13th Annual CEFIC-LRI Workshop

Brussels, November 17, 2011

N1: Tiered Approach to Testing and Assessment of Nanomaterial Safety to Human Health

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Fraunhofer ITEM

CEFIC-funded Project

Nanoscaled Zinc Oxide

Project Period M1-M24 (June 2009 – May 2011)

Sponsor
CEFIC
The European Chemical Industry Council

Sponsor's Study Monitor
Dr. Karin Wiench, Principal Toxicologist
BASF SE, GUP/PB-Z470

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Test/Reference Items

- **Z-COTE® HP1** **(nanoscaled ZnO; coated with triethoxycaprylylsilane; BASF)** → **cosmetics sector**
- **Z-COTE®** **(nanoscaled ZnO; uncoated; BASF)**
- **ZnO 205532** **(microscaled ZnO; Sigma-Aldrich)**

Overview on Testing Schedule

Part of study	Exposure path	Task to be done within the CEFIC-Fraunhofer N1 project
in vitro work		Biocompatible formulation of test and reference items – Genotoxicity tests
		Dermal corrosion test in human skin model
		Dermal penetration test ^{65}ZnO
		Chromosomal aberration
		Mouse lymphoma assay
in vivo work	Inhalative	14-day nose-only test + 14-day rec herein: MN test in vivo herein: Comet assay
		90-day nose-only test + 28-day rec
	Dermal	Acute toxicity test
		Dermal penetration ^{65}ZnO

INHALATIVE EXPOSURE PATH

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5-Day DRF Study: Summary and Conclusion

Clean air control, 0.5 mg/m³, 2 mg/m³, and 8 mg/m³ Z-COTE® HP1

Results Histopath:

- **(Multi)focal very slight to slight**
  Degeneration of the olfactory epithelium in the nasal cavities
  in the high dose group (statistically not significant) → adverse finding
- **Alveolar accumulation of particle-laden macrophages in lungs in the high dose group (statistically significant) → adaptative response to the particle exposure.**

Conclusion for main test:

Dosing scheme of 0.5, 2 and 8 mg Z-COTE® HP1/m³

14-Day nose-only Inhalation Study

Dose Group	Treatment	Aerosol concentration (mg/m^3)	Number of animals per group (males)	Number of animals per group (females)
14-Day inhalation: 6 hrs/day on 5 days/wk, for 2 wks Endpoints: see Table 5 (p.15)				
1	Clean air	Clean air	45 + 5*	5*
2	Z-COTE® HP1	0.5	45 + 5*	5*
3	Z-COTE® HP1	2	45 + 5*	5*
4	Z-COTE® HP1	8	45 + 5*	5*
5	Z-COTE®	8	45 + 5*	5*
6	Microscaled ZnO	8	45 + 5*	5*
7	Cyclophosphamide **p.o.**	Positive control	5*	5*
		Total:	305	35
		Overall total:	340	

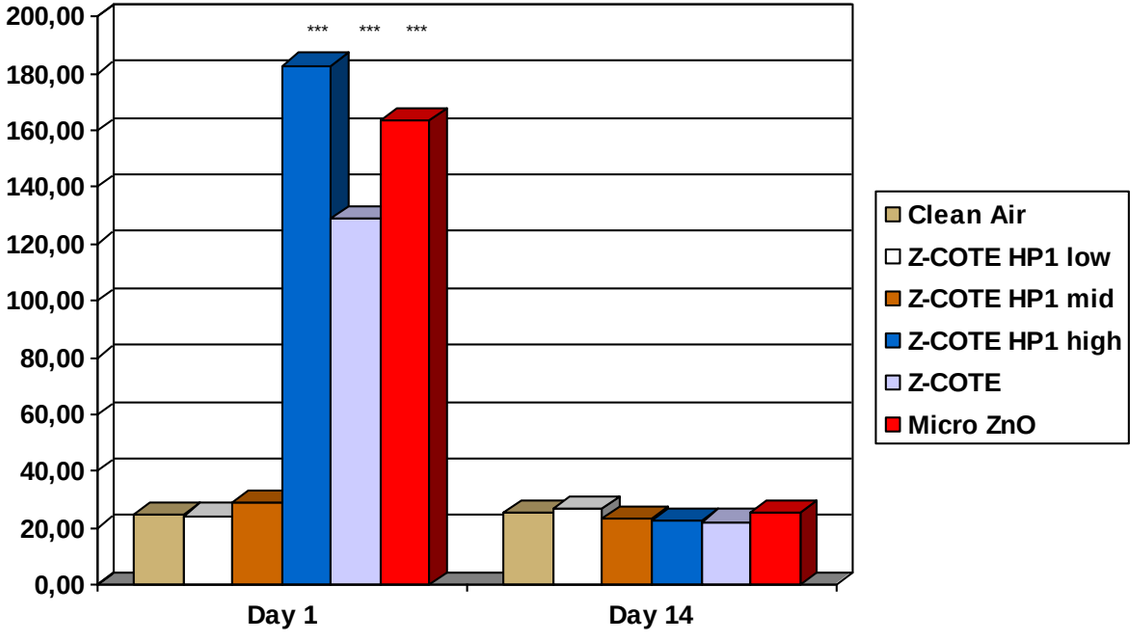
14-Day nose-only Inhalation Study

No significant changes in:

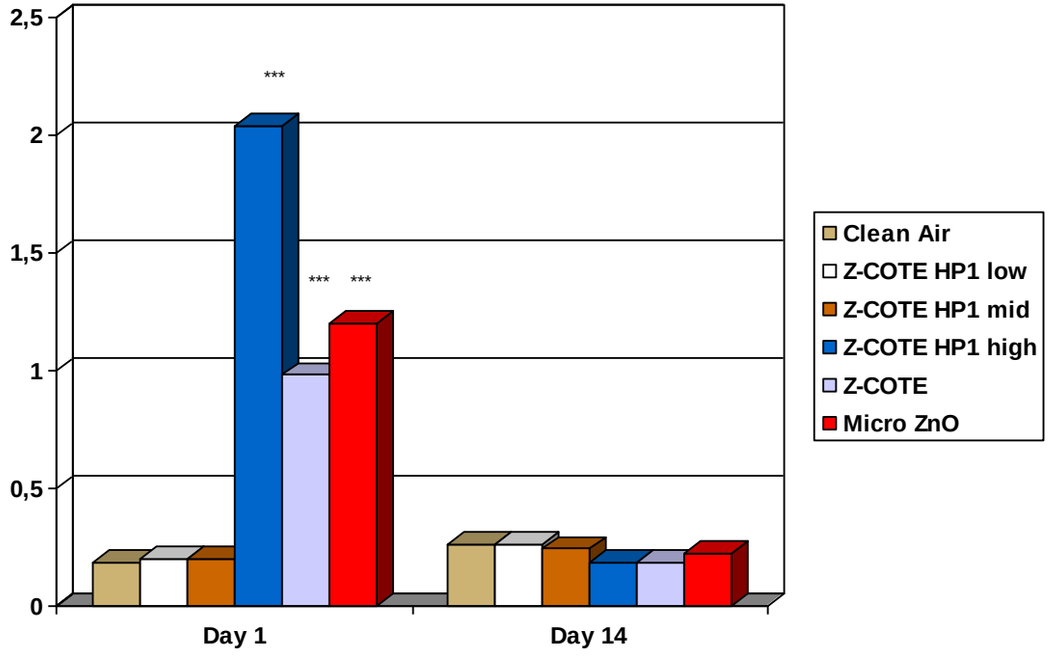
- **Body weights and food consumption**
- **Clinical chemistry**
- **Haematology**
- **Urinalysis**

14-Day Study: Bronchoalveolar Lavage

Lactic Dehydrogenase (U/l)

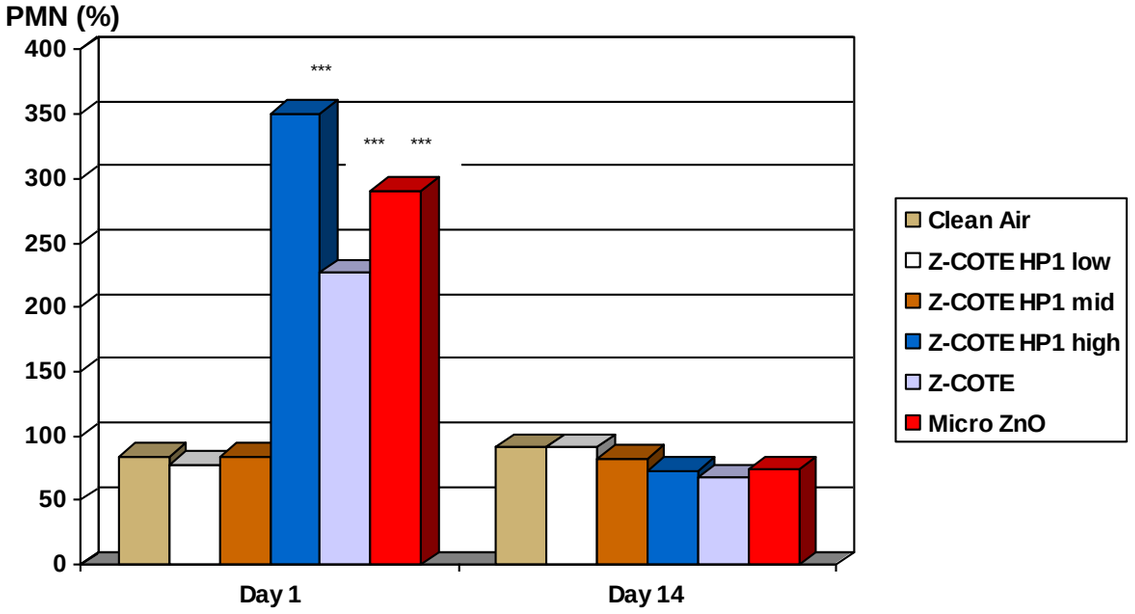


β-Glucuronidase (U/l)

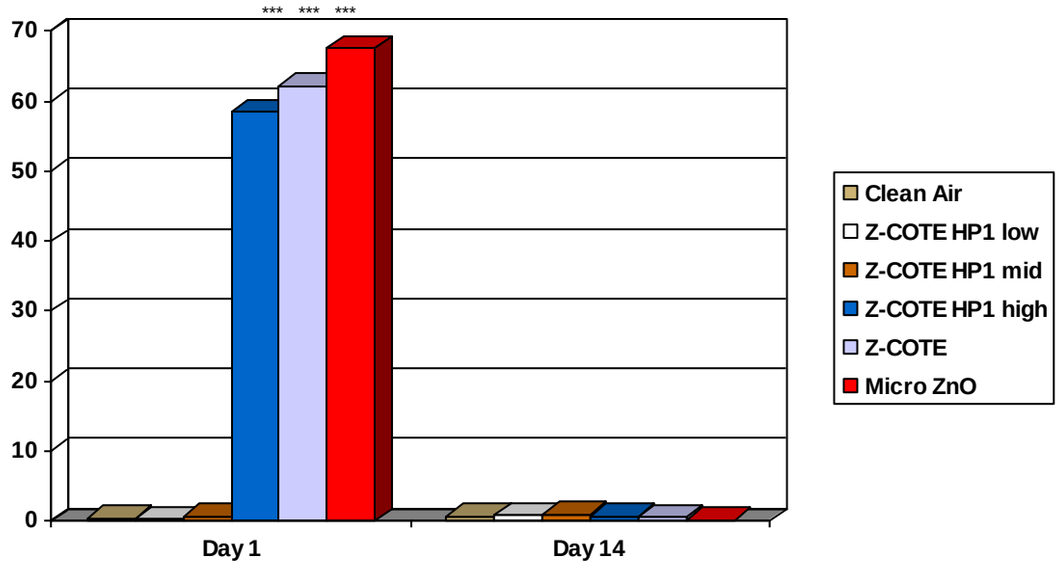


14-Day Study: Bronchoalveolar Lavage

Total Protein (mg/ml)



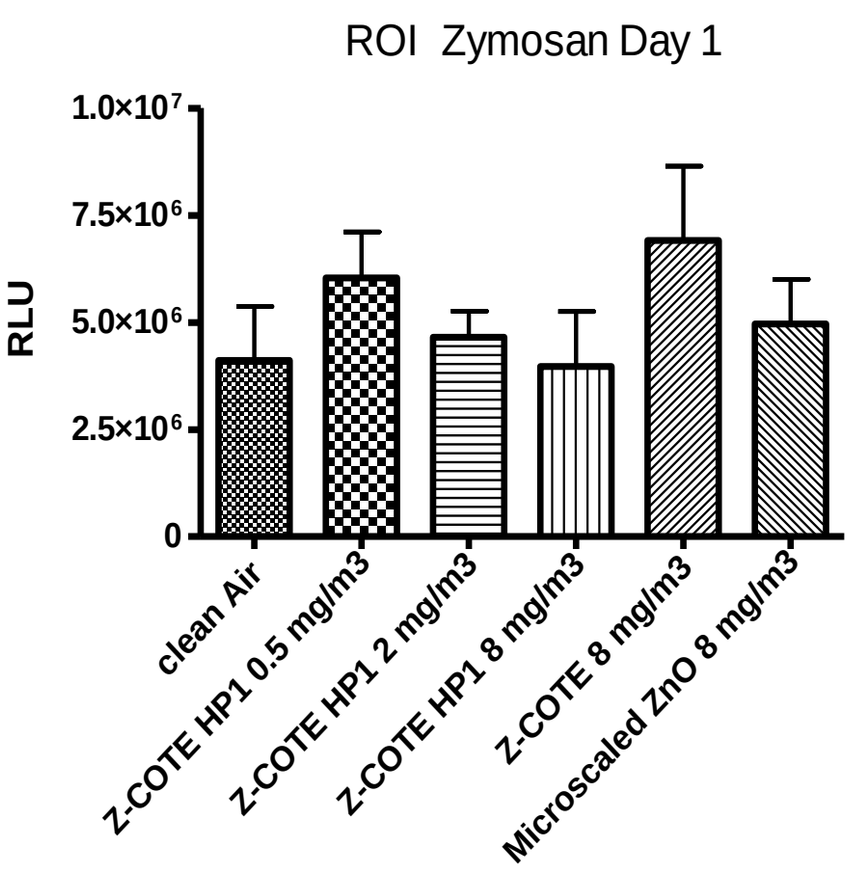
PMN (%)

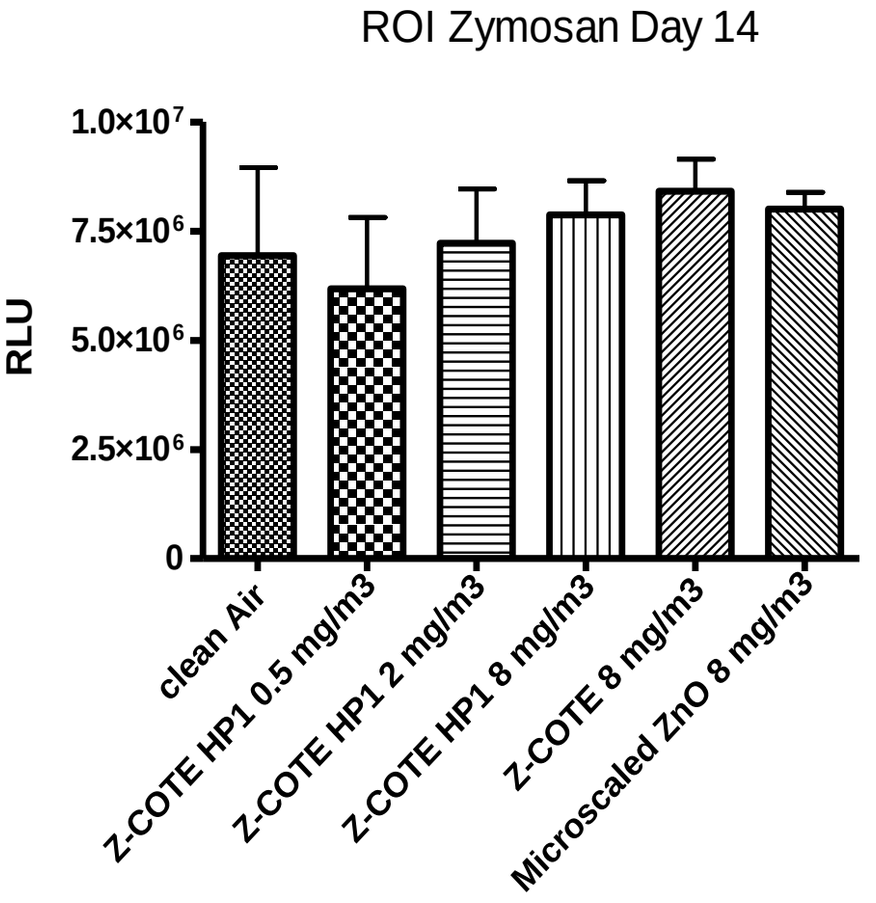


14-Day Study: Bronchoalvelar Lavage

- **Significant increases were detected in the high dose groups of Z-COTE® HP1, Z-COTE® and microscaled ZnO 1 day after exposure**
- **Effects were reversible within 14-day post-exposure period**

14-Day Study: Reactive Oxygen Species (ROS)

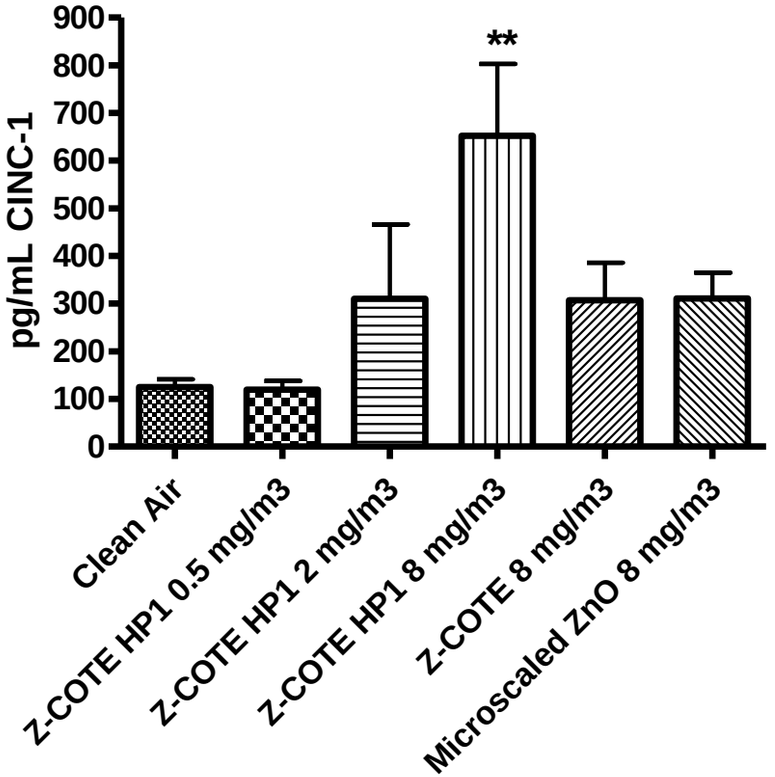




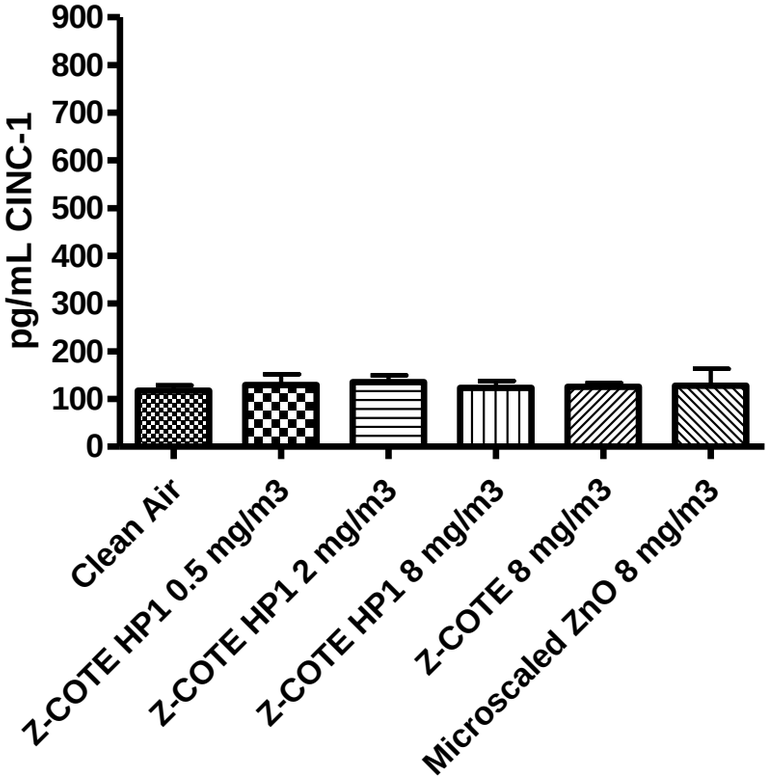
No significant differences

14-Day Study: CINC-1 – Chemotactic Factor

Day 1



Day 14



Chemotactic factor: induction of neutrophil influx → no increases after 14 days

14-Day Study: Other Parameters in BAL

IL-6 (pro-inlammatory cytokine)

TGF-β (regulatory active protein)

TNF-α (pro-inflammatory protein)

Showed significant increases after 1 and 14 days of recovery (albeit of low absolute values)

14-Day Study: Histopathology - Day 1

Nasal and Paranasal Cavities

8 mg/m³ Z-COTE HP1:

- **3 out of 5 males with (multi)focal very slight to slight degeneration of the olfactory epithelium (level 3 of the 4 nasal cavity sections)**
  → the only adverse effect in the present study

- **1 male with multifocal very slight (adaptive) mucous (goblet) cell hyperplasia affecting mainly the respiratory epithelial lining of the nasal septum.**

Lungs

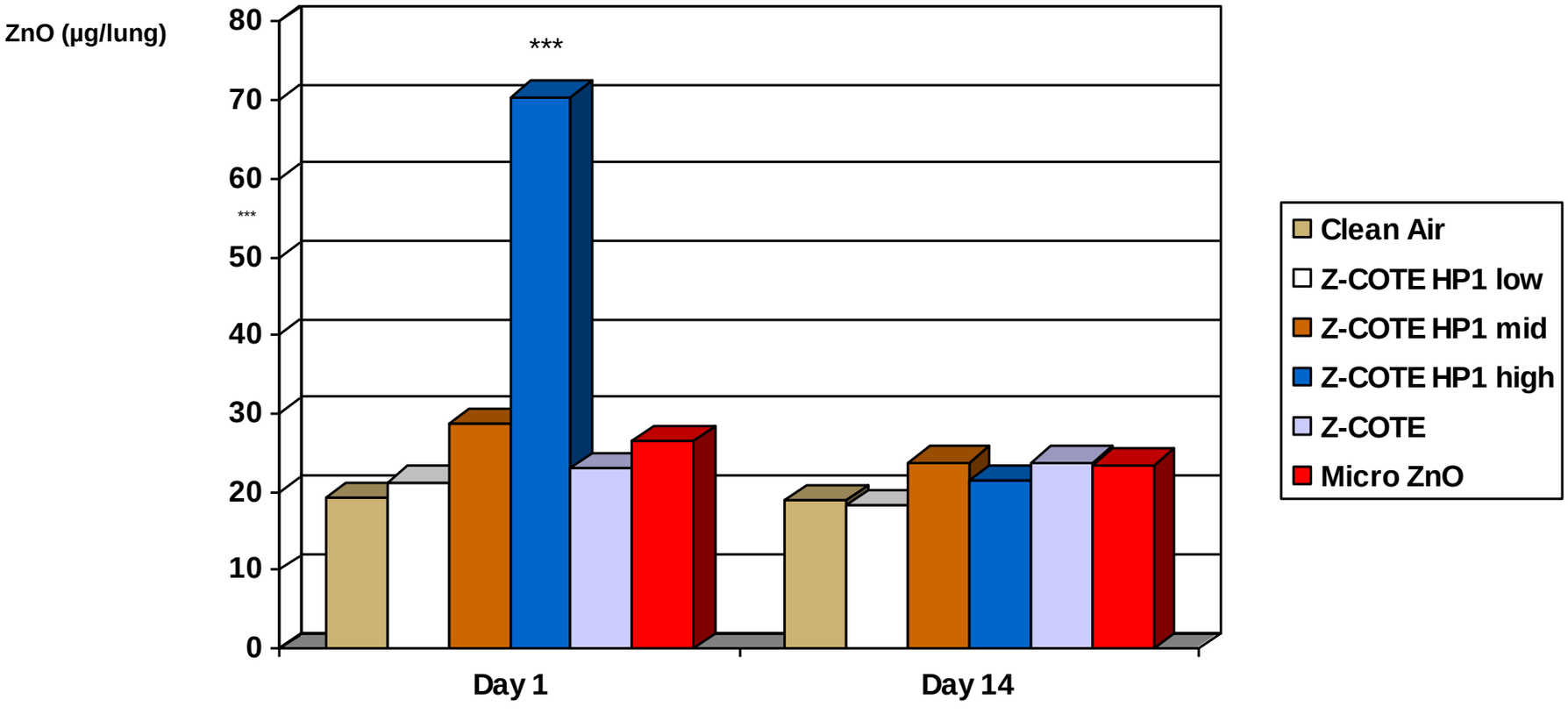
(Multi)focal very slight (minimal) alveolar accumulation of particle-laden macrophages was observed dose-dependently in 1/5, 3/5 and 5/5 males of the 0.5 mg/m³, 2 mg/m³ and 8 mg/m³ Z-COTE HP1 groups, resp. → adaptative

Lung-associated lymph nodes (LALN)

1/5 up to 3/5 males of the Z-COTE HP1 exposure groups only showed slight to moderate lymphoid hyperplasia as a probably exposure-related reactive finding

14-Day Study: Toxicokinetics – ZnO in Lungs

The only relevant significant increase observed on day 1 post-exposure in lungs

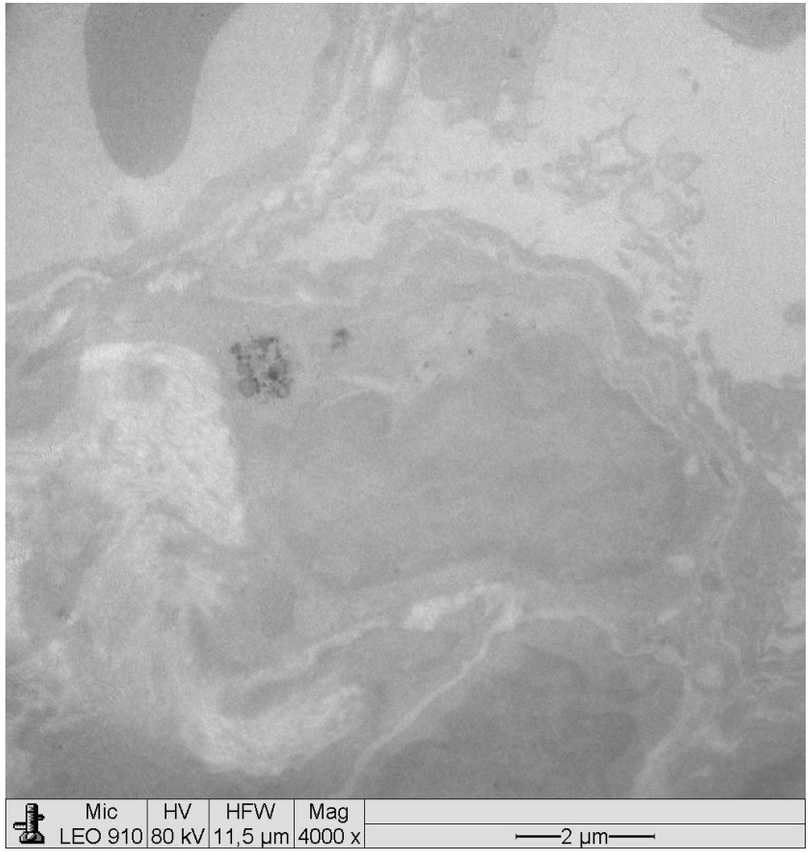


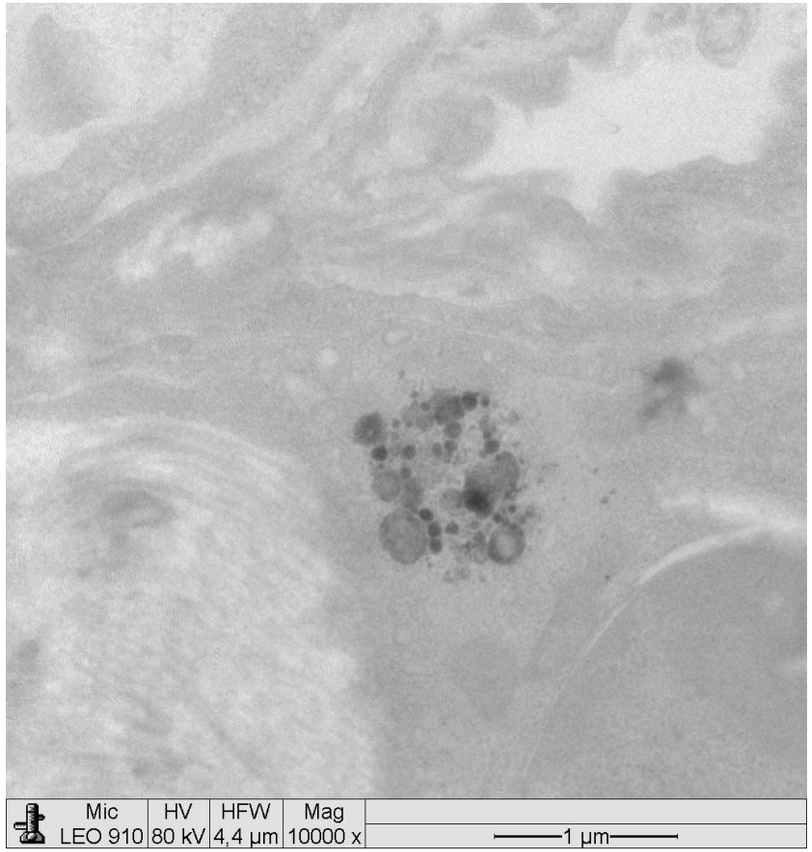
Solubility of Test Items in Various Media

test item	Matrix	pH	Solubility (%)
blank	Gambles S	4.5	< 0,01
		7.4	< 0,01
	Artificial LF	4.5	< 0,01
	Artificial AF	7.4	< 0,01
Z-COTE HP1	Gambles S	4.5	< 20
		7.4	< 0,05
	Artificial LF	4.5	> 90
	Artificial AF	7.4	< 0,05
Z-COTE	Gambles S	4.5	< 10
		7.4	< 0,05
	Artificial LF	4.5	> 90
	Artificial AF	7.4	< 0,05
Microscaled ZnO	Gambles S	4.5	< 20
		7.4	< 0,05
	Artificial LF	4.5	> 90
	Artificial AF	7.4	< 0,05

TEM Evaluation on Existing Particulate Material in Lungs

Possibly detected ZnO particles in MPh cytoplasm

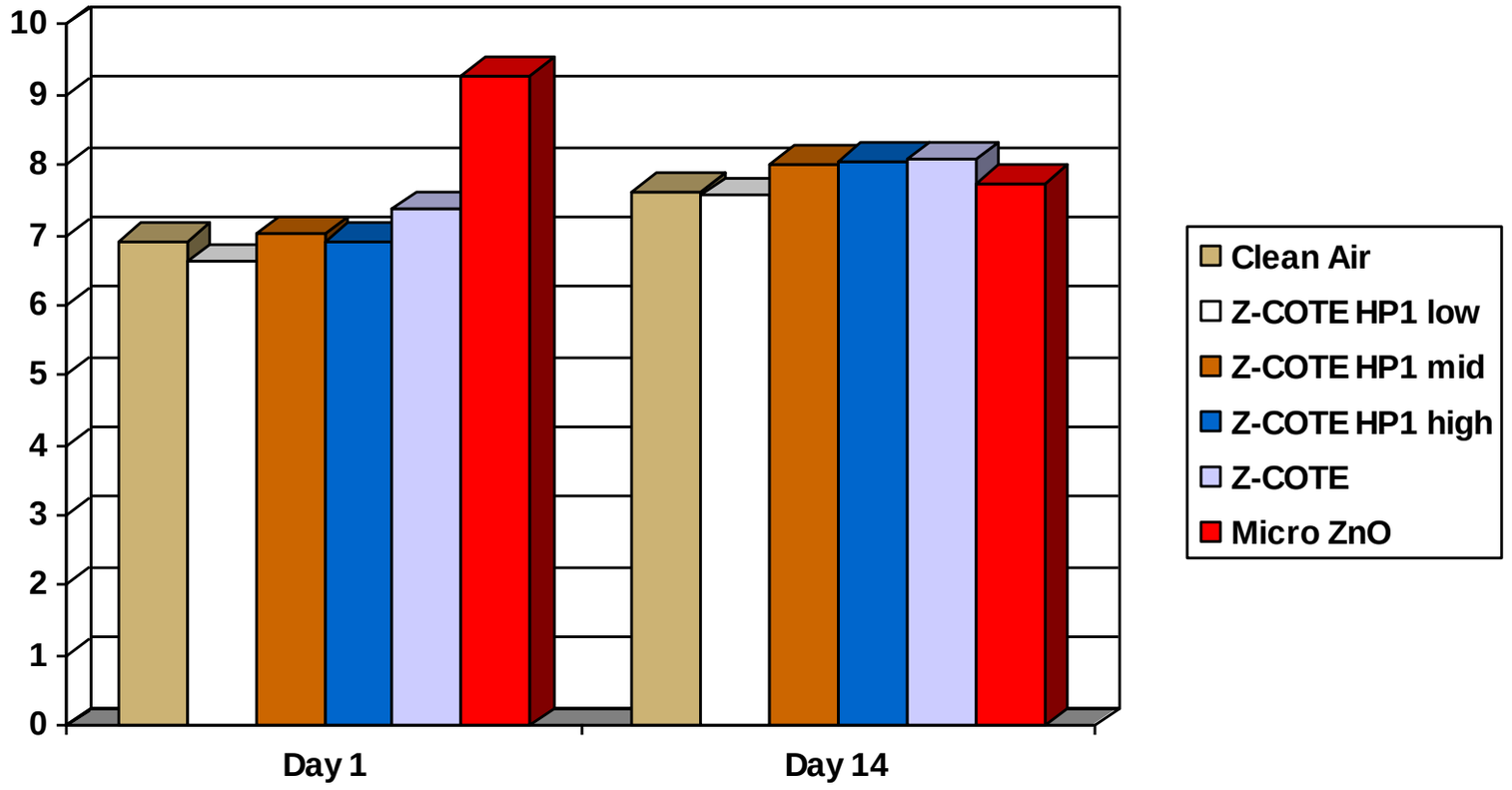




Oxidative damage: Immunohistochemical detection of 8-OH-dG in lung tissue

Immunohistochemistry in Lungs
8-OH-dG (positive nuclei per 10000 µm²)

No statistically significant changes



Genotoxicity Testing ZnO

Chromosomal aberration test / V79 cells OECD 473

→ negative

- **Mouse lymphoma mutation test / L5178Y/TK$^{+/-}$ cells OECD 476**

  → positive

- **Micronucleus test OECD 474**

  → negative

90-Day Nose-only Inhalation Test

→ **Aerosol concentrations:**

Z-COTE® HP1 0 – 0.3 – 1.5 – 4.5 mg/m³

Microscaled ZnO 4.5 mg/m³

90-Day Nose-only Inhalation Test + 28-day rec

Endpoint	Z-COTE® HP1 Low 0.3 mg/m³	Z-COTE® HP1 Mid 1.5 mg/m³	Z-COTE® HP1 High 4.5 mg/m³	Microscaled ZnO 4.5 mg/m³
Body weights	-	-	-	-
Food consumption	-	-	-	-
Organ weights Lungs	-	-	-	↑
Haematology, clinical chemistry	-	-	-	-
BAL PMN Day 1	-	-	-	↑
BAL Lymph. Day 1	-	-	-	↑
BAL LDH Day 1	-	-	↑	↑
BAL Protein Day 1	-	-	-	↑
Histopathology nasal cavities	-	-	-	↑
Histopathology lungs: bronch.-alveolar hyperplasia	-	-	↑	↑
Histopathology lungs: mononuclear cell infiltration	-	-	↑	↑
Cell proliferation	n.d.	n.d.	↓	↓
Toxicokinetics	Practically complete dissolution of the retained test item; no translocation			
TEM	No distinct ZnO particles detectable at any time-point			
		NOAEL	**LOAEL**	

↑ statistically significant increase
↓ statistically significant decrease
- no statistically significant change as compared to concurrent controls

DERMAL EXPOSURE PATH

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Dermal Toxicity Test *in vivo/in vitro*

in vivo

Acute Dermal toxicity test according to OECD 402

→ Not classified

in vitro

Skin corrosion test according to OECD 431

→ Not corrosive

Dermal Absorption of [^{65}Zn]-Z-COTE® HP1 *in vivo*

Experimental design according to OECD 427
^{65}Zn label by neutron activation

Not absorbed fraction (%) Swabs, O-rings, spacers, gauze and plasters	**Skin total (%)** Tesa strippings, skin at application size	**Absorbed fraction (%)** Urine/feces; carcass	**Recovery (%)**
70.3	24.1	0.01	94.5

Conclusions

INHALATIVE

- **14-day study: no systemic but local effects on olfactory epithelium and in lungs → reversible**
- **LOAEL: 8 mg/m³ - NOAEL: 2 mg/m³**

- **90-day study: Adverse effects restricted to high dose → reversible**
- **LOAEL: 4.5 mg/m³ - NOAEL: 1.5 mg/m³**

- **Effects independent of ZnO particle size**

DERMAL

- **Z-COTE® HP1 was not absorbed *in vivo***
- **Acute test → no effects**

GENOTOXICITY *in vitro/in vivo* → negative

CEFIC-funded Project

Amorphous Silicon Dioxide

Project Period M1-M24 (Nov 2010 – Oct 2012)

Sponsor
CEFIC
The European Chemical Industry Council

Sponsor's Study Monitor
Dr. Monika Maier, Principal Toxicologist
Evonik, Hanau

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Test/Reference Items

- **NM-200** **(precipitated amorphous silica; JRC)** → **food sector**
- **No microscaled reference**

Tests with SiO_2 already completed

28-Day oral limit test → no effects observed
14-day inhalation test → underway ($t_{1/2}$ SiO_2= approx. 14 days; lungs)

Chromosomal aberration test / V79 cells OECD 473

Mouse lymphoma mutation test / L5178Y/$TK^{+/-}$ cells OECD 476

Mouse micronucleus test OECD 474

→ all negative

Main Recommendations for Expanded Endpoint Pattern

- **Dissolution: Analysis in (mimicked) physiological fluids at various pH**
- **Toxicokinetics: a. chemical analysis b. TEM**
- **Immunohistochemistry (8-OH-dG) → oxidative damage on epithelial cells - Other genotoxicity tests**

- **Cytokines and ROI ? Value equivocal**
- ***in vitro/in vivo* tests complement each other, i.e concept of N1 was confirmed**