Finnish Institute of Occupational Health

Carbon nanotube -induced genotoxicity in mice:
Detection of DNA double strand breaks in histopathological lung specimens

Kukka Aimonen [1], Sauli Savukoski [1], Katriina Huumonen [1, 2], Annabrita Schoonenberg [3], Katja Välimäki [3], Silvana Libertini [4], Ulla Vogel [5], Henrik Wolff [1], Julia Catalán [1,6], Hannu Norppa [1]

[1] Finnish Institute of Occupational Health, Helsinki, Finland, e-mail: kukka.aimonen@ttl.fi
[2] Linnunmaa Oy, Joensuu / Finland, [3] Digital and Molecular Pathology Unit, Institute for Molecular Medicine Finland, Helsinki, Finland
[4] Novartis Institutes for BioMedical Research, Basel / Switzerland, [5] National Research Centre for the Working Environment, Copenhagen, Denmark
[6] Department of Anatomy, Embryology and Genetics, University of Zaragoza, Zaragoza / Spain

Multiwalled carbon nanotubes (MWCNTs) can induce DNA strand breaks in lung tissue, but the genotoxic potential of these materials varies considerably. In the present study phosphorylation of histone H2AX (γ-H2AX) at serine 139 was used as a biomarker of DNA double strand breaks allowing assessment of genotoxicity in histopathological tissue sections commonly prepared in animal toxicity studies.

Materials & Methods

We investigated the toxic effects of 10 forms of carbon nanotube (CNT) materials (4 MWCNTs and 6 single-walled CNTs) **(Table 1)** in female C57BL-6 mice, sampled 28 days after treatment by intratracheal instillation (54 µg/mouse). The immunofluorescent γ-H2AX-staining was performed on formalin-fixed paraffin-embedded lung samples with an autostainer using primary (rabbit monoclonal anti-gamma H2AX-phospho-Ser139) and secondary (goat anti-rabbit IgG) antibody incubations and tyramide amplification of the fluorescent signal (Alexa Fluor™ 488 Tyramide SuperBoost™ Kit; ThermoFisher Scientific) according to manufacturer's instructions. Samples were counterstained with DAPI (4',6-diamidino-2-phenylindole) and digitized with 20x fluorescent scanning **(Figure 1)**. For each sample, all nuclei in four randomly selected annotations (200 µm x 200 µm) were classified as negative, weak positive (≤ 3 foci), positive (> 3 foci), or apoptotic (pan-stained nucleus).

CNT Material			Diameter	Length	Surface area
NM-402	MWCNT	Pristine	7-20 nm	1372 ± 192 nm	226 m²/g
NM-403	MWCNT	Pristine	5-37 nm	443 ± 33 nm	135 m²/g
NM-411	SWCNT	Pristine	2 nm	1000 nm	861,0 m²/g
NRCWE-051	SWCNT	Pristine	1-2 nm	5000-30000 nm	442,6 m²/g
NRCWE-052	SWCNT	Pristine	1-2 nm	5000-30000 nm	405,7 m²/g
NRCWE-053	SWCNT	-OH (3.96wt%)	1-2 nm	5000-30000 nm	367,8 m²/g
NRCWE-055	SWCNT	Pristine	1-2 nm	1000-3000 nm	453,1 m²/g
NRCWE-056	SWCNT	-OH (3.96wt%)	1-2 nm	1000-3000 nm	356,7 m²/g
NRCWE-062	MWCNT	Pristine	< 8 nm	10000-30000 nm	443,2 m²/g
NRCWE-063	MWCNT	-OH (5.58wt%)	< 8 nm	10000-30000 nm	426,4 m²/g

Table 1. Material properties of 10 studied CNTs (4 MWCNTs and 6 SWCNTs) as reported in NANoREG (EU-FP7 grant agreement no. 310584).

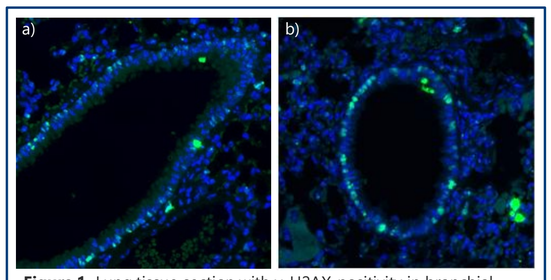

Figure 1. Lung tissue section with γ-H2AX-positivity in bronchial epithelium and alveolar space 28 d after exposure to a) MWCNTs (NM-402) and b) SWCNTs (NRCWE-051)

Results & discussion

From the 10 studied CNT materials, five (NM-402, NRCWE-051, NRCWE-052, NRCWE-055 and NRCWE-062) showed induction of γ-H2AX positivity 28 days after the treatment compared to the control animals **(Figure 2)**. Strongest γ-H2AX-positivity was detected after treatment with NRCWE-062 MWCNTs. The detection of γ-H2AX *in situ* enables the localization of the genotoxic effect in tissue-specific structures and even cell types. In the present study, majority of the γ-H2AX-positivity was seen in the bronchial epithelium.

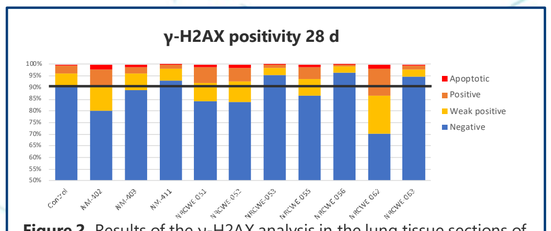

Figure 2. Results of the γ-H2AX analysis in the lung tissue sections of CNT exposed mice 28 d after exposure

Results of the of γ-H2AX analysis were compared to existing data from the same experiments. Genotoxicity was assessed in bronchoalveolar lavage fluid (BAL) and lung tissue cells by the comet assay **(Figure 3)** and systemic genotoxicity by the micronucleus assay in blood erythrocytes **(Figure 4)**. Inflammatory reaction in the CNT exposed mice was evaluated by the influx of neutrophils in BAL and serum amyloid A3 (SAA3) induction **(Figure 5)**. However, no clear correlation was seen between the results of the different assays.

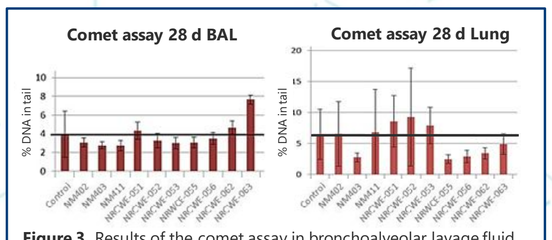

Figure 3. Results of the comet assay in bronchoalveolar lavage fluid (BAL) and lung cells 28 d after exposure: % of DNA in comet tail

CNTs can be modified by adding functional groups on their surface. This can enhance material properties, but functionalization may also alter the toxicity of CNT materials. In the present study CNT materials with hydroxyl groups (-OH) induced less γ-H2AX-positivity than the corresponding pristine CNTs. Similar effect, however, was not observed for the other assays.

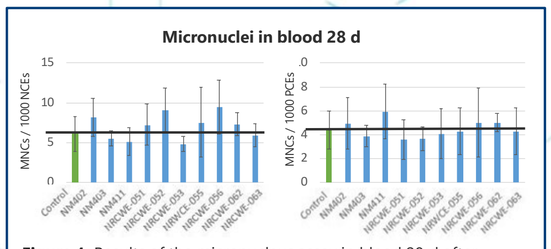

Figure 4. Results of the micronucleus assay in blood 28 d after exposure to CNTs: Micronucleated cells (MNCs) in normochromatic erythrocytes (NCEs) and polychromatic erythrocytes (PCEs)

The toxic potential of CNTs may be highly variable due to their heterogeneous physicochemical properties and further studies are still needed to clarify the mode of action of CNT genotoxicity. The immunofluorescent γ-H2AX-staining provides means to localize the genotoxic effect in histopathological lung tissue samples.

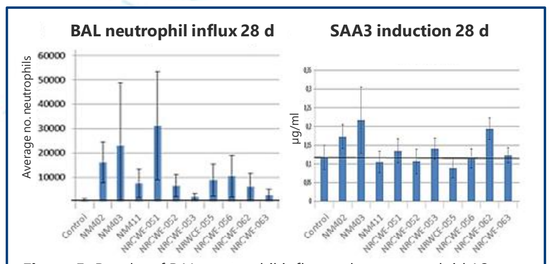

Figure 5. Results of BAL neutrophil influx and serum amyloid A3 (SAA3) induction 28 d after exposure to CNTs

Study shows that the immunofluorescent detection of γ-H2AX in histopathological tissue specimens can be used for post-experimental assessment of the genotoxicity of CNTs *in vivo*. Method allows new possibilities for studying genotoxic effects in tissue samples collected from past animal experiments and from exposed humans.

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