Graphistrength© C100 MultiWalled Carbon Nanotubes (MWCNT): thirteen-week inhalation toxicity study in rats with 13- and 52-week recovery periods combined with comet and micronucleus assays

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Abstract. Graphistrength© C100 provides superior electrical and mechanical properties for various applications and is one of the industrial MWCNT referenced in the OECD sponsorship program for the safety testing of nanomaterials. Graphistrength© C100 is formed of MWCNT (ca. 12 walls, outer mean diameter ca. 12 nm, length ca. 1 µm) agglomerated in particles with a granulometry centered on 400 µm. A general feature of MWCNT after inhalation or intratracheal exposures is the induction of an inflammatory reaction in the lungs sometimes associated with local genotoxic effects. Most of the in vitro and in vivo genotoxicity data available on Graphistrength© C100 are negative. However, a weak DNA damage activity in the in vitro and in vivo FPG-modified Comet assays and a weak clastogenic effect in the in vitro micronucleus test were reported. After investigating different parameters for the aerosol generation, male and female Wistar rats were exposed by nose-only inhalation (6h/day, 5d/week) to target concentrations of 0.05, 0.25 and 5.0 mg/m³ air of a respirable aerosol (MMAD < 3 µm) and sacrificed immediately after 4 and 13 weeks of exposure and 13 and 52 weeks of recovery after the 13-week exposure. Clinical, biological and histological evaluations were performed according to the OECD TG 413. Broncho-alveolar lavage fluid (BALF) was collected and analysed for cytokines and inflammatory parameters. Immediately after 13 weeks of exposure, chromosomal aberrations in the bone marrow cells of males and females were evaluated by the micronucleus test (OECD TG 474) and DNA damage in the lung, kidney and liver cells of males were assessed by both the standard and the human 8-oxoguanine DNA N-glycosylase 1 (hOGG1)-modified comet assay (OECD TG 489). Concentration-related deposition of black particles (MWCNT) was observed in lungs. At all sacrifice periods, an inflammatory lung reaction was observed in rats exposed to 5.0 mg/m³ associated with changes in the differential white blood cells counts. The lung inflammation was characterized by changes in the cytological, biochemical and cytokine parameters of the BALF, an increase of the lung weight, an interstitial...
inflammation mainly around the alveolar ducts at the bronchiole-alveolar junction and a cell hypertrophy/hyperplasia in the terminal and respiratory bronchioles. The slight changes in BALF parameters observed at 0.25 mg/m$^3$ recovered after the 13-week treatment-free period and were not associated with any of the histological changes observed in lungs at 5.0 mg/m$^3$. Signs of lung clearance of the MWCNT were observed at 0.05 and 0.25 mg/m$^3$. After a one year treatment-free period, the inflammatory lung reaction was slight and of similar intensity that at the earlier sacrifice periods. Additional findings were minimal/slight bronchiolar/alveolar cell hypertrophy/hyperplasia and focally extensive alveolar septal fibrosis. No other pathological change was observed, nor was there any brain translocation via the olfactory bulb. The microscopic observations of the pleura were unremarkable. Neither increase in the number of micronucleated polychromatic erythrocytes nor increase in percent DNA damage were observed at any concentration. In conclusion, a lung inflammation characteristic of an overload with insoluble particles was observed after a 13-week inhalation exposure to 5.0 mg/m$^3$ of Graphistrength© C100. A No-Observed Adverse Effect Concentration (NOAEC) of 0.25 mg/m$^3$ was established for the repeated-dose toxicity and Graphistrength© C100 appears of low concern in term of local and systemic genotoxicity.

1. Introduction

Graphistrength© C100 provides superior electrical and mechanical properties for various applications. Graphistrength© C100 is formed of entangled MWCNT agglomerated in particles with a granulometry centered on 400 µm and contains a residual amount (<0.23 %) of small agglomerates (<15 µm) [1]. These small particles are comparable to those observed in the atmosphere of our high safety laboratory dedicated to scientific experiments with MWCNT [2], indicating a possible inhalation exposure to these small particles in case of an insufficient workers protection.

A general feature of MWCNT after inhalation or intratracheal exposures is the induction of an inflammatory reaction in the lungs sometimes associated with local genotoxic effects [3]. Therefore, conducting an inhalation subchronic toxicity study combined to genotoxicity assays was judged to be a key feature in the safety assessment of Graphistrength© C100.

An aerosol generation procedure was developed [4] in order to perform a valid study which fulfils the requirement of the inhalation OECD test guideline no. 413 [5]. The micronisation process used is an enrichment of the small particle fraction and is allowing the worst case material to be used as expected by the regulatory authorities. Another important criterion was ensuring that the administered aerosol has physico-chemical properties similar to the original material. After a careful evaluation, the defined technical conditions for the generation of respirable aerosols from Graphistrength© C100 were assessed in a 5-day range finding inhalation toxicity study in rats with a 28-day recovery period [4]. Then, a 13-week inhalation toxicity study was performed in rats, including an interim sacrifice after 4 weeks of exposure, 13- and 52-week recovery periods and the evaluation of the pulmonary inflammation parameters. A part of this study have been previously published by the same authors [4], in addition, the results obtained after the 4-week exposure and the 52-week recovery periods are presented in this article for completeness.

There are a number of publications reporting *in vitro* and *in vivo* genotoxicity studies on Graphistrength© C100 [4] under the coded named NM 402 or JRCNM04002a [6]. A weak DNA damage activity was reported in the *in vitro* (C3A and HK-2 cells) [7][8] and the *in vivo* (lung cells) [9] FPG-modified Comet assay. A weak clastogenic effect was observed in the *in vitro* micronucleus test in human lymphocytes [10] and A549 cells [11][12]. On the other hand, negative results were also reported using the same *in vitro* and *in vivo* assays [11][12][13][14].

Therefore, the genotoxic potential of Graphistrength© C100 was evaluated in cells directly in contact, and at a distance in case material was translocated from the lungs. A standard and hOGG1-modified comet assay was performed on the lung, liver and kidney cells of the rats exposed by inhalation for 13 weeks to Graphistrength© C100. The hOGG1-modified comet assay [15] was chosen because it is more specific than the FPG (formamidopyrimidine glycosilase) comet assay for the identification of
oxidative DNA damage. A micronucleus assay was performed on the bone marrow cells of the rats exposed to Graphistrength© C100 in case of translocation from the lungs to the bone marrow.

2. Methods

The study was conducted in an AAALAC-accredited laboratory according to the OECD GLP Principles, the OECD test guidelines no. 413 [5], 474 [16] and 489 [17] and the OECD recommendations [18] for the revision of the tests guidelines applicable to the inhalation toxicity testing of nanomaterials.

2.1. Test materials

Graphistrength© C100 (Figure 1) is formed of tightly bound spherical, ovoid or irregular shaped agglomerates (granulometry centred on 400 μm) of entangled MWCNT (diameter ≈ 12 nm, wall number ≈ 12, length ≈ 1 μm, 92% C, 3% Al, 2.7% Fe).

Figure 1. Scanning Electron Microscopic images of Graphistrength© C100

Legend: (A) Magnification: 22 fold. (B) Magnification: 120 fold. (C) Magnification: 10’000 fold.

2.2. Aerosol generation and monitoring

Graphistrength© C100 was ground in a ceramic ball mill for 12 hours under an argon atmosphere to reduce oxidation. The high aerosol concentrations was generated from the milled and sieved Graphistrength© C100 using a SAG 410 Solid Aerosol Generator connected to a micronizing jet mill and a cyclone and two elutriators thereafter (Figure 2). The low and intermediate concentrations were achieved by serial dilution of the high concentration. Aerosol concentrations were determined by gravimetric analysis. The cumulative particle size distribution was determined using Mercer cascade impactors and a Wide Range Particle Spectrometer©.
Figure 2. Aerosol generation and animal exposure

2.3. Physico-chemical characterisations
The original Graphistrength© C100 and samples taken at different steps of the aerosol generation process during the method development and during the inhalation exposure were analyzed for physico-chemical parameters (see Table 2).

2.4. Animal exposure

2.4.1. Graphistrength© C100. Four groups of 35 8-week old male and female Wistar rats were exposed by nose-only inhalation, 6 h/day, 5 d/week for 4 or 13 weeks to a respirable aerosol (MMAD 1.62-2.30 μm) at target concentrations of 0, 0.05, 0.25 and 5.0 mg/m³ air and measured concentrations of 0, 0.06, 0.28 and 4.84 mg/m³.

2.4.2. Positive controls for the genotoxicity studies. For the micronucleus assay, cyclophosphamide monohydrate (CPA, 20 mg/kg) was administered orally 24 h before tissue sampling. For the Comet assay, methyl methanesulfonate (MMS) was administered orally three times at 100 mg/kg bw at approximately 24 h intervals.

2.5. Study design

2.5.1. Repeated dose toxicity. Clinical, biological (haematology, biochemistry and urinalysis), broncho-alveolar lavage (BAL), sperm, oestrus cycle and histopathological examinations were performed according to the OECD TG no. 413 [5]. Sacrifices were performed after 4 and 13 weeks of exposure and after 13- and 52-week treatment-free periods (Table 1). BAL fluid (BALF) was examined for cytological and biochemical parameters and cytokine levels (TNF-α, IL-1α, IL-1β and IL-5). The olfactory bulb was examined microscopically at the end of the 13-week exposure. Microscopic
examinations on the 13- and 52-week recovery rats were limited to the respiratory tracts, pleura, heart and/or aorta.

Table 1. Design of 4/13-week exposure and 13/52-week recovery study

| 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              |                        |              |                          |                          |
| Full histopathology                       |                        |              |                          |                          |
| Respiratory tract histopathology          |                        |              |                          |                          |
| Sperm analysis                            |                        |              |                          |                          |
| Genotoxicity assays                       |                        |              |                          |                          |

2.5.2. *Micronucleus test.* Femoral bone marrow was collected from the last 5 males and 5 females of each group of 10 rats, 24 h after the last day of the 13-week exposure to Graphistrength© C100 or the single administration of the positive control. Nucleated cells were separated from the erythrocytes using the method of Romagna [19]. The preparation and the scoring of the slides and the interpretation of the data were performed as recommended by the OECD TG 474 [16].

2.5.3. *Comet assay.* Parts of the lung, kidney and liver were collected from the first 5 males from each group of ten rats sacrificed 24 h after the last day of the 13-week exposure to Graphistrength© C100 or 6 hours after the last treatment with the positive control. The organs were minced using fine scissors in cold mincing buffer. The preparation and the scoring of the slides and the interpretation of the data were performed as recommended by the OECD TG 489 [17].

3. Results

3.1. *Physico-chemical analysis*

Minor changes (Table 2) were noted between the starting material and the ball milled and 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.
Table 2. 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^1 |
|---------------------------|-------------------------------|-----------------------------------------------|-------------------------------|
| Apparent Density (g/cm³) (mean ± sd) by porosimetry with Hg intrusion | 0.106 ± 0.06 (n = 3)^a | 0.2, 0.2^b | 0.17, 0.18^b |
| Elementary organic analysis by calcination | % C | 92.0, 91.6 | 91.1, 90.8 | 90.2, 90.1 |
|                            | % H, N, O | < LoD | < LoD | < LoD |
| Ash content (%) by calcination | | 8.2 ± 0.0 (n = 3) | nd | nd |
| Particle Size Distribution (µm) by laser | | | |
| D_{10} | 223 | 9.3 | |
| D_{50} | 418 | 27.0 | MMAD: 1.62-2.30^2 |
| D_{90} | 655 | 57.2 | |
| Specific area (m²/g) by BET | 225.6 | 244 | 242 |
| Metal Content by lithium tetraborate fusion | | | |
| 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 by TEM | | | |
| 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) by TEM | 12 ± 4 | 12 ± 5 | 12 ± 4 |
| Lenght (nm) by TEM | | | |
| mean ± sd | 1069 ± 1102 | 713 ± 537 | 750 ± 623 |
| D_{50} | 708 | 569 | 563 |
| Surface to Volume ratio (m⁻¹) | 2.4 · 10⁷ | 4.9 · 10⁷ | 4.2 · 10⁷ |
| Ends and alignment of carbon by TEM Nanotubes (% open tips) | 20 | nd | 25 |

^1collected in the inhalation exposure system
^2gravimetric determination

There was no apparent alteration by TEM of the MWCNT structure between the original, milled and aerosolized Graphistrength© C100 (Figure 3).
3.2. In life animal observations
No mortality, specific clinical signs and exposure-related adverse effects on body weight gain, food consumption, FOB parameters, blood pressure, ophthalmoscopic examinations, blood chemistry, estrus cycle and urinalysis parameters were observed.

An increase in relative and absolute blood neutrophil counts and slight decrease of the relative (but not absolute) lymphocyte counts were noted at all sacrifice periods in both sexes of rats exposed to 5.0 mg/m³.

3.3. Post-mortem observations

3.3.1. Semiology. No exposure-related adverse effects were observed on sperm counts, motility and morphology after 13 weeks of exposure and 13 and 52 weeks of recovery.

3.3.2. BALF. Presence of black particles was observed in the BALF of almost all exposed rats, from minimal at 0.05 mg/m³ to severe at 5.0 mg/m³.

At all sacrifice periods, a significant increase in neutrophils and lymphocytes with a concomitant decrease in macrophages were observed in BALF of rats exposed to 5.0 mg/m³ (Figure 4). At 0.25 mg/m³, the effect was slight after 13 weeks of exposure and reversible after 13 and 52 weeks of recovery.

From 4 weeks of exposure, significant changes in biochemical parameters were observed in rats exposed to 5.0 mg/m³ (Figure 4), maximal after 13 weeks of exposure and slightly improved during the
recovery periods. At 0.25 mg/m³, GGT increased after 13 weeks of exposure, fully recovered in males and partially in females.

TNF-α levels were increased at 0.25 and 5.0 mg/m³. Levels decreased after 13 weeks of recovery.

**Figure 4.** BALF parameters of male and female rats after 4 (A, B) and 13 weeks (C, D) of exposure to Graphistrength© C100
Figure 4 (continued). BALF parameters of male and female rats after 13 (E, F) and 52 (G, H) weeks of recovery

Changes are shown as x-fold differences compared to controls using a logarithmic scaling.Abbreviations: ALP: alkaline phosphatase, GGT: γ-glutamyltransferase, LDH: lactate dehydrogenase.

3.3.3. **Macroscopic findings.** Dark red discoloration of the lung at the 4-week interim sacrifice, and black brown discoloration of the lung and/or greenish foci at the other sacrifice periods were seen in most of the animals exposed to 5.0 mg/m³. Black discoloration was also recorded in the bronchial lymph nodes of most of these animals.

3.3.4. **Organ weights.** Increase of the absolute and relative lung weight, maximal at the 13-week recovery sacrifice, was observed at 5.0 mg/m³ (Figure 5).
3.3.5. Microscopic findings. Deposition of variably-sized and shaped black particles, localized in the lungs, within the alveolar macrophages, were observed in all rats exposed to 0.05 and 0.25 mg/m³ and within tissue macrophages or free within the alveolar lumen in rats exposed to 5.0 mg/m³ (Figure 6A and 6B).

Every time the pleura was unremarkable (Figure 6A and 7A).

Adverse histological changes in the respiratory tract were only observed in rats exposed to 5.0 mg/m³:
- minimal alveolar granulocyte infiltration in lungs (all sacrifice periods) (Figure 6B),
- minimal/moderate intra-alveolar eosinophilic material deposition in lungs, considered to be the result of macrophages membrane cell rupture (Figure 6C),
- minimal/slight interstitial inflammation in lungs (all sacrifice periods) (Figure 6A),
- minimal/slight focal/multifoca/focally extensive alveolar septa fibrosis in lungs (Figure 7B),
- minimal/slight granulomatous fibrosing inflammation in lungs (Figure 7C),
- minimal/slight bronchiolar cell hypertrophy/ hyperplasia in lungs (Figure 7D),
- Moderate/marked cytoplasmic eosinophilic globules (inclusions) in the respiratory and nasal epithelial cells (Figure 7E).

Minimal to marked concentration-related deposition of black particles in cortex/paracortex of the tracheobronchial lymph nodes was observed in rats exposed to 0.25 and 5.0 mg/m³ (Figure 6D and 7F) consistent with continuous drainage of black particles from the lungs.

Specifically, no histological lesions were observed in aorta, heart and olfactory bulb and no deposit of MWCNT aggregate was observed in the liver, kidneys and bone marrow and other organs of the exposed animals.
Figure 6. Histological changes after 13 weeks of exposure to 5.0 mg/m³.

Legend: (A) Presence of black particles within the alveolar macrophages (bleu arrow) and tissue macrophages (yellow arrow) around a blood vessel. The pleura overlying the parenchymal inflammation is unremarkable (green arrow). (B) Presence of black particles within the alveolar macrophages (bleu arrow) and tissue macrophages (red arrow). Note the interstitial inflammation around the alveolar duct with macrophages arranged as a small nodule-like structure (black arrow), forming concentric layers around black particles. (C) Presence of black particles within alveolar macrophages (blue arrow) or free within the alveolar lumen, admixed with the eosinophilic material (black arrow). (D) Accumulation of black particles associated with increased lymphocytes in the cortex/paracortex of the tracheobronchial lymph nodes.
**Figure 7.** Histological changes 52 weeks after 13 weeks of exposure to 5.0 mg/m³.

Legend: (A) Presence of black particles within the alveolar macrophages (bleu arrow). The pleura overlying the parenchymal inflammation is unremarkable (green arrow). (B) Focally extensive alveolar septal fibrosis (arrows) (C) Focal subpleural granulomatous fibrosing inflammation. Note the presence of cholesterol-like clefts (arrows). (D) Bronchiolar/alveolar cell hypertrophy/hyperplasia (arrows) associated with the presence of intra-alveolar eosinophilic material (E) Eosinophilic globules are observed within nasal epithelial cells (arrow) (F) severe accumulation of black particles in the cortex/paracortex of tracheobronchial lymph nodes in relation to the pulmonary clearance process and the black discoloration observed at necropsy.
3.4. Genotoxicity assays

3.4.1. Comet assay. No increase in the tail intensity (mean of median), in absence and presence of hOGG1 (Table 3), was observed in isolated lung, liver and kidney cells from male rats exposed to Graphistrength© C100 for 13 weeks.

Table 3. Results of the h-OGG1-modified comet assay in the lung, kidney and liver of male rats.

| Test groups (n=5/group) | hOGG1 | Air control | Graphistrength© C100 (mg/m³) | Positive control¹ |
|------------------------|-------|-------------|------------------------------|-------------------|
|                        |       |             | 0.05 | 0.25 | 5.0 |                      |
| LUNG                   |       |             |      |      |    |                      |
| % of DNA in tail¹      | -     | 8.8 ± 5.5   | 3.4 ± 1.5* | 8.2 ± 5.1 | 4.7 ± 3.7 | 48.7 ± 3.0** |
|                        | +     | 20.2 ± 5.2  | 16.7 ± 5.7 | 22.3 ± 11.8 | 17.9 ± 5.9 | 79.1 ± 7.9** |
| Relative ratio of ghost cell² | - | - | 1.0 | 1.2 | 0.8 | 0.6** |
|                        | + | - | 2.1** | 2.0** | 1.3 | 1.1 |
| KIDNEYS                |       |             |      |      |    |                      |
| % of DNA in tail¹      | -     | 7.4 ± 4.4   | 7.5± 4.2 | 8.2± 4.2 | 9.7± 3.1 | 72.5± 5.8** |
|                        | +     | 22.8 ± 7.4  | 22.4 ± 7.3 | 23.4 ± 8.4 | 24.3 ± 8.0 | 74.0 ± 5.9** |
| Relative ratio of ghost cell² | - | - | 1.6 | 0.9 | 1.5 | 2.8** |
|                        | + | - | 1.1 | 1.6* | 1.7** | 0.6* |
| LIVER                  |       |             |      |      |    |                      |
| % of DNA in tail¹      | -     | 3.8 ± 3.1   | 3.4 ± 1.6 | 1.8 ± 1.0 | 5.5 ± 2.9 | 71.2 ± 9.1** |
|                        | +     | 11.6 ± 6.4  | 7.5 ± 1.5 | 7.7 ± 1.1 | 9.6 ± 3.0 | 78.6 ± 3.7** |
| Relative ratio of ghost cell² | - | - | 0.2 | 0.5 | 2.8** | 4.9** |
|                        | + | - | 0.5* | 0.2** | 1.1 | 1.7** |

¹Mean of median ± sd
²Mean value in treated groups/mean control value,
³MMS
⁴/**: Non-parametric Mann-Whitney test significant <0.05 5% (*), <0.01 (**)  

3.4.2. Micronucleus assay. No increase in the frequency of micronucleated polychromatic erythrocytes (PCE) (Table 4) was observed in the bone marrow of male and female rats exposed to Graphistrength© C100 for 13 weeks.
Table 4. Results of the micronucleus assay in the bone marrow of male and female rats.

| Test groups                  | Concentration (mg/m³) | PCEs with micronuclei (%) | Range² | PCE per 2000 erythrocytes |
|------------------------------|-----------------------|----------------------------|--------|---------------------------|
| **MALES**                    |                       |                            |        |                           |
| Air control                  | 0                     | 0.340                      | 2 - 17 | 1099                      |
|                              | 0.05                  | 0.440                      | 5 - 11 | 1073                      |
| Graphistrength© C100         | 0.25                  | 0.280                      | 2 - 10 | 1094                      |
|                              | 5.00                  | 0.210                      | 3 - 8  | 1081                      |
| Positive control¹             | 20                    | 0.833*                     | 10 - 26| 806                       |
| **FEMALES**                  |                       |                            |        |                           |
| Air control                  | 0                     | 0.290                      | 3 - 9  | 1155                      |
|                              | 0.05                  | 0.210                      | 1 - 8  | 1223                      |
| Graphistrength© C100         | 0.25                  | 0.190                      | 1 - 5  | 1147                      |
|                              | 5.00                  | 0.220                      | 2 - 9  | 1091                      |
| Positive control¹             | 20                    | 0.750**                    | 8 - 28 | 813                       |

*/**: Non-parametric Mann-Whitney test significant <0.05 (*) or <0.01 (**),
¹CPA, 20 mg/kg
²per 2000 PCEs per animal

4. Discussion and conclusion

This 13-week inhalation toxicity study on MWCNT Graphistrength© C100 was performed after a careful tuning of the conditions for the generation of a respirable aerosol which respect the physicochemical properties of the MWCNT [4]. A concentration-related increase of black inclusions (regarded to be MWCNT) were observed in the alveolar space and the cytoplasm of infiltrated macrophages, indicating an adequate exposure of the lungs.

The signs of systemic effects were limited to an increase in neutrophil counts and a concomitant decrease in lymphocyte counts in blood of rats exposed to 5.0 mg/m³. After 4 and 13 weeks of exposure to 5.0 mg/m³ and 13 and 52 weeks post-exposure, a black brown discoloration of the lung and/or greenish foci were seen in most of the rats. This was associated with an increase of the lung weights maximal at the 13-week recovery sacrifice. The pulmonary reaction to the overload with these insoluble particles was revealed by changes in the cytological, biochemical and cytokine parameters of BALF, slight and reversible at 0.25 mg/m³ but marked at 5.0 mg/m³.

Histological changes were only observed in rats exposed to 5.0 mg/m³. Inflammatory changes in the lungs and eosinophilic globules in the nasal epithelium were observed at all sacrifice periods. After 13 and 52 weeks of recovery, minimal or slight focal collagen deposition was also observed within alveolar septae. Even one year post exposure to 5.0 mg/m³, no microscopic changes were observed in pleura, heart and aorta.
Twenty-four hours after a 13-week inhalation exposure to MWCNT Graphistrength© C100, in the presence of a clear inflammatory reaction in the lungs of the rats exposed to 5.0 mg/m³, no primary and hOGG1-sensitive oxidative DNA damage was detected by the comet assay, either in the lung cells directly in contact with the MWCNT or systemically in the liver and kidney cells and no increase of the micronucleus frequency was detected in the bone marrow.

These results differs significantly to those reported on the thick and long fibre-like MWCNT-7 (70-170 nm x ≈ 5 μm from Hodogaya Chemical ) which induced lung carcinomas in rats after a chronic inhalation exposure [20] and is classified by IARC [21] as possibly carcinogenic to humans (Group 2B). In the lung cells of ICR [22] and C57Bl/6 [23] mice instilled intratracheally with MWCNT-7, DNA damage, analysed by the comet assay, increased in a dose-dependent manner. Moreover, DNA oxidative damage, indicated by 8-oxo-7,8-dihydro-2′-deoxyguanosine and heptanone ethenodeoxyribonucleosides occurred in the lungs [22]. Increases of DNA strand breaks in lung and bronchoalveolar lavage (BAL) cells and of micronucleated alveolar type II cells were also observed in mice exposed to aerosolized MWCNT-7 (8.2 ± 1.7 mg/m³), for 4 days (4 h/day) [23]. In contrast, thin and tangled MWCNT (8-15 nm x 0.37 μm from Cheap Tubes) like Graphistrength© C100, did not increase the DNA damages in BAL and lung cells of mice after a single pharyngeal aspiration (1-200 μg/mouse) and an inhalation exposure (17.5±2.0 mg/m³, 4 h/day for 4 days) [23].

Therefore, MWCNT Graphistrength© C100 appeared of low concern in terms of local and systemic genotoxicity even in presence of a pulmonary inflammation. Considering the limited and reversible effects on the BALF parameters and the lack of adverse pathological changes in the lungs of rats exposed to 0.25 mg/m³ up to one year after the end of exposure, this concentration was considered to be the No-observed Adverse Effect Concentration (NOAEC).

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