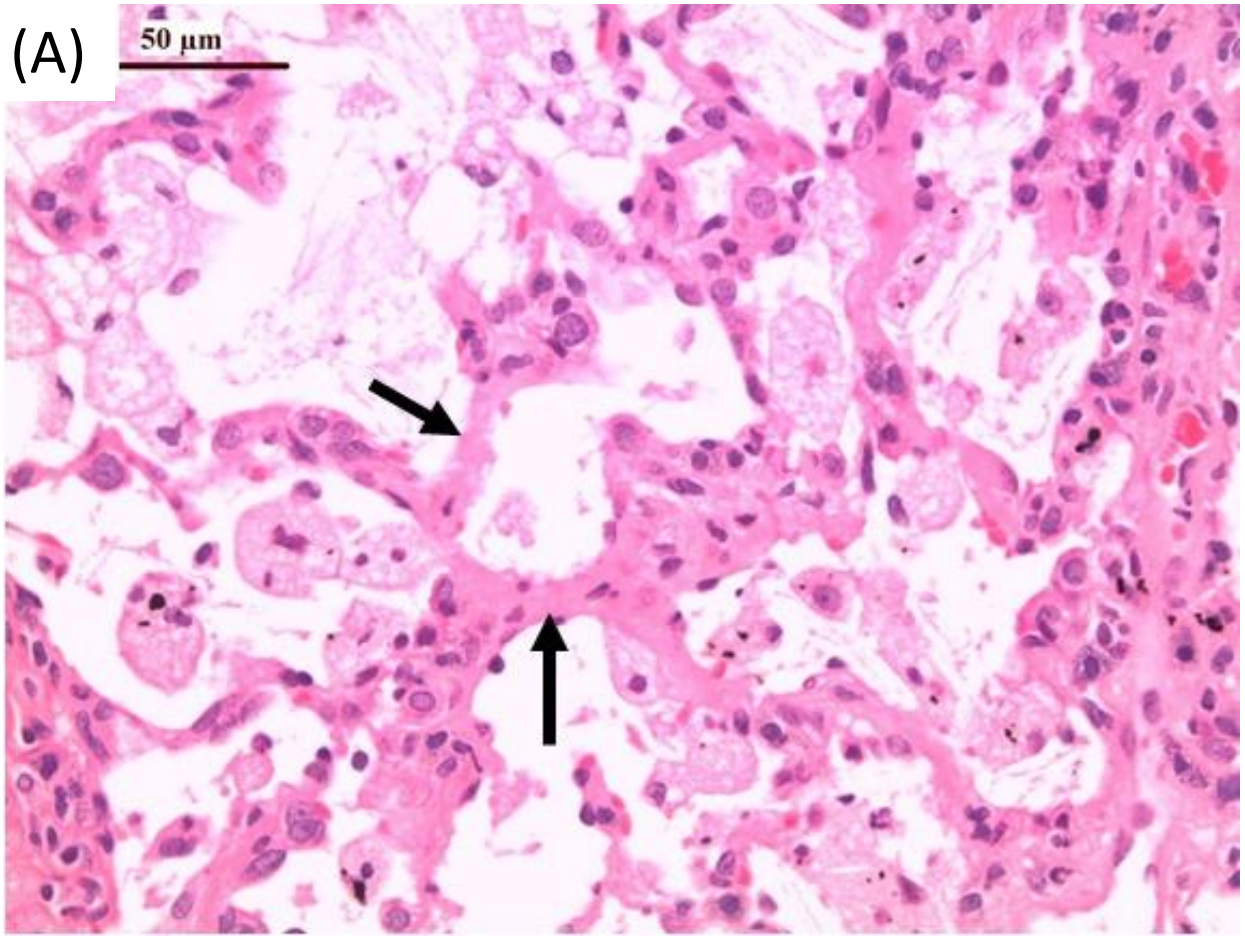
(B) 5.0 mg/m^3, after 52 weeks of recovery

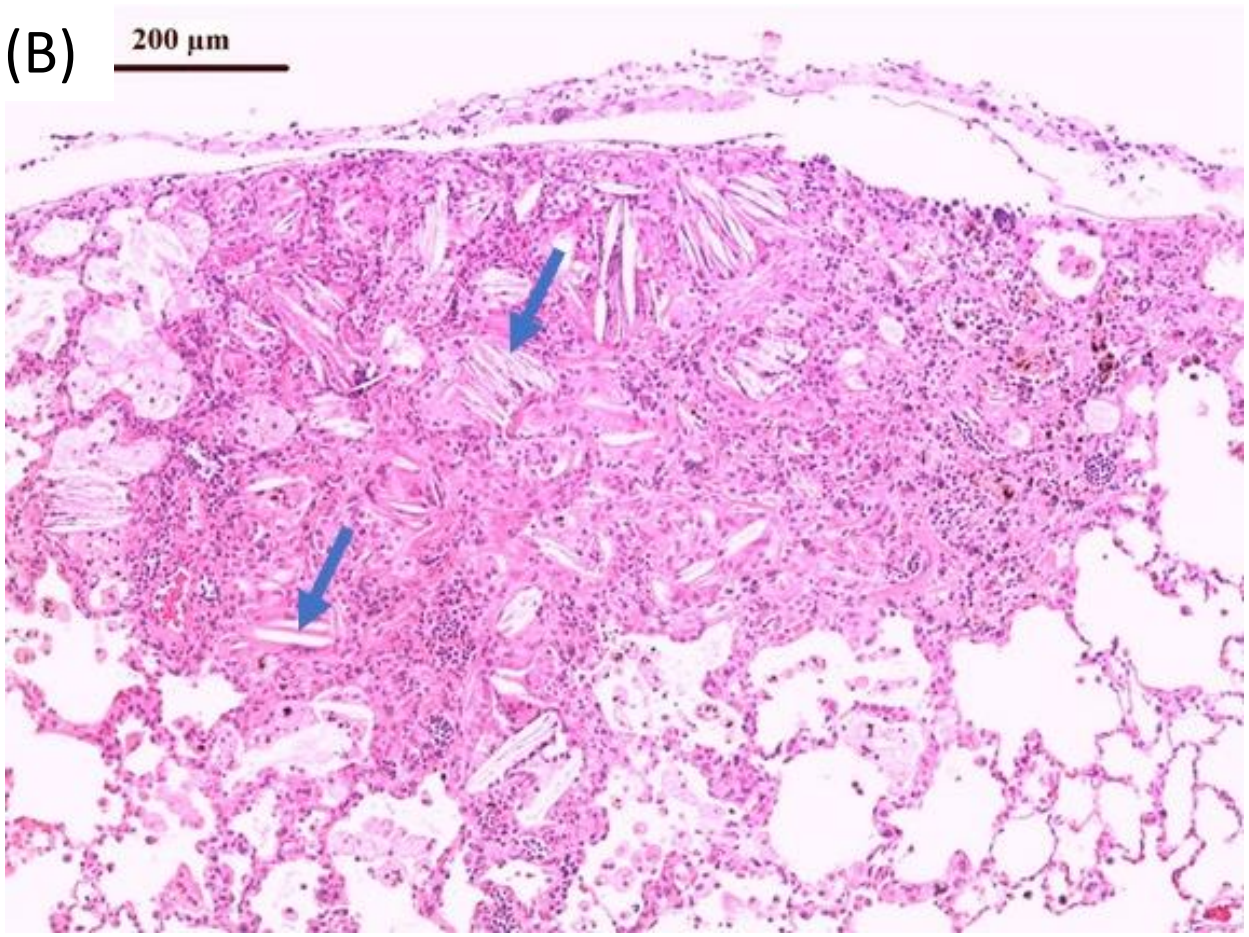




RESULTS

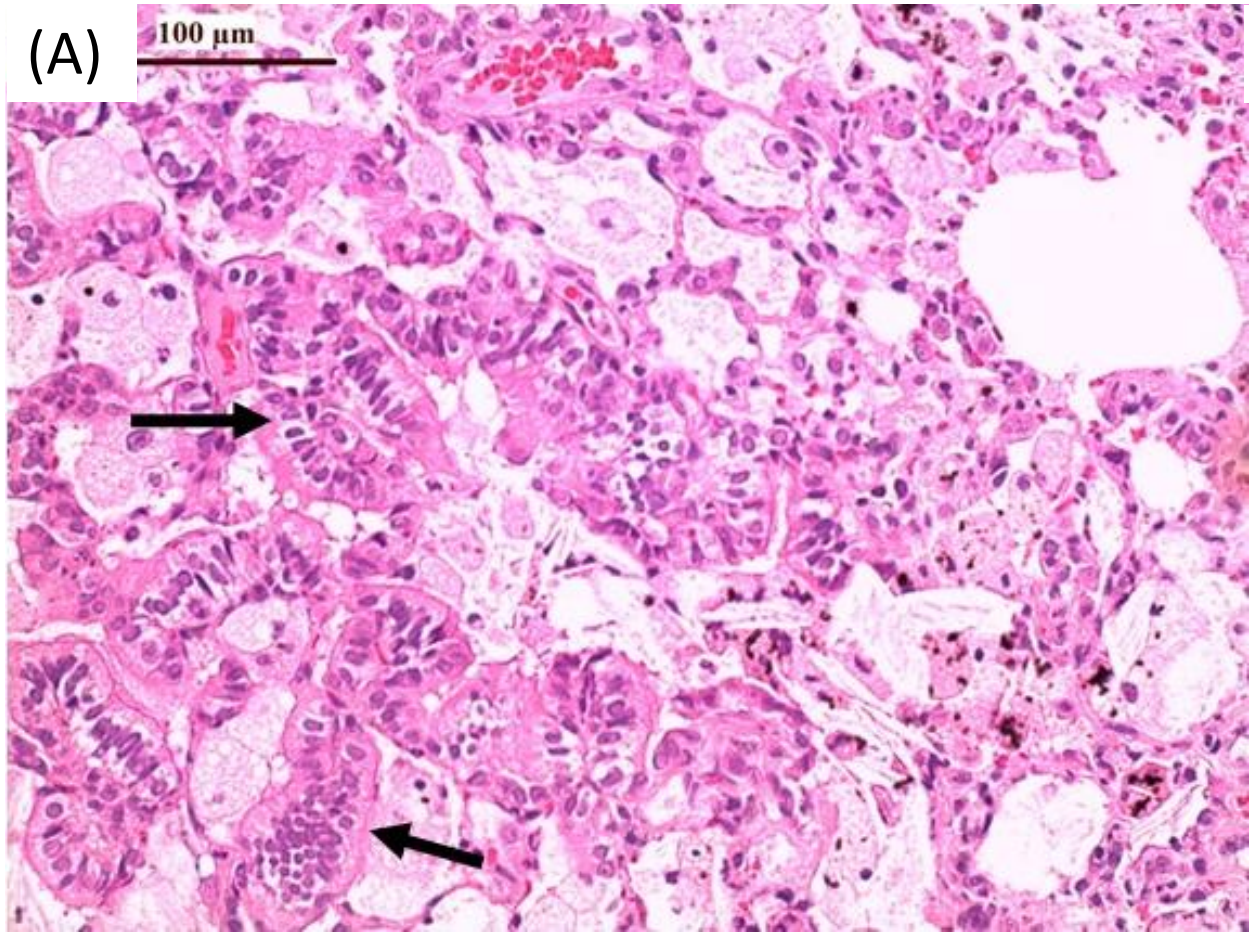
5.0 mg/m^3, after 52 weeks of recovery

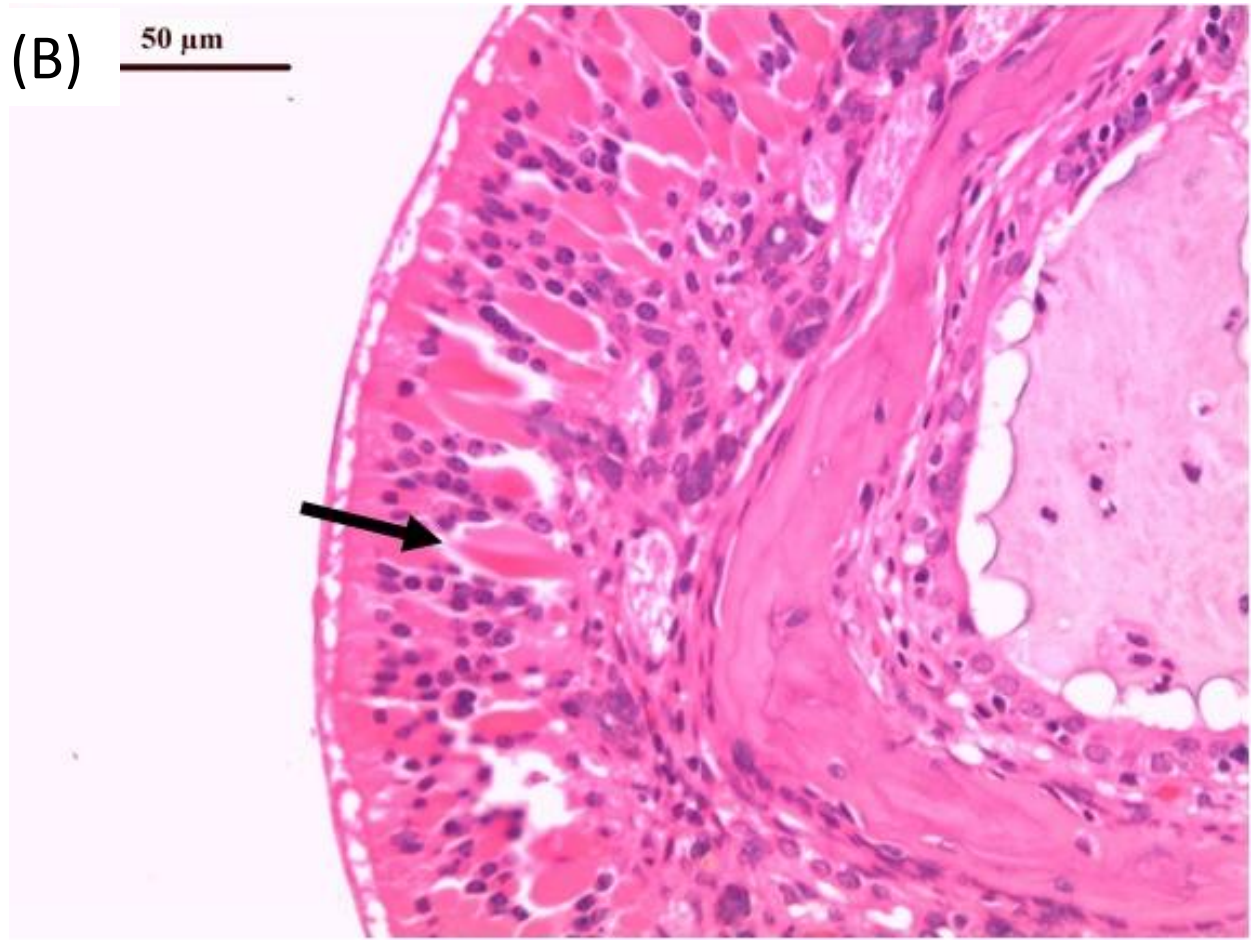




RESULTS

Lung (A) and nasal epithelium (B), 52 weeks after 13 weeks of exposure to 5.0 mg/m^3

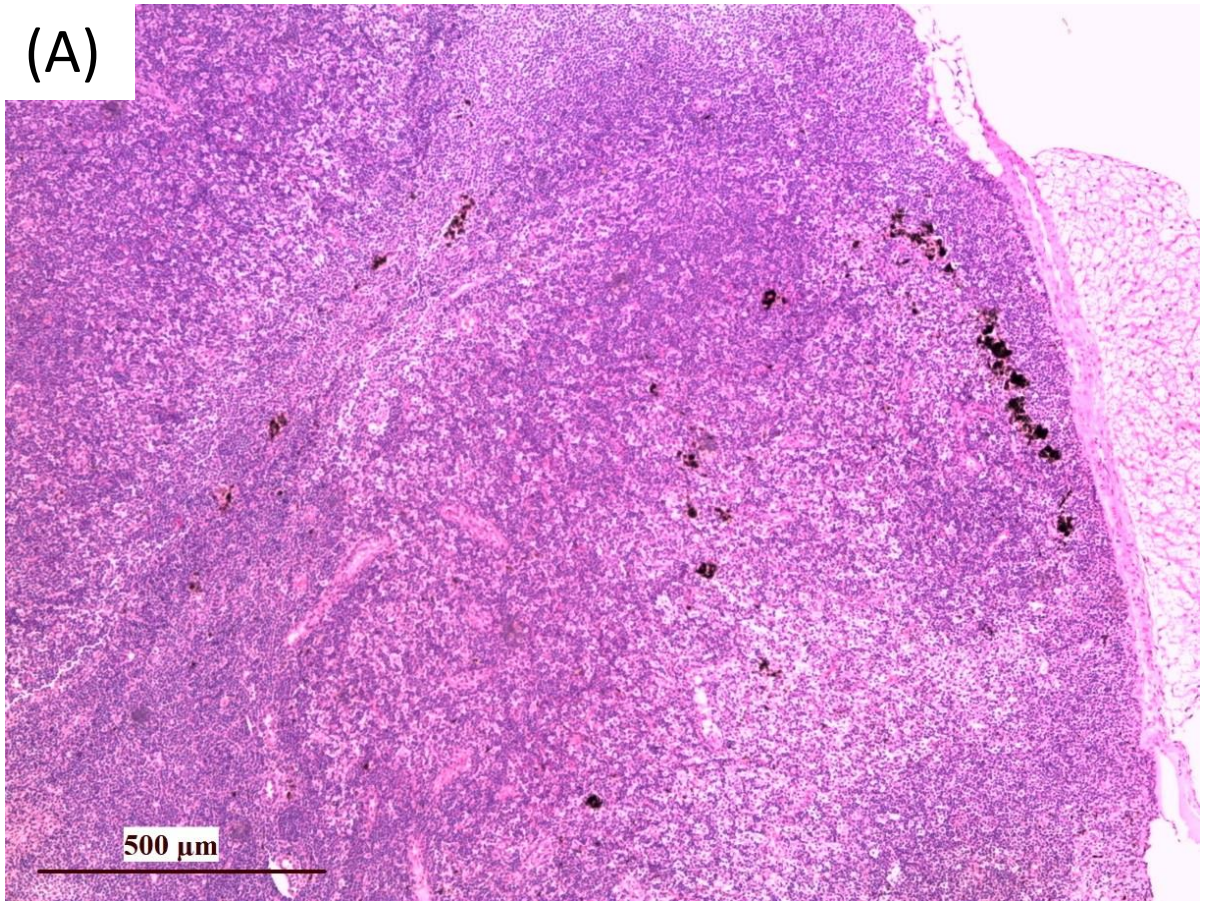




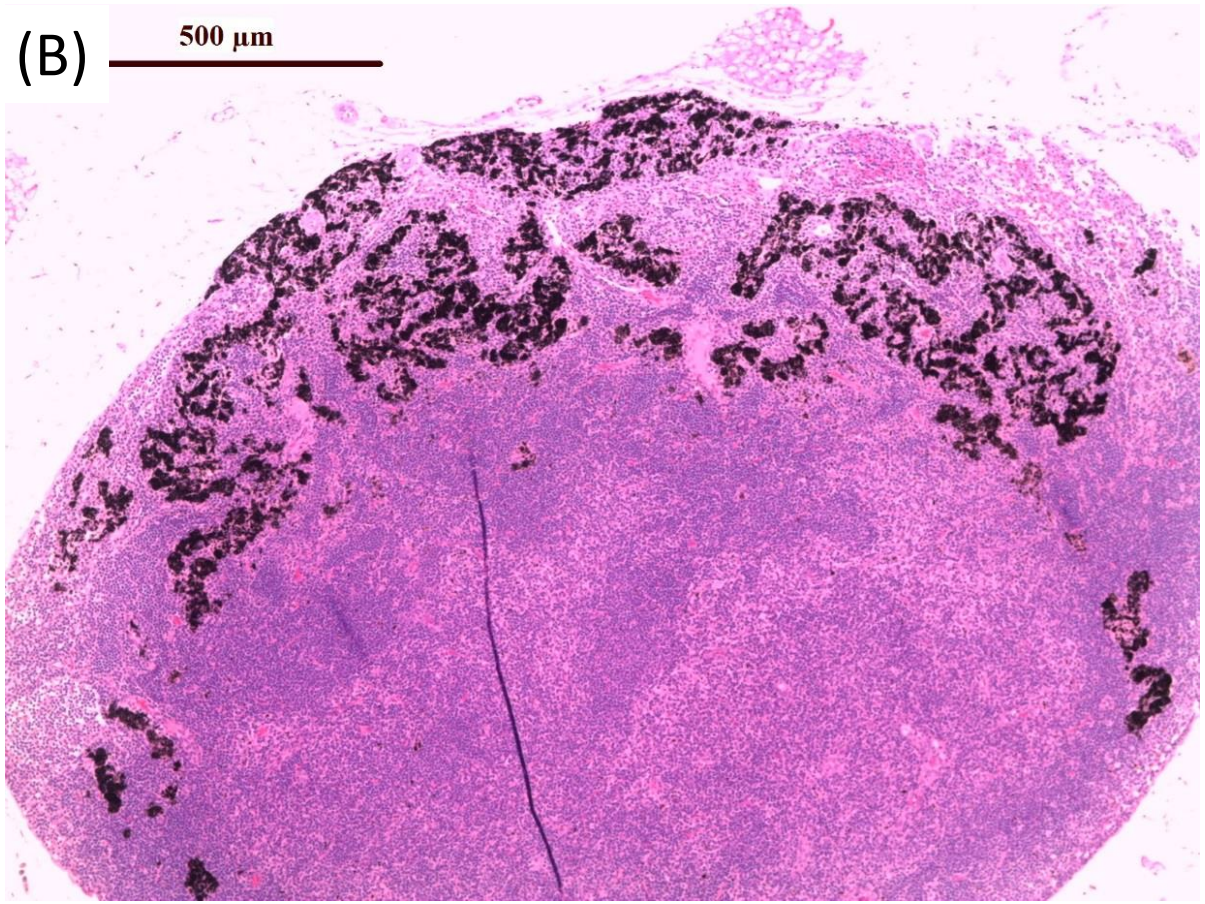
RESULTS

Tracheobronchial lymph nodes, 24 hours after 13 weeks of exposure to 5.0 mg/m^3 (A) and 52 weeks of recovery (B)

(A)

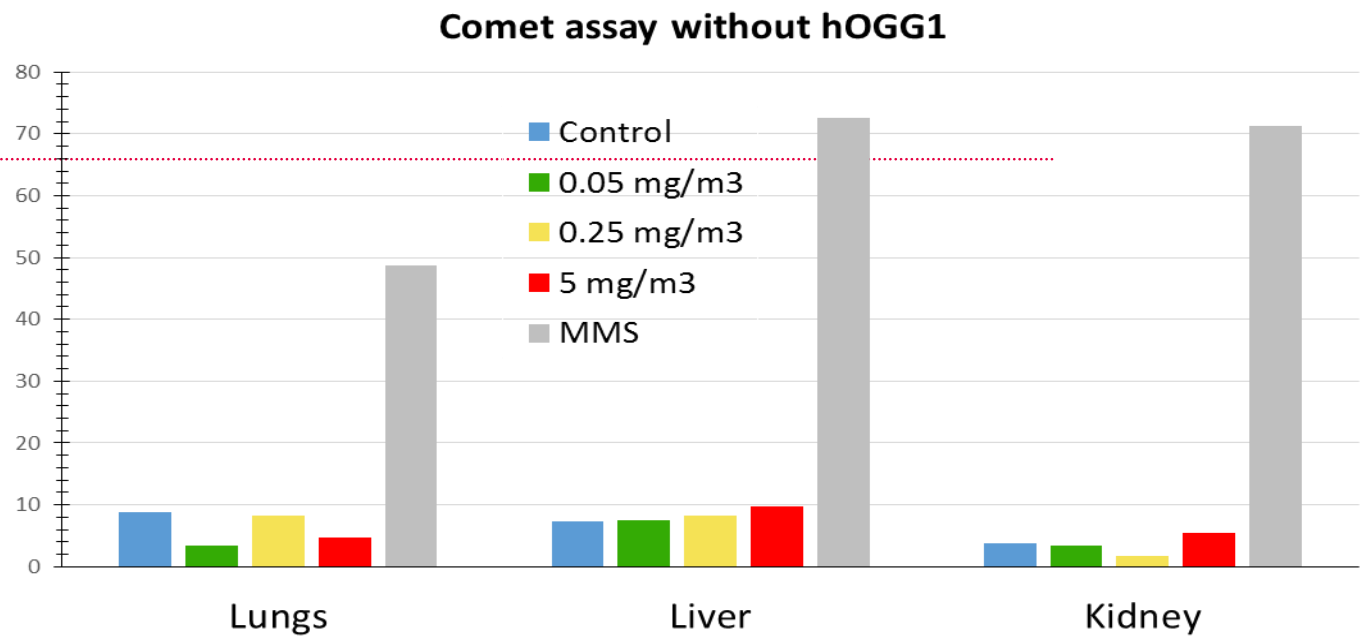


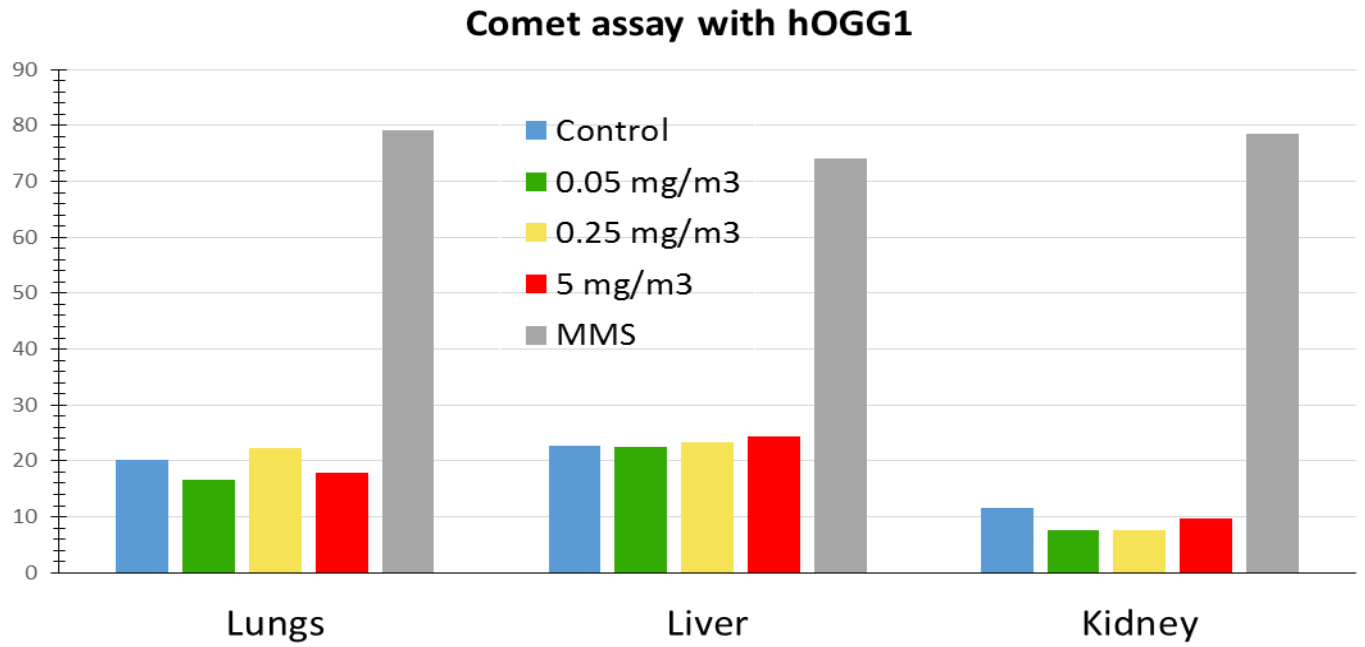
(B)



Comet assay

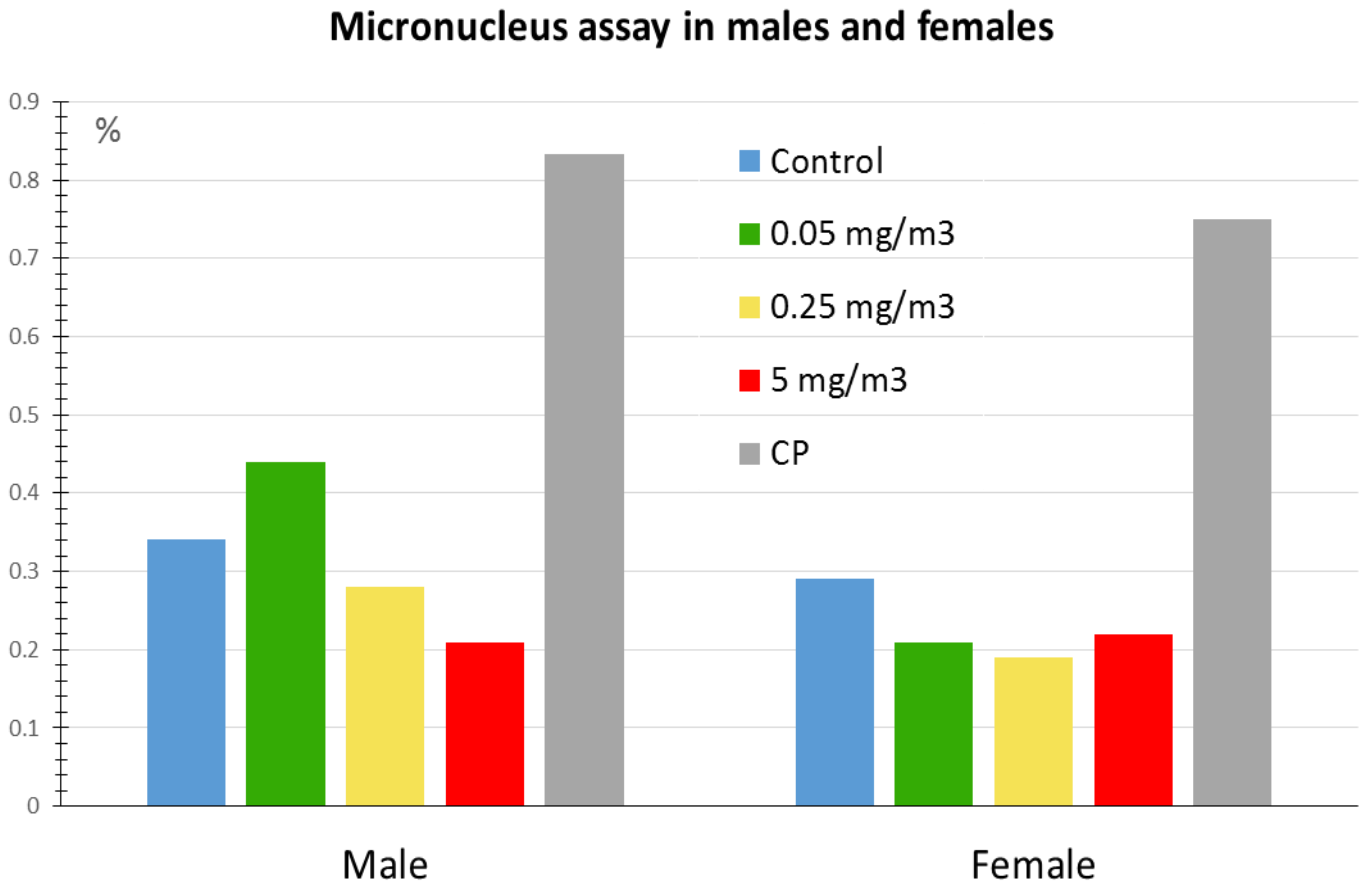
- No increase in the tail intensity, in absence and presence of hOGG1, in isolated lung, liver and kidney cells of male rats





Micronucleus assay

- No increase in the frequency of micronucleated polychromatic erythrocytes (PCE) in the bone marrow of male and female rats



CONCLUSION

Inhalation exposure

- the physicochemical properties of the MWCNT were not altered
- the particle size allowed the exposure of all relevant regions of the respiratory tract
- concentration-related deposition of black inclusions in lungs, indicating an adequate exposure

Systemic toxicity

- 5.0 mg/m^3: increase in neutrophil counts and a concomitant decrease in lymphocyte counts in blood

Respiratory tract effects

- 5.0 mg/m^3: pulmonary inflammatory reaction to the overload with insoluble particles
  - increase of the lung weights maximal at the 13-week recovery sacrifice
  - changes in the cytological, biochemical and cytokine parameters of BALF
  - inflammatory changes in the lungs and eosinophilic globules in the nasal epithelium
  - slight interstitial fibrosis 52 weeks post exposure
  - no microscopic changes in pleura, heart and aorta
- 0.05 and 0.25 mg/m^3: no adverse histological change

Genotoxicity

- No local and systemic genotoxicity

No-observed Adverse Effect Concentration (NOAEC): 0.25 mg/m^3

Pothmann *et al. Particle and Fibre Toxicology* (2015) 12:21
DOI 10.1186/s12989-015-0096-2

PARTICLE AND FIBRE TOXICOLOGY

RESEARCH Open Access

Lung inflammation and lack of genotoxicity in the comet and micronucleus assays of inc Graphistrength® C100 after a 90-day nose-only inhalation exposure of rats

Thank you for your attention

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Physico-chemical characterization of Graphistrength© C100 before and after aerosol generation

	Graphistrength© C100 original	**Graphistrength© C100** 12-h ball-milled under Argon	**Graphistrength© C100** aerosol
Apparent Density (g/cm^3) (mean ± sd)	0.106 ± 0.06 (n = 3)[B]	0.2, 0.2[B]	0.17, 0.18[B]
Elementary organic analysis			
% C	92.0, 91.6	91.1, 90.8	90.2, 90.1
% H, N, O	< LoD	< LoD	< LoD
Ash content (%)	8.2 ± 0.0 (n = 3)	nd	nd
Particle Size Distribution (µm)			
D_{10}	223	9.3	MMAD < 3µm
D_{50}	418	27.0	
D_{90}	655	57.2	
Specific area (m²/g)	225.6	244	242
Metal Content			
Al (% w/w)	3.0 ± 1.5 (n = 4)	2.9, 3.0	3.0, 3.0
Fe (% w/w)	2.7 ± 0.6 (n = 4)	2.2, 2.3	2.1, 2.1
Chemical Surface Analysis by XPS			
C (% w/w)	99.5 ± 0.2 (n = 14)	99.1 ± 0.2 (n = 4)	99.2 ± 0.3 (n = 4)
O (% w/w)	0.54 ± 0.20 (n = 14)	0.70 ± 0.12 (n = 4)	0.62 ± 0.22 (n = 4)
N (% w/w)	< 0.2 (n = 14)	< 0.2 (n = 4)	< 0.2 (n = 4)
Al (% w/w)	< 0.2 (n = 14)	0.17 ± 0.06 (n = 4)	0.13 ± 0.08 (n = 4)
Fe (% w/w)	< 0.2 (n = 14)	<0.1 (n = 4)	<0.1 (n = 4)
Diameters			
External Diameters (nm) (mean ± sd)	12.1 ± 3.5	12.1 ± 3.5	11.8 ± 3.0
Internal Diameters (nm) (mean ± sd)	4.4 ± 1.5		
Walls number (mean ± sd)	12 ± 4	12 ± 5	12 ± 4
Lenght (nm)			
mean ± sd	1069 ± 1102	713 ± 537	750 ± 623
D_{50}	708	569	563
Surface to Volume ratio (m^{-1})	2.4 ·10^7	4.9 · 10^7	4.2 · 10^7
Ends and alignment of Carbon Nanotubes (% open tips)	20	nd	25