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

< 0.2 % of fragments of pellets < 15 µm
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 recovery 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
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
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/m³ air

→ Study design

<table>
<thead>
<tr>
<th>Examinations</th>
<th>4-week interim animals</th>
<th>Main animals</th>
<th>13-week recovery animals</th>
<th>52-week recovery animals</th>
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<tbody>
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<td>Clinical signs, body weight, food consumption</td>
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<td>Ophthalmology</td>
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<td>Oestrus cycle</td>
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<td>Functional observation battery</td>
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<td>Blood pressure</td>
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<td>Hematology, blood chemistry and urinalysis</td>
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<td>Bronchoalveolar lavage fluid (cytology, biochemistry, cytokines)</td>
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<td>Full histopathology</td>
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<td>Respiratory tract histopathology</td>
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<td>Sperm analysis</td>
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<tr>
<td>Genotoxicity assays (comet and micronucleus assays)</td>
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</table>
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/m³ to severe at 5.0 mg/m³.
- Increase in neutrophils and lymphocytes with a concomitant decrease in macrophages at 5.0 mg/m³. Slight effect at 0.25 mg/m³ after 13 weeks of exposure, reversible after 13 and 52 weeks of recovery.
RESULTS

→ BALF examinations

• **5.0 mg/m$^3$:** changes in all biochemical parameters, maximal after 13 weeks of exposure and slightly improved during the recovery periods.

• **0.25 mg/m$^3$:** slight increase of GGT after 13 weeks of exposure, fully recovered in males, partially in females
RESULTS

BALF examinations

- increases in TNF-α at 0.25 and 5.0 mg/m³. Levels decreased after 13 weeks of recovery

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

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/m³: within the alveolar macrophages
    - 5.0 mg/m³: within tissue macrophages or free within the alveolar lumen
  • 5 mg/m³: significant microscopic changes
RESULTS

Lung, after 13 weeks of exposure to 5.0 mg/m³
RESULTS

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

(B) 5.0 mg/m$^3$, after 52 weeks of recovery
5.0 mg/m³, after 52 weeks of recovery
RESULTS

Lung (A) and nasal epithelium (B), 52 weeks after 13 weeks of exposure to 5.0 mg/m³
RESULTS

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

Comet assay

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

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\(^3\)

Régnier JF et al. Nanosafe 2016, Grenoble
Lung inflammation and lack of genotoxicity in the comet and micronucleus assays of
\textit{inc} Thank you for your attention
\textit{G}raphistrength = 1.00 after a 90-day nose-only inhalation exposure of rats

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<table>
<thead>
<tr>
<th></th>
<th>Graphistrength© C100 original</th>
<th>Graphistrength© C100 12-h ball-milled under Argon</th>
<th>Graphistrength© C100 aerosol</th>
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</thead>
<tbody>
<tr>
<td>Apparent Density (g/cm³) (mean ± sd)</td>
<td>0.106 ± 0.06 (n = 3)</td>
<td>0.2, 0.2</td>
<td>0.17, 0.18</td>
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<td>Elementary organic analysis</td>
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<tr>
<td>% C</td>
<td>92.0, 91.6</td>
<td>91.1, 90.8</td>
<td>90.2, 90.1</td>
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<tr>
<td>% H, N, O</td>
<td>&lt; LoD</td>
<td>&lt; LoD</td>
<td>&lt; LoD</td>
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<tr>
<td>Ash content (%)</td>
<td>8.2 ± 0.0 (n = 3)</td>
<td>nd</td>
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<td>Particle Size Distribution (µm)</td>
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<tr>
<td>D₁₀</td>
<td>223</td>
<td>9.3</td>
<td>MMAD &lt; 3µm</td>
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<tr>
<td>D₅₀</td>
<td>418</td>
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<td>D₉₀</td>
<td>655</td>
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<tr>
<td>Specific area (m²/g)</td>
<td>225.6</td>
<td>244</td>
<td>242</td>
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<td>Metal Content</td>
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<tr>
<td>Al (% w/w)</td>
<td>3.0 ± 1.5 (n = 4)</td>
<td>2.9, 3.0</td>
<td>3.0, 3.0</td>
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<tr>
<td>Fe (% w/w)</td>
<td>2.7 ± 0.6 (n = 4)</td>
<td>2.2, 2.3</td>
<td>2.1, 2.1</td>
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<td>Chemical Surface Analysis by XPS</td>
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<td>C (% w/w)</td>
<td>99.5 ± 0.2 (n = 14)</td>
<td>99.1 ± 0.2 (n = 4)</td>
<td>99.2 ± 0.3 (n = 4)</td>
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<tr>
<td>O (% w/w)</td>
<td>0.54 ± 0.20 (n = 14)</td>
<td>0.70 ± 0.12 (n = 4)</td>
<td>0.62 ± 0.22 (n = 4)</td>
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<td>N (% w/w)</td>
<td>&lt; 0.2 (n = 14)</td>
<td>&lt; 0.2 (n = 4)</td>
<td>&lt; 0.2 (n = 4)</td>
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<tr>
<td>Al (% w/w)</td>
<td>&lt; 0.2 (n = 14)</td>
<td>0.17 ± 0.06 (n = 4)</td>
<td>0.13 ± 0.08 (n = 4)</td>
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<td>Fe (% w/w)</td>
<td>&lt; 0.2 (n = 14)</td>
<td>&lt; 0.1 (n = 4)</td>
<td>&lt; 0.1 (n = 4)</td>
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<tr>
<td>Diameters</td>
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<tr>
<td>External Diameters (nm) (mean ± sd)</td>
<td>12.1 ± 3.5</td>
<td>12.1 ± 3.5</td>
<td>11.8 ± 3.0</td>
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<td>Internal Diameters (nm) (mean ± sd)</td>
<td>4.4 ± 1.5</td>
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<td>Walls number (mean ± sd)</td>
<td>12 ± 4</td>
<td>12 ± 5</td>
<td>12 ± 4</td>
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<td>Length (nm)</td>
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<tr>
<td>mean ± sd</td>
<td>1069 ± 1102</td>
<td>713 ± 537</td>
<td>750 ± 623</td>
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<tr>
<td>D₅₀</td>
<td>708</td>
<td>569</td>
<td>563</td>
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<tr>
<td>Surface to Volume ratio (m⁻¹)</td>
<td>2.4 · 10⁷</td>
<td>4.9 · 10⁷</td>
<td>4.2 · 10⁷</td>
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<td>Ends and alignment of Carbon</td>
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<tr>
<td>Nanotubes (% open tips)</td>
<td>20</td>
<td>nd</td>
<td>25</td>
</tr>
</tbody>
</table>

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

Régnier JF et al. Nanosafe 2016, Grenoble