CEFIC-LRI N1 Project: GENOTOXICITY OF A SYNTHETIC AMORPHOUS SILICA (SAS) IN RATS

C. Ziemann, O. Creutzenberg, J. Knebel

Fraunhofer Institute for Toxicology and Experimental Medicine (ITEM), Hannover, Germany

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Background

There is still a debate about potential direct genotoxicity of nanoparticles. Due to their high specific surface area, nanostructured materials are of special interest in this regard. Thus, genotoxicity was one focus in the expanded toxicity testing of NM-200, a synthetic amorphous, precipitated silica available at the European Union JRC nanomaterials repository.

Objectives

- To investigate under GLP-conditions the genotoxic potential of NM-200 both *in vivo* and *in vitro* using established OECD Guideline methods, not specifically adapted to nanomaterials.
- To evaluate new approaches for genotoxicity testing of particles/nanomaterials.

Methods

- All *in vivo* assays were part of a 14-day nose-only inhalation toxicity study with Wistar rats (strain: Crl:WU). For details and characterization of NM-200 see poster board 459.
- The “Mammalian Erythrocytes Micronucleus Test” with polychromatic erythrocytes of the bone marrow (OCED No. 474), the “*In vitro* Mammalian Chromosome Abberration Test” with V79 cells (OECD No. 473), and the “*In vitro* Mammalian Cell Gene Mutation Test” with L5178Y/TK$^{+/-}$ cells (OECD No. 476) were performed under GLP and according to the respective guidelines.
- Both the *in vivo* and *in vitro* hOGG1 modified alkaline Comet assays to detect both DNA-strand breaks and oxidative DNA lesions were performed with cells isolated by bronchoalveolar lavage (BAL cells). For methodological details of the used Comet assay version see e.g. Ziemann et al. (2014).
- The oxidative DNA lesion 8-hydroxydeoxyguanosine (8-OH-dG) was analysed in formalin-fixed lung tissue by immunohistochemistry using a 8-OH-dG-specific mouse monoclonal antibody N45.1 (Abcam, Cambridge, UK, ab48508). See Rittinghausen et al. (2013).
- Lactate dehydrogenase (LDH) release was determined in the incubation supernatant using the “Cytotoxicity Detection Kit (LDH) from Roche Applied Science (Mannheim, Germany).

Results

Table 1: Red blood cell formation and micronucleus induction in bone marrow of rats, after 14 days of exposure to clean air, cyclophosphamide monohydrate or NM-200 (OECD No. 474).

Treatment group	Concentration, sampling time	PCE/200 RBC	PCE:NCE	MN/2000 PCE	% MN PCE
Negative control, **Clean air**	24 h	♂ 67 ♀ 94	♂ 0.52 ♀ 0.93	♂ 4.0 ♀ 5.4	♂ 0.20 ♀ 0.27
Positive control, **CP**	20 mg/kg b.w., p.o., 24 h	♂ 60 ♀ 68**	♂ 0.43 ♀ 0.51	♂ 40.8** ♀ 35.4**	♂ 2.04** ♀ 1.77**
Test item, **NM-200**	1 mg/m^3, 24 h	♂ 75 ♀ 100	♂ 0.61 ♀ 1.04	♂ 5.2 ♀ 6.0	♂ 0.26 ♀ 0.30
Test item, **NM-200**	5 mg/m^3, 24 h	♂ 75 ♀ 88	♂ 0.60 ♀ 0.80	♂ 5.8 ♀ 9.6	♂ 0.29 ♀ 0.48
Test item, **NM-200**	25 mg/m^3, 24 h	♂ 69 ♀ 87	♂ 0.51 ♀ 0.78	♂ 5.2 ♀ 7.2	♂ 0.26 ♀ 0.36

PCE: polychromatic erythrocytes; NCE: normochromatic erythrocytes; RBC: red blood cells; MN: micronuclei; % MN PCE: percent micronucleated PCE; significantly different from negative controls: **: $P \leq 0.01$, U-test according to Mann-Whitney (two-group comparisons versus negative control). 5 males and females per group.

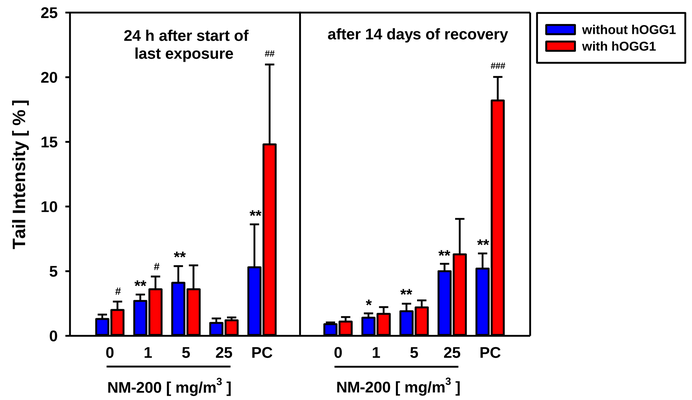

Figure 1: hOGG1-modified *in vivo* Comet assay. DNA-damage in BAL cells from male Wistar rats (strain: Crl:WU) 24 h after start of the last inhalation exposure or after 14 days of recovery. Data represent group means ± SD of 5 males per treatment. Tail intensities of 100 nuclei per animal and slide treatment (± hOGG1 incubation) were analysed. */**Significantly different from clean air control: $P \leq 0.05$ / $P \leq 0.01$, Mann-Whitney Rank Sum Test. #/##/### Significantly different from samples incubated without hOGG1: $P \leq 0.05$ / $P \leq 0.01$/ $P \leq 0.001$, Student’s *t*-test for paired values. PC = positive control for oxidative DNA-damage, $KBrO_3$, 3 h, 250 mg/kg, i.p., in physiological saline.

Table 2: 8-OH-dG positive nuclei per 10000 µm^2 area in terminal bronchioles and lung parenchymal cells.

Treatment group	Concentration	24h after start of last exposure		After 14 days of recovery	
		Group mean	SD	Group mean	SD
Negative control, **Clean air**	0 mg/m^3	5.80	0.32	6.17	0.45
Test item, **NM-200**	1 mg/m^3	7.67*	1.01	8.70**	0.74
Test item, **NM-200**	5 mg/m^3	7.55*	1.09	8.58**	0.54
Test item, **NM-200**	25 mg/m^3	7.60*	1.10	7.67**	0.64

5 males per group. */** Significantly different from clean air controls: $p \leq 0.05$ / $p \leq 0.01$, Tukey's studentized range test.

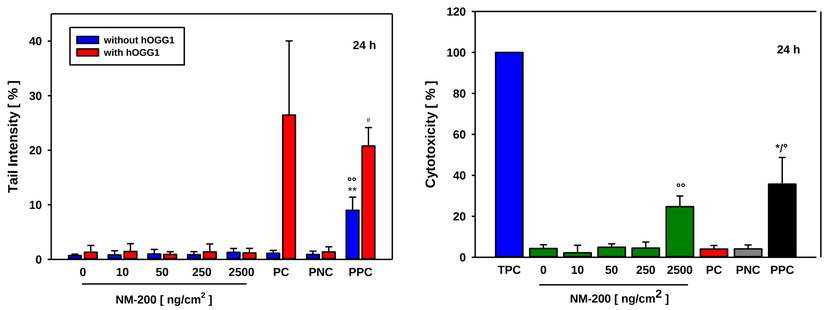

Figure 2: hOGG1-modified *in vitro* Comet assay (left graph) and LDH assay (right graph) with primary rat alveolar macrophages. Cells were pre-cultured for 24 h and then incubated for 24 h ± NM-200. Data represent means ± SD of 3 independent experiments with 100 nuclei analysed per slide and treatment (± hOGG1) in the oxidative Comet assay. */** or °/°° Significantly different from medium control or PNC: $P \leq 0.05$ / $P \leq 0.01$, Student’s *t*-test for unpaired values. # Significantly different from samples incubated without hOGG1: $P \leq 0.05$, Student’s *t*-test for paired values. TPC: technical positive control, Triton X-100 (set to 100%); PC: positive control $KBrO_3$ (0.5mM, 4h); PNC: particulate negative control, Al_2O_3 (2500 ng/cm^2, 24h); PPC: particulate positive control, quartz DQ12 (25 µg/cm^2, 4h).

Table 3: *In vitro* mammalian cell gene mutation test with mouse lymphoma L5178Y/TK$^{+/-}$ cells (OECD No. 476). Four hours of incubation without and with S9-mix. Mean values of 2 independent cultures per treatment.

Treatment group	µg/ml	S9	SG	RTG	RS (Survivor II) [%]	MF
Negative control	-	-	20.63	1.00	100.0	99.5
		+	17.23	1.00	100.0	101.2
Positive control, **MMS**	10	-	14.14	0.45	62.4	770.2*
		+	-	-	-	-
Positive control, **CP**	2.5	-	-	-	-	-
		+	16.27	0.64	67.8	476.3*
Test item, **NM-200**	10	-	19.31	0.87	91.1	92.7
		+	19.17	0.98	88.1	145.9
Test item, **NM-200**	100	-	18.91	1.02	110.2	102.7
		+	18.01	1.18	112.6	99.2
Test item, **NM-200**	300	-	22.04	1.09	98.6	100.3
		+	17.08	1.29	131.0	91.9
Test item, **NM-200**	900	-	17.93	0.92	104.2	98.4
		+	18.63	1.58	145.6	87.9
Test item, **NM-200**	2700	-	12.84	0.72	112.8	119.0
		+	15.63	1.11	121.2	115.9
Test item, **NM-200**	5000	-	4.56	0.20	91.4	163.3
		+	1.67	0.07	97.3	112.6

SG = Suspension Growth: (Cell Counts $_{day\ 1}$ / Cell Setup $_{day\ 0}$) x (Cell Counts $_{day\ 2}$ / Cell Setup $_{day\ 1}$)
RTG = Relative Total Growth: (SG $_{test\ item}$ / SG $_{negative\ control}$) x (PE $_{test\ item}$ / PE $_{negative\ control}$)
RS = Relative Survival: (PE $_{test\ item}$ / PE $_{negative\ control}$) x 100
MF = Mutant Frequency (total colonies): (PE $_{mutant}$ / PE $_{viable}$) x 10^6
PE $_{mutant}$ = Plating Efficiency TFT Selection Plates: -ln (Number of Empty Wells / Number of Total Wells Plated) / 2000
PE $_{viable}$ = Plating Efficiency Survivor II: -ln (Number of Empty Wells / Number of Total Wells Plated) / 1.6
* Relevant increase based on the "Global Evaluation Factor" concept (Moore et al., 2003) in both of the two cultures

Summary and Conclusions

- NM-200 was not clastogenic/aneugenic in polychromatic bone marrow erythrocytes of rats after inhalation exposure (OECD No. 474; Table 1).
- In particular after 14 days of recovery BAL cells from NM-200-treated rats exhibited slight concentration-dependent increase in DNA-strand breaks, but not in oxidative DNA-damage, as compared to clean air controls (Figure 1).
- But, NM-200 was negative in *in vitro* Comet assays with primary rat alveolar macrophages after 24 h of incubation with *in vivo* adapted, lung relevant concentrations, speaking against a direct DNA-damaging potential of NM-200.
- Slight, but significant increase in 8-OH-dG positive nuclei was noted in lung epithelial cells after inhalation exposure to NM-200 (Table 2).
- In a chromosome aberration study (OECD No. 473) NM-200 treated cultures exhibited aberration frequencies in the range of historic negative controls.
- NM-200 did not exhibit substance-specific mutagenic potential up to the limit concentration of 5 mg/ml in a mouse lymphoma assay (OECD No. 476; Table 3).

➢ **There was no evidence for substance-specific, direct systemic or local genotoxic activity of NM-200.**

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