Research

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Histopathological investigation of specimens from a long term inhalation study (Histopathologische Untersuchung von Proben aus einer Langzeitinhalationsstudie)

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Histopathological investigation of specimens from a long term inhalation study

Abstract

Though the amount of information about acute and subacute toxicity of nanomaterials is constantly growing, data about the long-term effects of nanomaterials is still fragmentary. Studies dealing with the chronic inhalation of nanomaterials were only performed using nano-titanium dioxide or carbon black. These studies showed that high doses lead to inflammation in the lung and tumor induction. Furthermore, DNA damages were detectable when using dosages, which resulted in tumor formation later on. However, the processes which are causative for the tumor induction are still not known. If nanomaterials cause effects when inhaled in low doses, is still not known. Furthermore, the systemic distribution and the excretion of nanomaterials following chronic inhalation is mostly unknown.

To fully reveal relevant human health hazards of nano-CeO_2, putative lung carcinogenicity and putative systemic effects of low-dose life-time inhalation exposure, BASF Experimental Toxicology performed a long-term inhalation study co-financed by and in cooperation with the Federal Ministry for the Environment, Nature Conservation and Nuclear Safety, the German Environment Agency, the Federal Institute for Occupational Safety and Health, the German Federal Institute for Risk Assessment and the EU commission (Project NanoReg, FP 7/2007-2013, grant agreement number 310584). The goal of this study is to draw conclusions about the long-term effects of this selected nanomaterial after inhalation. A specific focus was laid on the investigation of low dose effects, which may gain environmental and work place relevance in case of exposure. Nano-cerium dioxide (CeO_2) was chosen as test material, since CeO_2 has a commercial relevance. It is used as ultraviolet absorber in dyes and plastic, as part of polishing and grinding material for silicon wafers, which are required by the electronical industry for ultra modern chip systems and solar cells, as well as as fuel additive. However, it has only been tested in short-term toxicity studies. The test material, NM212, was sourced – due to the large quantities which were required for the Long-term study - directly from the respective producer. The characterization of the test material NM212 is available (link EU SCIENCE HUB)

This whole-body inhalation study was performed according to OECD test guideline no. 453 with several protocol extensions. Female rats from the strain Han: WIST (100/group) were exposed to nano-CeO_2 (NM212) at four different dosages (0.1; 0.3; 1; 3 mg/m³) for two years and for two years plus a 6-month recovery period. A control group was exposed to clean air. The in-life part of the study was performed at BASF, Ludwigshafen. The German Federal Institute for Risk Assessment investigated the lung burden at different time points. Furthermore, an extended histopathological investigation of the lungs after two years as well as two years plus a 6-month recovery was done at the Fraunhofer Institute for Toxicology and Experimental Medicine ITEM, Hannover, which was funded by the German Environment Agency in another project.

In this project the respiratory tract and all required organs by the OECD test guideline no. 453 were investigated histopathologically from the animals following 12 months inhalation. Furthermore, except the lung all remaining required organs by the OECD test guideline no. 453 from animals after two years and after two years plus a 6-month recovery period were also investigated.

Key words:

Ceriumdioxide, long-term inhalation, nanoparticle, nanotoxicology

Histopathologische Untersuchung von Proben aus einer Langzeitinhalationsstudie

Kurzreferat

Während zur akuten und subakuten Toxizität von Nanomaterialien zunehmend Daten zur Verfügung stehen, gibt es erhebliche Datenlücken zu Langzeiteffekten von Nanomaterialien. In Bezug auf die inhalative Exposition wurden bisher lediglich mit nano-Titandioxid und Industrieruß chronische Studien durchgeführt. Hier wurden bei hoher Belastung Lungenentzündung und Tumorbildung festgestellt. Im tumorerzeugenden Dosisbereich traten darüber hinaus DNS-Schädigungen im Lungengewebe auf. Es ist gegenwärtig strittig, welche Prozesse ursächlich für die nachgewiesenen Tumoren sind. In diesem Zusammenhang steht auch die Frage, ob und welche Wirkungen von Nanomaterialien bei chronischer Exposition im umweltrelevanten Niedrigdosisbereich zu erwarten sind. Zudem sind die systemische Verteilung von Nanomaterialien nach chronischer Inhalation und die Geschwindigkeit der Ausscheidung weitgehend unbekannt und müssen ebenfalls untersucht werden.

Unter der Schirmherrschaft von und mittels Kofinanzierung durch das Bundesministerium für Umwelt, Naturschutz und nukleare Sicherheit wurde deshalb ein Kooperationsprojekt mit der BASF und den Bundesoberbehörden Umweltbundesamt, Bundesanstalt für Arbeitsschutz und Arbeitsmedizin sowie Bundesinstitut für Risikobewertung zur Durchführung und Auswertung einer chronischen Inhalationsstudie mit Nanomaterialien gestartet. Ziel dieser Studie war es, fundierte Aussagen über die Langzeitwirkung ausgewählter Nanomaterialien ableiten zu können. Ein besonderer Fokus lag dabei auf der Untersuchung von Wirkungen im Bereich niedriger Belastungen, die für eine Umwelt-oder Arbeitsplatzexposition am ehesten relevant sind. Als Prüfsubstanz wurde nano-Cerdioxid (CeO_2) festgelegt, da dieses Material kommerziell relevant ist (Verwendung als UV-Absorber in Lacken und Plastik, Polier- und Schleifmittel in der Halbleitertechnik, Kraftstoffadditiv), bisher aber nur in toxikologischen Kurzzeitstudien geprüft wurde. Die Testsubstanz, NM212, wurde – da die Langzeitstudie großer Mengen bedurfte – direkt von dem Hersteller bezogen. Die Charakterisierung der Testsubstanz NM212 ist verfügbar (Link: EU SCIENCE HUB).

Die über 24 Monate angelegte Inhalationsstudie mit zusätzlicher 6-monatiger Recoveryphase wurde mit dem nanoskaligen CeO_2 (NM212) an weiblichen Ratten vom Stamm Han: WIST (100/Gruppe) mit 4 verschiedenen Dosen (0,1; 0,3; 1; 3 mg/m³) nach den Prüfvorgaben der OECD (OECD Richtlinie 453) zur Ermittlung der chronischen Toxizität und eventueller Tumorerzeugung (Kanzerogenität) bei der BASF durchgeführt. Dabei wurde eine weitere Gruppe als Kontrolle Reinluft ausgesetzt. Das Bundesinstitut für Risikobewertung hat die Lungen- und die Organbeladung mit Cerium untersucht. Es erfolgte eine erweiterte histopathologische Untersuchung der Lunge am Fraunhofer Institut für Toxikologie und Experimentelle Medizin ITEM, welche vom Umweltbundesamt innerhalb eines anderen Projektes finanziert wurde.

In diesem Projekt wurden der Respirationstrakt sowie alle weiteren nach der OECD Richtlinie 453 geforderten Organe von den Tieren nach einer 12-monatigen Inhalation histopathologisch untersucht. Weiterhin wurden bis auf die Lunge alle weiteren Organe, die von der OECD Richtlinie 453 gefordert sind, von den Tieren nach 24-monatiger Inhalation bzw. nach 24-monatiger Inhalation mit zusätzlicher 6-monatiger Recoveryphase untersucht.

Schlagwörter:

Ceriumdioxid, Langzeitinhalation, Nanopartikel, Nanotoxikologie

1 Introduction

Though the amount of information about acute and subacute toxicity of nanomaterials is constantly growing, data about the long-term effects of nanomaterials is still fragmentary. Studies dealing with the chronic inhalation of nanomaterials were only performed using nano-titanium dioxide or carbon black. These studies showed that high doses lead to inflammation in the lung and tumor induction. Furthermore, DNA damages were detectable when using dosages, which resulted in tumor formation later on. However, the processes which are causative for the tumor induction are still not known. If nanomaterials cause effects when inhaled in low doses, is also still not known. Furthermore, the systemic distribution and the excretion of nanomaterials following chronic inhalation is mostly unknown.

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In this project the respiratory tract and all required organs by the OECD test guideline no. 453 were investigated histopathologically from the animals following 12 months inhalation. Furthermore, apart from the lung all remaining required organs by the OECD test guideline no. 453 from animals after two years and after two years plus a 6-month recovery period were also investigated.

2 Histopathological investigations after 12-months exposure to CeO_2

2.1 Material and Methods (12 months CeO_2 exposure)

2.1.1 Experimental animals and test group design

The experimental animals of the 12-months CeO_2 exposure at BASF (satellite groups 40-44) were allocated to one control and 4 exposure groups (Table 1).

Table 1: Test groups and animal numbers of the 12-months CeO_2 exposure

Test group	Substance	Concentration (mg/m^3)	No. of animals	Animal No.
40	Air Control	0	10	701 - 710
41	CeO_2	0.1	10	711 - 720
42	CeO_2	0.3	10	721 - 730
43	CeO_2	1	10	731 - 740
44	CeO_2	3	10	741 - 750

2.1.2 Organ/tissue fixation, sample shipment and histotechnical processing

Following sacrifice of the animals after 12 months of CeO_2 exposure at BASF, all organs and tissues were fixed and stored in 4% buffered formaldehyde. The lungs were transferred to 70% ethanol following a 24-48h fixation time in formaldehyde. The wet tissues - together with the individual macroscopic findings - were shipped to Fraunhofer Institute for Toxicology and Experimental Medicine (Fraunhofer ITEM) in 2 batches (batch 1: trachea, tracheobronchial and mediastinal lymph nodes, lungs, aorta and esophagus; batch 2: all other extra-pulmonary organs). Histotechnical processing was performed at Fraunhofer ITEM. All organs/tissues according to Table 2 were trimmed according to Ruehl-Fehlert et al. (2003), Kittel et al. (2004), Morawietz et al. (2004) as well as according to SOPs of Fraunhofer ITEM, dehydrated, embedded in paraffin wax and sectioned at a nominal thickness of 3-4 µm. Bones were decalcified prior to trimming. All sections were stained with hematoxylin and eosin (H&E). An additional section of the left lung lobe from all animals was stained with Masson trichrome for assessment of fibrotic changes.

2.1.3 Examination by light microscopy and assessment of findings

Light microscopical examination of all hematoxylin-eosin stained slides and a correlation between gross lesions and histopathological findings was performed by the undersigned Fraunhofer ITEM pathologist (Principal Investigator). All gross lesions were recorded and tabulated at BASF. All macroscopic findings were entered into the ITEM pathology software system (Provantis) for correlation of macro/micro findings.

Histologic alterations were described, wherever possible, according to their distribution (focal, multifocal, diffuse), severity (grades) and morphologic character.

Table 2: Organs/tissues of animals sacrificed after 12 months of CeO_2 exposure

Organs	**Test group**	**Test group**	**Test group**	**Test group**	**Test group**
	40	**41**	**42**	**43**	**44**
1. All gross lesions	A2	A2	A2	A2	A2
2. Adrenal glands	A1				A1
3. Aorta	A1				A1
4. Bone marrow (femur)	A1				A1
5. Brain	A1				A1
6. Cecum	A1				A1
7. Cervix	A1				A1
8. Colon	A1				A1
9. Duodenum	A1				A1
10. Esophagus	A1				A1
11. Eyes + optic nerve	A1				A1
12. Extraorbital lacrimal glands	A1				A1
13. Femur + knee joint	A1				A1
14. Harderian glands	A1				A1
15. Heart	A1				A1
16. Ileum	A1				A1
17. Jejunum	A1				A1
18. Kidneys	A1				A1
19. Larynx (3 levels)	A1	A1	A1	A1	A1
20. Liver	A1				A1
21. Lungs	A1/T1	A1/T1	A1/T1	A1/T1	A1/T1
22. Lymph nodes (tracheo-bronchial, mediastinal)	A1	A1	A1	A1	A1
23. Lymph nodes (axillary, mesenter.)	A1				A1
24. Mammary gland	A1				A1
25. Nasal cavity (4 levels)	A1	A1	A1	A1	A1
26. Ovaries	A1				A1

Organs	Test group	Test group	Test group	Test group	Test group
	40	41	42	43	44
27. Oviducts	A1				A1
28. Pancreas	A1				A1
29. Parathyroid glands	A1				A1
30. Pharynx	A1				A1
31. Pituitary gland	A1				A1
32. Rectum	A1				A1
33. Salivary glands (parotid, mandibular, sublingual)	A1				A1
34. Sciatic nerve	A1				A1
35. Spinal cord (cervical, thoracic, lumbar)	A1				A1
36. Spleen	A1				A1
37. Stomach (forestomach, glandular stomach)	A1				A1
38. Sternum with marrow	A1				A1
39. Skeletal muscle	A1				A1
40. Skin	A1				A1
41. Teeth	A1				A1
42. Thymus	A1				A1
43. Thyroid glands	A1				A1
44. Tongue	A1				A1
45. Trachea	A1	A1	A1	A1	A1
46. Ureter	A1				A1
47. Urethra	A1				A1
48. Urinary bladder	A1				A1
49. Uterus	A1				A1
50. Vagina	A1				A1

Methods (scope of examinations):

A= Hematoxylin-Eosin stain

T= Masson-Trichrome stain

1= All animals per test group

2= All animals affected per test group

2.1.4 Grades used at histopathology finding level

The grades were used for a grading system that takes into consideration either the severity or the number or the size of a microscopic finding (Table 3). The severity of each lesion was graded on a scale of very slight to very severe, indicating the approximate fraction of the organ/tissue or organ structure to be involved.

Table 3: Grading system

Grade	Severity	Percentage	Number	Size
1	Very slight (Minimal)	= 1-5%	Very few	Very small
2	Slight (Mild)	= 6-20%	Few	Small
3	Moderate	= 21-50%	Moderate number	Moderate size
4	Severe (Marked)	= 51-74%	Many	Large
5	Very Severe (Massive)	= 75-100%	Extensive number	Extensive size

2.1.5 Data compilation

Macroscopic data were recorded together with the microscopic findings by the undersigned Fraunhofer ITEM pathologist using on-line input into the Provantis computer system (version 8.3; INSTEM Life Science Systems, UK). All macroscopic and microscopic observations are presented for each rat in the 'Individual Animal Reports' (Appendix x.3). The incidences of macroscopic and microscopic findings are also presented in tabular form (Appendices x.2 – x.3). Incidence tables were created by the Provantis computer system.

2.1.6 Statistics of histopathology

The statistical analysis was performed with the Provantis system for each sex using a Chi-squared and 2-sided Fisher's Exact test. The significance of difference between the control and treatment groups is marked in the tables.

2.1.7 Peer review of histopathological findings

Following the initial examination by the Principal Investigator, an internal peer review of all target organs, all neoplastic and pre-neoplastic lesions as well as of 10% randomly selected animals (animals 703, 716, 724, 733, 745) from all test groups was performed according to Fraunhofer ITEM SOP 050708 by PD Dr. Susanne Rittinghausen (Fraunhofer ITEM, Hannover, Germany). Lungs, mediastinal and tracheobronchial lymph nodes of all animals were reviewed because they were indicated as target organs. Nasal cavities and larynges of all animals of the control group and 3 mg/m^3 CeO_2 group were reviewed because exposure-related findings have been observed in some animals. The results of the peer review are documented in the finding tables and will be archived as raw data.

Results presented in this report reflect the consensus opinion of the study pathologist and the peer review pathologist.

2.1.8 Archivation

All wet tissues, slides, blocks and data sheets containing the macroscopic findings will be sent back to BASF and will be archived for at least the period of time specified in the GLP principles.

The signed final pathology phase report and the signed individual animal reports (raw data) will be sent to BASF. Copies of the study plan, the final pathology phase report, the histopathology incidence tables and the individual animal reports on macroscopic and microscopic findings are maintained in the histology archive of Fraunhofer ITEM.

2.2 Results (12 months CeO_2 exposure)

2.2.1 Gross lesions

The **tracheobronchial** and **mediastinal lymphnodes** of all 10 animals of test group 44 revealed a white-beige to white-yellow discoloration and were moderately enlarged. The same findings were observed in 9 (discoloration) and 3 animals (enlarged) respectively, of test group 43. Few animals of test groups 41, 43, 44 showed a single focus in the **lungs**.

All other findings were single observations or equally distributed over the test groups. They were considered to be incidental or spontaneous in origin and without any relation to treatment.

2.2.2 Histopathology of the respiratory tract

Exposure-related microscopic changes were observed in the nasal cavity, larynx, lungs, tracheobronchial and mediastinal lymph nodes.

2.2.2.1 Nasal cavity

The presence of (multi)focal intracytoplasmic **eosinophilic globules** within the olfactory epithelium was increased in incidence and grade in the 3 mg/m^3 CeO_2 exposure group (test group 44: 9/10; 5/10 very slight, 3/10 slight, 1/10 moderate) as compared to the 1 mg/m^3 CeO_2 exposure group (test group 43: 4/10; 3/10 very slight, 1/10 slight), the 0.3 mg/m^3 CeO_2 exposure group (test group 42: 2/10; 1/10 very slight, 1/10 slight), the 0.1 mg/m^3 CeO_2 exposure group (test group 41: 3/10; 2/10 very slight, 1/10 slight) and the clean air control (test group 40: 6/10; 5/10 very slight, 1/10 slight). A similar trend was observed for eosinophilic globules in the respiratory epithelium. The incidence in test group 40 was 5/10 (all very slight), in test group 41 3/10 (all very slight), in test group 42 1/10 (very slight) and in test group 43 3/10 (all very slight) whereas 9/10 (7/10 very slight, 2/10 slight) females in test group 44 were affected. Although the difference between the control and CeO_2 high-dose test group was statistically not significant, the increase in incidence and severity of this change in both types of epithelium is considered to be exposure-related. The same is true for (multi)focal very slight subepithelial **(mixed) inflammatory cell infiltration** which occurred in 3/10, 3/10, 2/10, 4/10 and 7/10 females of test group 40, 41, 42, 43 and 44, respectively.

Further exposure-related findings such as (multi)focal very slight **accumulation of particle-laden macrophages within the NALT** (nasal mucosa-associated lymphoid tissue) were diagnosed in 1/10, 0/10, 4/10 and all (10/10) animals of test group 41, 42, 43 and 44, respectively. Moreover, multifocal very slight amounts of **intraepithelial (intracytoplasmic) particles** were observed in all animals of test group 44. Occasional particles were seen not only in the respiratory and olfactory epithelium, but also in epithelial cells of the submucosal glands (Bowman's glands).

Incidental findings in the nasal cavity which were considered to be unrelated to particle exposure included dilatation of submucosal glands, mucous cell hyperplasia, subepithelial mononuclear cell infiltration and subepithelial mineralization and were seen in up to 3/10 animals in all test groups.

2.2.2.2 Larynx

In 4/10 animals of the 3 mg/m^3 CeO_2 exposure test group, (multi)focal subepithelial **accumulation of particle-laden macrophages** (3/10 very slight, 1/10 slight) was observed as exposure-related finding.

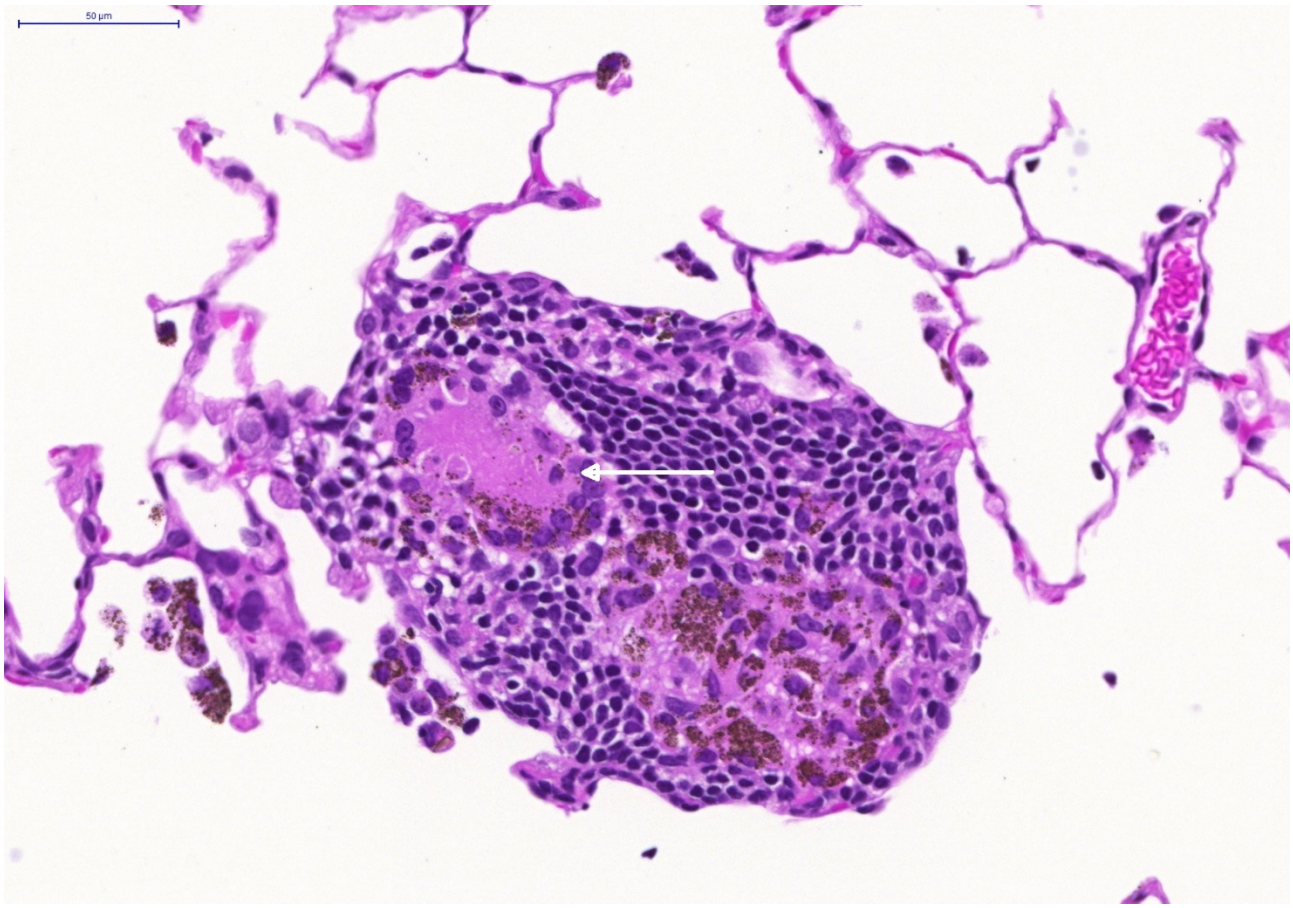
Spontaneous findings included very slight to slight subepithelial mononuclear cell infiltration in up to 5/10 animals as well as very slight to slight dilatation of submucosal glands in 2/10 females of test group 41, 42 and 44.

2.2.2.3 Lungs

CeO_2 exposure-related pulmonary findings (figure 4) included accumulation of particle-laden macrophages and giant cells, cell-free intra-alveolar agglomerations of CeO_2 particles and bronchiolo-alveolar hyperplasia of the bronchiolar type (alveolar bronchiolization).

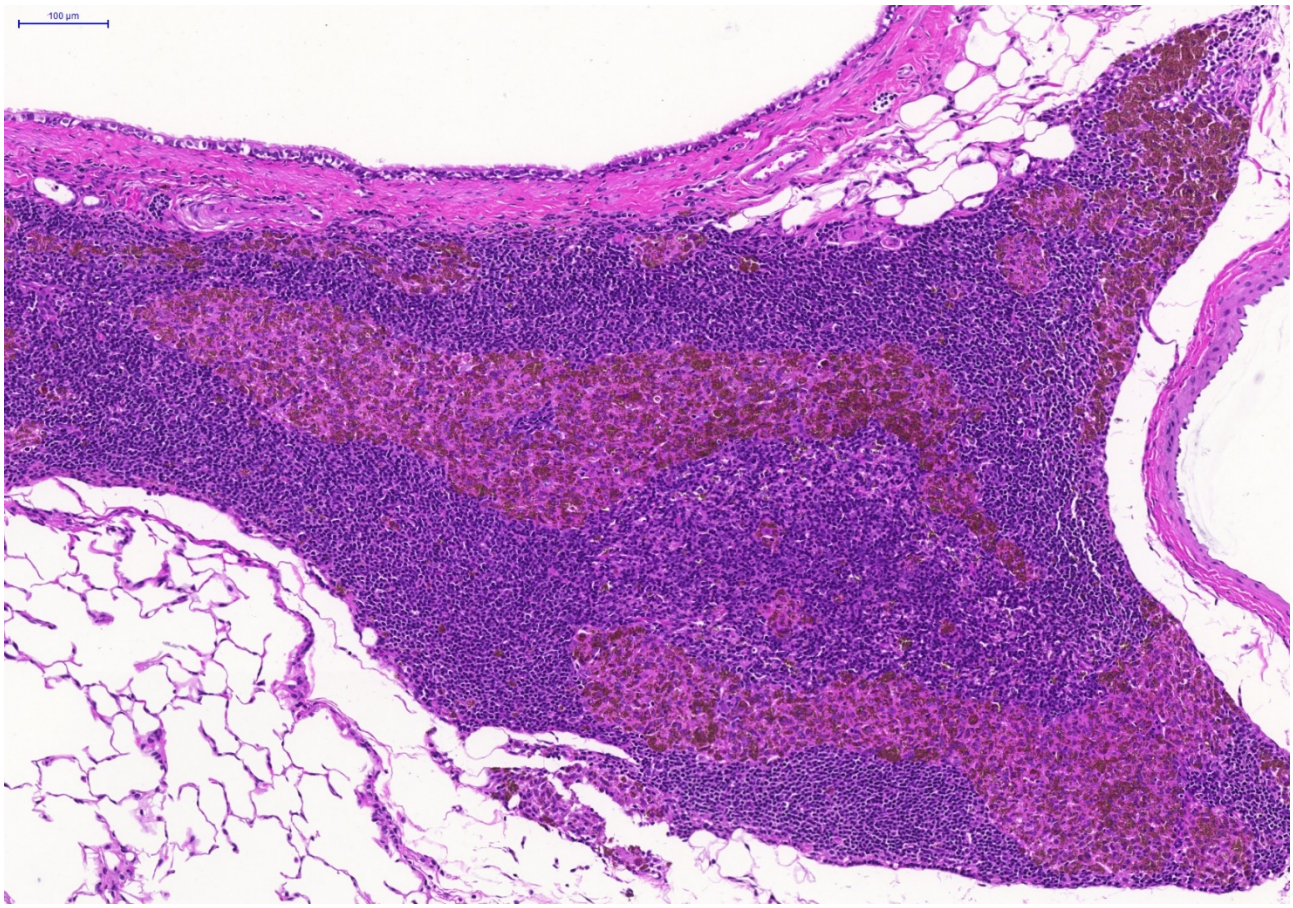
(Multi)focal **alveolar/interstitial accumulation of particle-laden macrophages** was observed dose-dependently in 10/10 females each of the 0.1 mg/m^3 (test group 41: 8/10 very slight, 2/10 slight), 0.3 mg/m^3 (test group 42: 8/10 very slight, 2/10 slight), 1 mg/m^3 (test group 43: 1/10 very slight, 9/10 slight) and 3 mg/m^3 (test group 44: 7/10 slight, 3/10 moderate) CeO_2 exposure test groups. Deposits of particle-laden macrophages were present not only in alveoli but also in interstitial (intraseptal, peribronchiolar and perivascular) compartments. In addition, agglomerates of CeO_2 particles were lying freely within alveoli at very slight to slight degrees in 3/10 animals of test group 41 and in 10/10 females each of exposure test groups 42-44. (Multi)focal aggregates of particle-laden macrophages were also observed dose-dependently within the bronchus-associated lymphoid tissue (BALT) at incidences of 10/10 each in test groups 41 (all very slight), 42 (8/10 very slight, 2/10 slight), 43 (8/10 slight, 2/10 moderate) and 44 (1/10 slight, 7/10 moderate, 2/10 severe). **Syncytial giant cells** - mainly particle-laden - were present in the BALT of 3/10, 9/10 and 10/10 females of test groups 42, 43 and 44, respectively. The amount of the intracellular particle-load in both single-nucleated macrophages and multinucleated giant cells corresponded well to the used CeO_2 exposure dose.

Figure 1: Granulomatous inflammation in a high dose cerium dioxide exposure group animal



Hematoxylin and eosin stained tissue slide of the lung of a rat exposed for 12 months with 3.0 mg/m^3 CeO_2 showing the granulomatous inflammation with particle-laden macrophages. Arrow depicts a particle-laden multinucleated syncytial giant cell.

Figure 2: Bronchus-associated lymphoid tissue of a high dose cerium dioxide exposure group animal



Hematoxylin and eosin stained tissue slide of the lung of a rat exposed for 12 months with 3.0 mg/m^3 CeO_2 showing particle-laden macrophages in the bronchus-associated lymphoid tissue.

(Multi)focal **bronchiolo-alveolar hyperplasia of the bronchiolar type** (Synonym: **alveolar bronchiolization**) was observed in a single animal of test group 41 (very slight) and in 2/10 (all very slight), 10/10 (all very slight) and 10/10 (9/10 very slight, 1/10 slight) females of test groups 42, 43 and 44, respectively.

Besides these reactive/adaptive (= non-adverse) pulmonary findings, several adverse changes were also diagnosed (figure 4). These included alveolar/interstitial (mixed) inflammatory cell infiltration, alveolar/interstitial granulomatous inflammation, interstitial fibrosis, alveolar lipoproteinosis and cholesterol granuloma(s). Except alveolar lipoproteinosis and cholesterol granuloma(s), all changes were seen at dose-dependent incidences and severity grades in all CeO_2 exposure test groups.

(Multi)focal **alveolar/interstitial (mixed) inflammatory cell infiltration** occurred in a single control animal (very slight) as a spontaneous finding, in 4/10 females of test group 41 (all very slight) and in 10/10 animals each of exposure test groups 42 (9/10 very slight, 1/10 slight), 43 (7/10 very slight, 3/10 slight) and 44 (4/10 very slight, 6/10 slight). In test groups 42-44, the difference to the control was statistically significant.

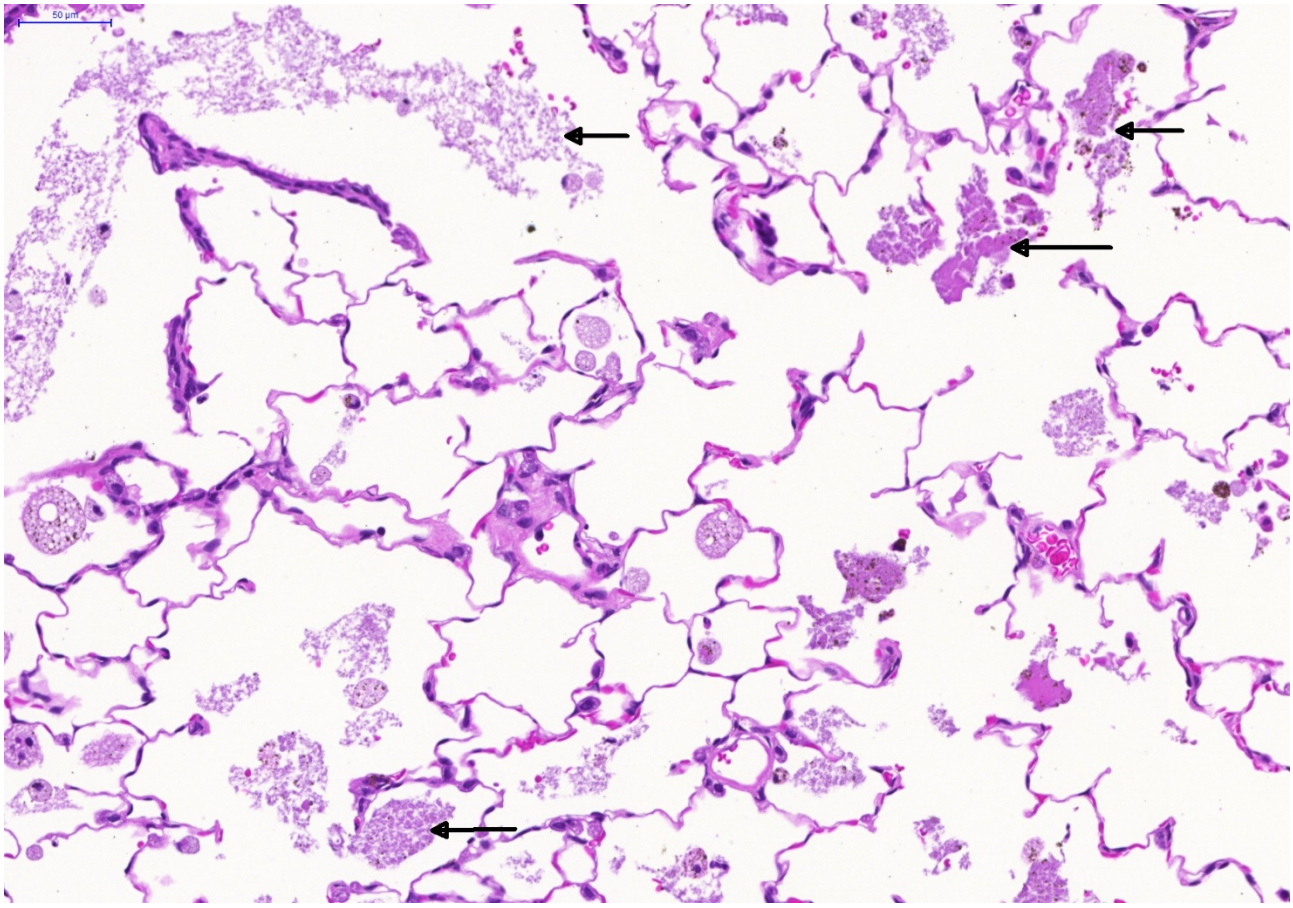
Probably as correlate to the macroscopic finding ‘focus in the lung’, multifocal **alveolar/interstitial granulomatous inflammation** was observed in 1/10 females of test group 41 (very slight), in 3/10 females of test group 42 (all very slight) and at significantly increased incidences in 10/10 animals

each of test groups 43 (7/10 very slight, 3/10 slight) and 44 (4/10 very slight, 6/10 slight). The term ‘granulomatous inflammation’ was used only, if (mixed) inflammatory cell infiltration, syncytial giant cells and interstitial fibrosis were present in conjunction to form a granuloma-like focal lesion.

(Multi)focal very slight **interstitial fibrosis** (mainly intraseptal) was diagnosed in 3/10, 4/10, 10/10 and 10/10 females of test groups 41, 42, 43 and 44, respectively. For test groups 43 and 44, the difference to control group 40 was statistically significant.

Multifocal **alveolar lipoproteinosis** was seen exclusively in 4/10 animals of the 3 mg/m^3 CeO_2 test group 44 (2/10 very slight, 1/10 slight, 1/10 severe). The intra-alveolar lipoproteinaceous material was mainly granular, eosinophilic and mixed with particle agglomerations reflecting basically an origin from degenerating particle-laden macrophages. A similar pathogenesis can be assumed for development of focal **cholesterol granuloma(s)** occurring in a single female each of test groups 43 (very slight) and 44 (slight).

Figure 3: Lung with lipoproteinosis of a high dose cerium dioxide exposure group animal



Hematoxylin and eosin stained tissue slide of the lung of a rat exposed for 12 months with 3.0 mg/m^3 CeO_2 showing the alveolar lipoproteinosis (arrows).

Incidental pulmonary findings occurring in single animals of different exposure groups as well as in control group 40 consisted of focal very slight osseous metaplasia, focal very slight neuroendocrine cell hyperplasia and focal very slight hair granuloma. In addition, 4/10 control animals revealed focal very slight alveolar macrophage aggregation. All these findings were considered to be unrelated to particle exposure.

After 12 month of inhalation exposure neither neoplastic nor pre-neoplastic findings were seen in the lungs of CeO_2 exposed animals.

2.2.2.4 Tracheobronchial and mediastinal lymph nodes

As correlate to the macroscopic findings ‘enlargement’ and ‘discoloration’, the lymph nodes at both sites showed a dose-dependent (multi)focal very slight to severe **accumulation of particle-laden macrophages**. Regarding the tracheobronchial lymph node, the incidences were 8/8 (all very slight) in test group 41, 9/9 (1/9 very slight, 7/9 slight, 1/9 moderate) in test group 42 and 10/10 each in test group 43 (2/10 slight, 8/10 moderate) and 44 (5/10 moderate, 5/10 severe). In addition, particle-laden **syncytial (multinucleated) giant cells** were present in the tracheobronchial lymph node of 1/8, 6/9, 10/10 and 10/10 females of test groups 41, 42, 43 and 44, respectively.

The incidences of (multi)focal accumulation of particle-laden macrophages in the mediastinal lymph nodes were 6/10 (all very slight) in test group 41, 10/10 (all very slight) in test group 42, 9/9 (3/9 slight, 6/9 moderate) in test group 43 and 10/10 (5/10 moderate, 5/10 severe) in test group 44, while syncytial giant cells were only observed in 9/9 and 10/10 females of test groups 43 and 44, respectively.

2.2.2.5 Histopathology of the remaining organs of the respiratory tact

Within the remaining organs of the respiratory tract such as trachea and nasopharynx no lesions were detected in any investigated group.

2.2.3 Histopathology of the other organs

Several sporadic neoplastic and non-neoplastic findings were observed in the other organs examined histopathologically. These occurred either incidentally or were similar in distribution pattern and severity in control rats compared to the CeO_2 high-dose test group. Sporadic findings in the other CeO_2 exposure groups were recorded only as correlates of macroscopic findings. All of the observed findings were considered to be without any relation to CeO_2 exposure.

A total number of 6 neoplasms were observed: an adenoma of the pars distalis in the pituitary gland of single females each of group 40, 41 and 42, a sebaceous adenoma and a lipoma of the skin/subcutaneous tissue in single animals of test group 42, and an endometrial stromal polyp of the uterus in a female control animal.

Findings such as epithelial degeneration (incidence up to 6/10 rats per test group) and interstitial inflammation (incidence up to 7/10 rats per test group) of the Harderian glands are most likely considered to be related to the blood sampling procedure. Further common spontaneous findings included (multi)focal very slight intratubular mineralization of the kidneys (incidence up to 8/10 rats per test group), (multi)focal very slight mononuclear cell infiltration of the liver (incidence up to 7/10 rats per test group), chondromucinous degeneration of sternebral cartilage (incidence up to 7/10 rats per test group), epithelial hyperplasia (incl. hyperplasia of the type ‘epithelial tubules and cords’) at incidences of up to 8/10 rats per test group in the thymus and acinar cell hypertrophy of the salivary glands (incidence up to 4/10 rats per test group). Estrous cycle-depending luminal dilatation of the uterus, C-cell hyperplasia of the thyroids, and parasites (nematodes) in the rectum, colon and/or cecum were observed in up to 3/10 animals per test group.

In addition, various other incidental findings occurred in single or in up to 2/10 rats per test group.

2.2.4 Summary and Conclusion (12 months CeO_2 exposure)

CeO_2 exposure-related findings were exclusively observed in the respiratory tract and included reactive/adaptive changes such as accumulation of particle-laden macrophages in the nasal cavity, larynx, lungs, tracheobronchial and mediastinal lymph nodes. In the nasal cavity, the incidence of age-related intra-epithelial eosinophilic globules was increased in the 3 mg/m^3 high-dose CeO_2 exposure group as compared to the control group and associated with minimal inflammatory cell infiltration. The adverse and non-adverse histopathological findings observed in the lungs are summarized in figure 4 and figure 5.

Non-adverse findings consisted of accumulation of particle-laden macrophages in the alveolar/interstitial areas and in the BALT as well as particle-laden syncytial giant cells in the BALT. In addition, bronchiolo-alveolar hyperplasia of the bronchiolar type graded no more than “very slight” (grade 1) or “slight” (grade 2) was considered as a non-adverse finding.

Adverse effects in the lungs included dose-dependent alveolar/interstitial inflammatory cell infiltration, alveolar/interstitial granulomatous inflammation and interstitial fibrosis. Alveolar lipoproteinosis was observed in the 3 mg/m³ high-dose CeO_2 exposure group only and cholesterol granulomas occurred in a single female each of the 1 and 3 mg/m³ CeO_2 exposure groups.

After 12 month of inhalation exposure neither neoplastic nor pre-neoplastic treatment-related findings were seen in the lungs of CeO_2-exposed animals.

Figure 4: Summary of incidences of lung changes related to CeO_2 exposure

Lung / No. of animals (♀)	Clean Air 10	CeO_2 0.1 mg/m³ 10	CeO_2 0.3 mg/m³ 10	CeO_2 1 mg/m³ 10	CeO_2 3 mg/m³ 10	
Accumulation, Particle-Laden Macrophages, Alveolar/Interst., *grade 1–3*	0	10*	10*	10*	10*	non-adverse lesion
Accumulation, Particle-Laden Macrophages, BALT, *grade 1–4*	0	10*	10*	10*	10*	
Giant Cells, Syncytial, BALT, *present, no grade*	0	0	3	9*	10*	
Hyperplasia, Bronchiolo-Alveolar; Bronchiolar type, *grade 1–2*	0	1	2	10*	10*	
Infiltration, Inflammatory Cell, Alveolar/Interstitial, *grade 1–2*	1	4	10*	10*	10*	adverse lesion
Inflammation, Granulomatous, Alveolar/Interstitial, *grade 1–2*	0	1	3	10*	10*	
Fibrosis, Interstitial, *grade 1*	0	3	4	10*	10*	
Lipoproteinosis, Alveolar, *grade 1–4*	0	0	0	0	4	
Granuloma, Cholesterol, *grade 1–2*	0	0	0	1	1	

* = p < 0.001, Chi-Quadrat/Fisher-Test, two-sided

Figure 5: Summary of grade of lesions of lung changes related to CeO_2 exposure

Lung / 10 animals per group (♀)	Clean Air	CeO_2 0.1 mg/m³	CeO_2 0.3 mg/m³	CeO_2 1 mg/m³	CeO_2 3 mg/m³
Accumulation, Particle-Laden Macrophages, Alveolar/Interstitial	0	1.2★	1.2★	1.7★	2.9★
Accumulation, Particle-Laden Macrophages, BALT	0	1★	1.2★	2.2★	3★
Giant Cells, Syncytial, BALT	0	0	0.3	0.9★	1★
Hyperplasia, Bronchiolo-Alveolar; Bronchiolar type	0	0.1	0.2	1★	1.1★
Infiltration, Inflammatory Cell, Alveolar/Interstitial	0.1	0.4	1.1★	1.3★	1.6★
Inflammation, Granulomatous, Alveolar/Interstitial	0	0.1	0.3	1.3★	1.6★
Fibrosis, Interstitial	0	0.3	0.4	1★	1★
Lipoproteinosis, Alveolar	0	0	0	0	0.8
Granuloma, Cholesterol	0	0	0	0.1	0.2

* = p < 0.001, Chi²-test/Fisher-test, two-sided

Although statistically not significant, some adverse effects such as alveolar/interstitial inflammatory cell infiltration, alveolar/interstitial granulomatous inflammation, and interstitial fibrosis have already been observed in the 0.1 mg/m³ low-dose CeO_2 exposure group. Thus, a NOAEL (no observed adverse effect level) could not be established for the lungs after 12 months of exposure to the present CeO_2 nanoparticle concentrations.

3 Histopathological investigations after 24-months exposure to CeO_2

3.1 Material and methods (carcinogenicity study, 24 months and 30 months)

3.1.1 Experimental animals and test group design

The main group animals of the 24 months CeO_2 exposure at BASF were allocated to one control and 4 exposure groups (table 4).

Table 4: Main group animals (carcinogenicity, 24 months)

Test group	Substance	Concentration (mg/m³)	No. of animals	Animal No.
00	Air Control	0	50	1 - 50
02	CeO_2	0.3	50	201 - 250
04	CeO_2	3	50	401 - 450

The post-exposure group animals which had a recovery period of 6 months following 24 months CeO_2 exposure at BASF were also allocated to one control and 4 exposure groups (table 5).

Table 5: Post-exposure group animals (carcinogenicity, 30 months)

Test group	Substance	Concentration (mg/m³)	No. of animals	Animal No.
50	Air Control	0	49	51 – 99
52	CeO_2	0.3	49	251 – 299
54	CeO_2	3	50	451 - 500

3.1.2 Organ/tissue fixation, sample shipment and histotechnical processing

Following sacrifice of the animals after 24 months of CeO_2 exposure at BASF or after the post-exposure period at 30 months, all organs and tissues were fixed and stored in 4% buffered formaldehyde. All wet tissues from groups 00-04 and 50-54 together with the individual macroscopic findings were shipped to the Fraunhofer Institute for Toxicology and Experimental Medicine ITEM for histotechnical processing. Histotechnical processing of the organs included trimming according to Ruehl-Fehlert et al. (2003), Kittel et al. (2004), Morawietz et al. (2004).

The investigation of all organs but the lung, was the purpose of the research project described here, whereas the investigation of the lung was subject to a separate research project.

Table 6: Organs/tissues of animals sacrificed after 24 months of exposure and after 30 months

Organs	**Test group**	**Test group**	**Test group**	**Test group**	**Test group**
	00 50	**01 51**	**02 52**	**03 53**	**04 54**
1. All gross lesions[1)]	A2	A2	A2	A2	A2
2. Adrenal glands	A1	A1	A1	A1	A1
3. Aorta	A1	A1	A1	A1	A1
4. Bone marrow (femur)	A1	A1	A1	A1	A1
5. Brain	A1	A1	A1	A1	A1
6. Cecum	A1	A1	A1	A1	A1
7. Cervix	A1	A1	A1	A1	A1
8. Colon	A1	A1	A1	A1	A1
9. Duodenum	A1	A1	A1	A1	A1
10. Esophagus	A1	A1	A1	A1	A1
11. Eyes and optic nerve	A1	A1	A1	A1	A1
12. Extraorbital lacrimal glands	A1	A1	A1	A1	A1
13. Femur and knee joint	A1	A1	A1	A1	A1
14. Harderian glands	A1	A1	A1	A1	A1
15. Heart	A1	A1	A1	A1	A1
16. Ileum	A1	A1	A1	A1	A1
17. Jejunum	A1	A1	A1	A1	A1
18. Kidneys	A1	A1	A1	A1	A1
19. Larynx (3 levels)	A1	A1	A1	A1	A1
20. Liver	A1	A1	A1	A1	A1
21. Lungs	T1	T1	T1	T1	T1
22. Lymph nodes (tracheobron-chial, mediastinal)	A1	A1	A1	A1	A1
23. Lymph nodes (axillary, mes-enteric)	A1	A1	A1	A1	A1
24. Mammary gland (females)	A1	A1	A1	A1	A1
25. Nasal cavity (4 levels)	A1	A1	A1	A1	A1
26. Olfactory bulb	A1	A1	A1	A1	
27. Ovaries	A1	A1	A1	A1	A1

Organs	Test group	Test group	Test group	Test group	Test group
	00 50	01 51	02 52	03 53	04 54
28. Oviducts	A1	A1	A1	A1	A1
29. Pancreas	A1	A1	A1	A1	A1
30. Parathyroid glands	A1	A1	A1	A1	A1
31. Pharynx	A1	A1	A1	A1	A1
32. Pituitary gland	A1	A1	A1	A1	A1
33. Rectum	A1	A1	A1	A1	A1
34. Salivary glands (parotid, mandibular, sublingual)	A1	A1	A1	A1	A1
35. Sciatic nerve	A1	A1	A1	A1	A1
36. Spinal cord (cervical, thoracic, lumbar)	A1	A1	A1	A1	A1
37. Spleen	A1	A1	A1	A1	A1
38. Stomach (forestomach, glandular stomach)	A1	A1	A1	A1	A1
39. Sternum with bone marrow	A1	A1	A1	A1	A1
40. Skeletal muscle	A1	A1	A1	A1	A1
41. Skin	A1	A1	A1	A1	A1
42. Teeth	A1	A1	A1	A1	A1
43. Thymus	A1	A1	A1	A1	A1
44. Thyroid glands	A1	A1	A1	A1	A1
45. Tongue	A1	A1	A1	A1	A1
46. Trachea	A1	A1	A1	A1	A1
47. Ureter	A1	A1	A1	A1	A1
48. Urethra	A1	A1	A1	A1	A1
49. Urinary bladder	A1	A1	A1	A1	A1
50. Uterus	A1	A1	A1	A1	A1
51. Vagina	A1	A1	A1	A1	A1

[1)] Gross lesions, which were detected during the necropsy of the animals were processed separately

Methods (scope of examinations):

A= Hematoxylin-Eosin stain

T= Lungs were investigated in another research project

1= All animals per test group

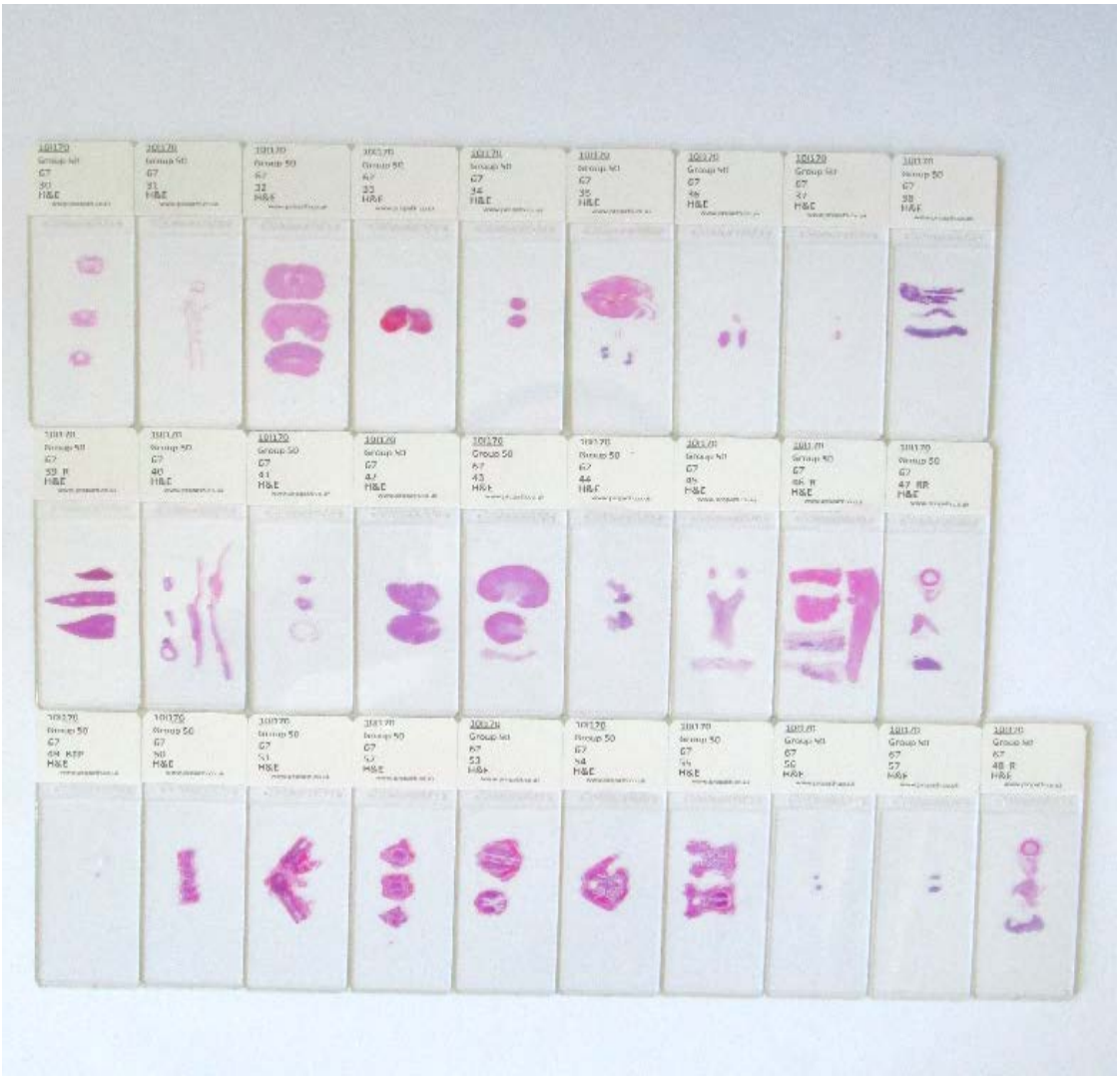
2= All animals affected per test group

3.1.3 Examination by light microscopy and assessment of findings

Overall more than 56 organs were investigated per animal summing up to more than 27720 investigated organs in this project. Light microscopical examination of all hematoxylin-eosin stained slides and a correlation between gross lesions and histopathological findings was performed by the undersigned Fraunhofer ITEM pathologist (Principal Investigator, PI). All gross lesions were recorded and tabulated at BASF. All macroscopic and microscopic findings were entered into the Fraunhofer ITEM pathology software system (Provantis). Macroscopic findings were correlated to microscopic changes, whenever possible.

Histologic alterations were described, wherever possible, according to their distribution (focal, multifocal, diffuse), severity (grades) and morphologic character.

Figure 6: Example of tissue slides of one animal



3.1.4 Grades and nomenclature used for histopathology

The grades were used for a grading system that takes into consideration either the severity or the number or the size of a microscopic finding (Table 7). The severity of each lesion was graded on a scale of very slight to very severe, indicating the approximate fraction of the organ/tissue or organ structure to be involved.

The nomenclature used was according to INHAND [International Harmonization of Nomenclature and Diagnostic Terms].

Table 7: Grading system of histopathology

Grade	Severity	Percentage	Number	Size
Grade 1	Very slight (Minimal)	= 1-5%	Very few	Very small
Grade 2	Slight (Mild)	= 6-20%	Few	Small
Grade 3	Moderate	= 21-50%	Moderate number	Moderate size
Grade 4	Severe (Marked)	= 51-74%	Many	Large
Grade 5	Very Severe (Massive)	= 75-100%	Extensive number	Extensive size

3.1.5 Statistics of histopathology

The statistical analysis was performed with the Provantis system using a Chi-squared and 2-sided Fisher's Exact test.

3.2 Peer review of histopathological findings

Following the initial examination by the Principal Investigator, an internal pathology peer review was performed according to Fraunhofer ITEM SOP 050708 by PD Dr. Susanne Rittinghausen (Fraunhofer ITEM, Hannover, Germany) including all neoplastic and pre-neoplastic lesions of animals from test groups 00-04 and 50-54. In addition, a pathology working group was installed thereafter consisting of three internationally recognized experts in this research area to peer review lesions in the respiratory tract.

Results presented in this report reflect the consensus opinion of the study pathologist, the peer review pathologist and the pathology working group.

3.3 Archiving

All wet tissues, slides, blocks and data sheets containing the macroscopic findings will be sent back to BASF and will be archived for at least the period of time specified in the GLP principles. The signed final pathology phase report and the signed individual animal reports (raw data) will also be sent to BASF. Copies of the study plan, the final pathology phase report, the histopathology incidence tables and the individual animal reports on macroscopic and microscopic findings are maintained in the histology archive of Fraunhofer ITEM.

3.4 Results of the CeO_2 exposure groups (carcinogenicity, 24 months and 30 months)

Exposure-related microscopic changes were observed only in the respiratory tract specifically in the nasal cavity, larynx, trachea, tracheobronchial and mediastinal lymph nodes. The lung showed exposure-related findings as well. These results were reported within another research project.

Within the remaining organs investigated according OECD test guideline 453 no exposure-related findings and no increased neoplastic or pre-neoplastic lesions compared to the clean air control were detected.

3.4.1 General tumor incidences (carcinogenicity, 24 months and 30 months)

Overall within 88.8 %, 91.0%, 90.9%, 90.9% and 84.0% of the animals investigated of the clean air control group, 0.1 mg/m³ CeO_2 exposure group, 0.3 mg/m³ CeO_2 exposure group, 1 mg/m³ CeO_2 exposure group and 3 mg/m³ CeO_2 exposure group, respectively, tumors were detected (see table 8). The percentage of animals with benign tumors were 76.7, 81.7, 79.7, 82.8 and 77.0 for the clean air control group, 0.1 mg/m³ CeO_2 exposure group, 0.3 mg/m³ CeO_2 exposure group, 1 mg/m³ CeO_2 exposure group and 3 mg/m³ CeO_2 exposure group, respectively. The percentage of animals with malignant tumors were 31.3, 32.0, 36.3, 31.3 and 27.0 for the clean air control group, 0.1 mg/m³ CeO_2 exposure group, 0.3 mg/m³ CeO_2 exposure group, 1 mg/m³ CeO_2 exposure group and 3 mg/m³ CeO_2 exposure group, respectively. The organs with the highest tumor incidences were pituitary gland, mammary gland, uterus and liver. All the detected tumors represent commonly found tumors within this rat strain and were all interpreted to be unrelated to the exposure with CeO_2.

Table 8: General tumor incidences

Treatment	Clean Air	CeO_2 0.1 mg/m³	CeO_2 0.3 mg/m³	CeO_2 1 mg/m³	CeO_2 3 mg/m³
Animals examined	99	98	99	98	100
Tumor bearing animals	87.8%	91.0%	91.0%	89.8%	84.0%
Animals with malignant tumors	28.2%	32.0%	34.3%	31.3%	26.0%
Animals with benign tumors	76.7%	81.7%	79.7%	81.8%	77.0%

3.4.2 Histopathology of the respiratory tract (carcinogenicity, 24 months)

Exposure-related microscopic changes were observed in the nasal cavity, larynx, trachea, tracheobronchial and mediastinal lymph nodes.

3.4.2.1 Nasal cavity

Exposure-related findings such as (multi)focal very slight **accumulation of particle-laden macrophages within the NALT** (nasal mucosa-associated lymphoid tissue) were diagnosed in 4/50, 5/50, 16/50 and 31/50 animals of test group 01, 02, 03 and 04, respectively. One animal of the high dose group (3 mg/m³ CeO_2 exposure group, test group 04) exhibited multifocal very slight, particles intraepithelial.

In contrast to the investigation after 12 months of exposure, no exposure-related increase of **eosinophilic globules** was observed. There were (multi)focal intracytoplasmic **eosinophilic globules** within the olfactory epithelium in the 3 mg/m³ CeO_2 exposure group (test group 04: 35/50;

17/50 very slight, 11/50 slight, 5/50 moderate, 2/50 severe), in 1 mg/m^3 CeO_2 exposure group (test group 03: 29/50; 11/50 very slight, 6/50 slight, 8/50 moderate, 4/50 severe), the 0.3 mg/m^3 CeO_2 exposure group (test group 02: 38/50; 11/50 very slight, 10/50 slight, 10/50 moderate, 7/50 severe), the 0.1 mg/m^3 CeO_2 exposure group (test group 01: 33/50; 9/50 very slight, 9/50 slight, 10/50 moderate, 5/50 severe) and the clean air control (test group 00: 35/50; 13/50 very slight, 11/50 slight, 8/50 moderate, 3/50 severe).

The eosinophilic globules in the respiratory epithelium were not dose-dependent but exposure related increased resulting in the incidence in test group 00 of 9/50 (7/50 very slight, 2/50 slight), in test group 01 15/50 (11/50 very slight, 4/50 slight), in test group 02 14/50 (8/50 very slight, 6/50 slight), in test group 03 17/50 (15/50 very slight, 2/50 slight) and in test group 04 16/50 (14/50 very slight, 2/50 slight).

Similarly, (multi)focal **hyperplasia/metaplasia of mucous cells** were observed in 37/50 (24/50 very slight, 13/50 slight), in 47/50 (38/50 very slight, 9/50 slight), in 48/50 (32/50 very slight, 16/50 slight), in 41/50 (32/50 very slight, 9/50 slight) and in 49/50 (32/50 very slight, 15/50 slight, 2/50 moderate) of test group 00, 01, 02, 03 and 04, respectively.

Incidental findings in the nasal cavity which were considered to be unrelated to particle exposure included corpora amylacea, dilatation of submucosal glands, focal erosion, subepithelial infiltration of mixed inflammatory cells, acute inflammation, chronic active inflammation, subepithelial mononuclear cell infiltration, hyperplasia of the respiratory epithelium and were seen in single up to 7/50 animals in the different test groups.

Single animals showed an infiltration of the nasal cavity by a skin tumor or lymphoma cells.

3.4.2.2 Larynx

In 6/50 animals of the 3 mg/m^3 CeO_2 exposure test group, (multi)focal subepithelial **accumulation of particle-laden macrophages** (5/50 very slight, 1/50 slight) was observed as exposure-related finding.

Spontaneous findings included very slight to slight (multi)focal subepithelial mononuclear cell infiltration in up to 10/50 animals, very slight to slight dilatation of submucosal glands in up to 6/50 and very slight to slight focal epithelial alteration in up to 5 females of the different test groups as well as multifocal very slight mixed inflammatory cell infiltration and focal slight hyperplasia of the respiratory epithelium in single animals.

One animal showed a metastasis from a primary tumor in the mediastinum.

3.4.2.3 Trachea

As an exposure-related finding (multi)focal subepithelial **accumulation of particle-laden macrophages** was detected at the bifurcation in 3/50 (3/50 very slight), in 5/50 (5/50 very slight), in 8/50 (8/50 very slight) and in 15/50 (14/50 very slight, 1/50 slight) animals of the test group 01, 02, 03 and 04, respectively. Furthermore, very slight (multi)focal subepithelial accumulation of particle-laden macrophages was seen in 5/50, 1/50, 3/50 and 1/50 animals of test group 01, 02, 03 and 04, respectively, at another location in the trachea. There was a focal subepithelial infiltration of mononuclear cells at the bifurcation in 5/50 (5/50 very slight), 10/50 (8/50 very slight, 2/50 slight), 11/50 (11/50 very slight) and 4/50 (3/50 very slight, 1/50 slight) in rats of test group 01, 02, 03 and 04, respectively.

Spontaneous findings included very slight to slight (multi)focal subepithelial mononuclear cell infiltration at a different location than the bifurcation in a single animal and slight dilatation of submucosal glands in a single animal.

Single animals showed a metastasis from a primary tumor in the mediastinum or uterus or showed an infiltration by lymphoma cells.

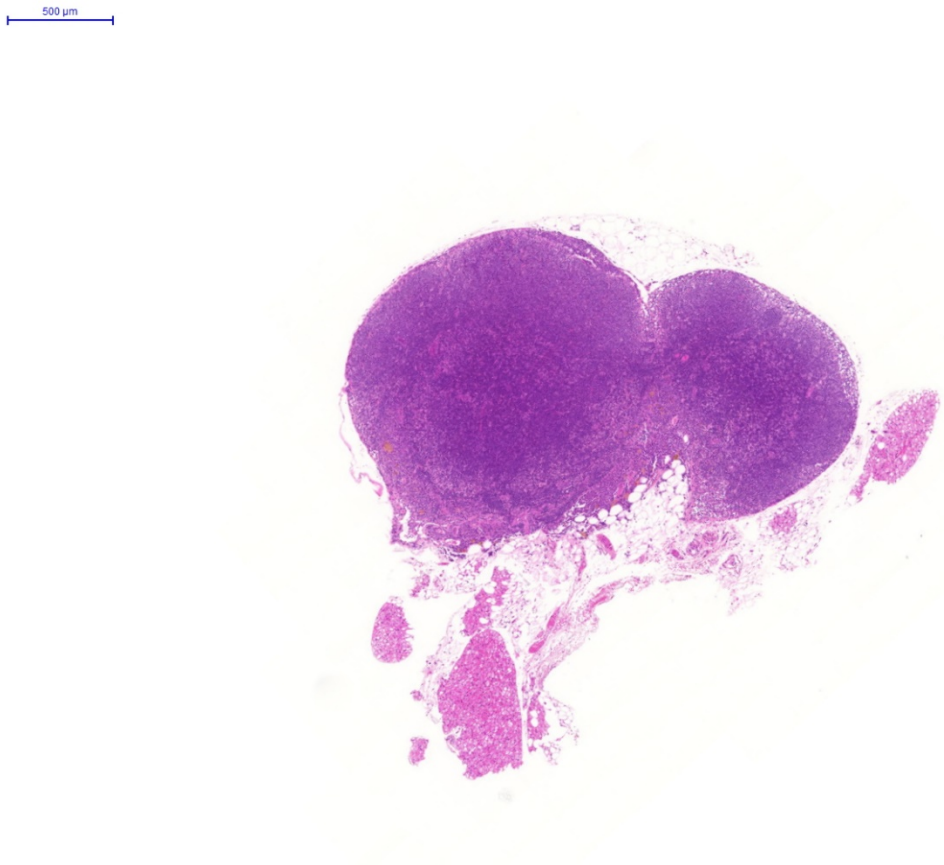
3.4.2.4 Tracheobronchial and Mediastinal lymph nodes

As correlate to the macroscopic findings ‘enlargement’ and ‘discoloration’, the lymph nodes at both sites showed a (multi)focal very slight to severe **accumulation of particle-laden macrophages** (see figure 8 and 9). Regarding the tracheobronchial lymph node, the incidences were 43/50 (18/50 very slight, 23/50 slight, 2/50 moderate), 39/50 (2/50 very slight, 17/50 slight, 19/50 moderate, 1/50 severe), 49/50 (1/50 slight, 23/50 moderate, 25/50 severe), 45/50 (2/50 slight, 5/50 moderate, 29/50 severe, 9/50 very severe) of test groups 01, 02, 03 and 04, respectively. **Syncytial (multinucleated) giant cells** were detected in 9/50 (9/50 very slight), 25/50 (24/50 very slight, 1/50 slight), 34/50 (26/50 very slight, 8/50 slight), 31/50 (29/50 very slight, 2/50 slight) of test groups 01, 02, 03 and 04, respectively.

The incidence of (multi)focal accumulation of particle-laden macrophages in the mediastinal lymph nodes were 12/50 (8/50 very slight, 4/50 slight), 24/50 (5/50 very slight, 8/50 slight, 10/50 moderate, 1/50 severe), 23/50 (2/50 very slight, 15/50 slight, 6/50 moderate), 42/50 (4/50 slight, 10/50 moderate, 13/50 severe, 15/50 very severe) of test groups 01, 02, 03 and 04, respectively. **Syncytial (multinucleated) giant cells** (see figure 5) were detected in 2/50 (2/50 very slight), 12/50 (12/50 very slight), 11/50 (8/50 very slight, 3/50 slight), 28/50 (27/50 very slight, 1/50 slight) of test groups 01, 02, 03 and 04, respectively.

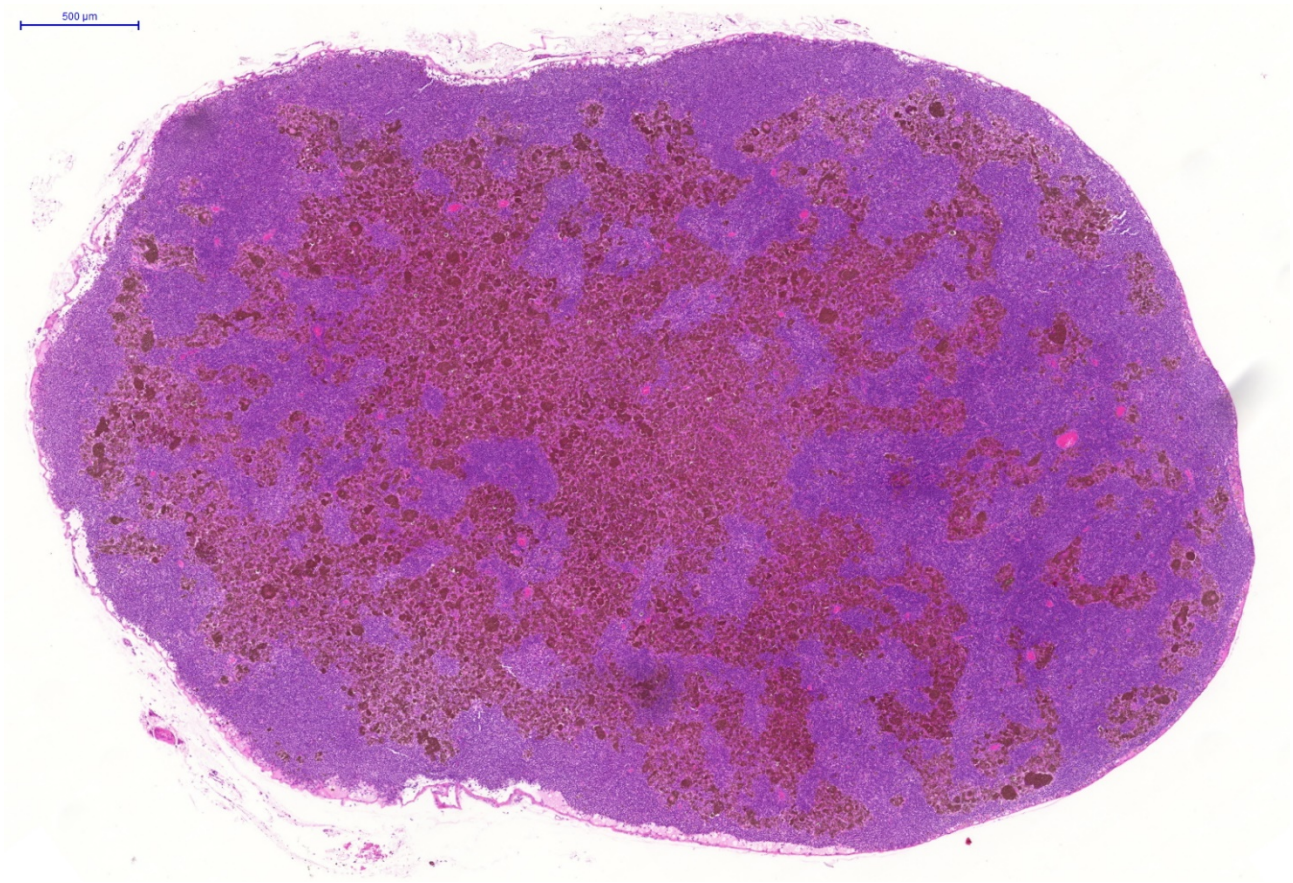
Metastasis from malignant tumors such as lymphoma or uterine carcinoma were detected in few animals without any correlation to the exposure.

Figure 7: Tracheobronchial lymph node of a clean air control animal



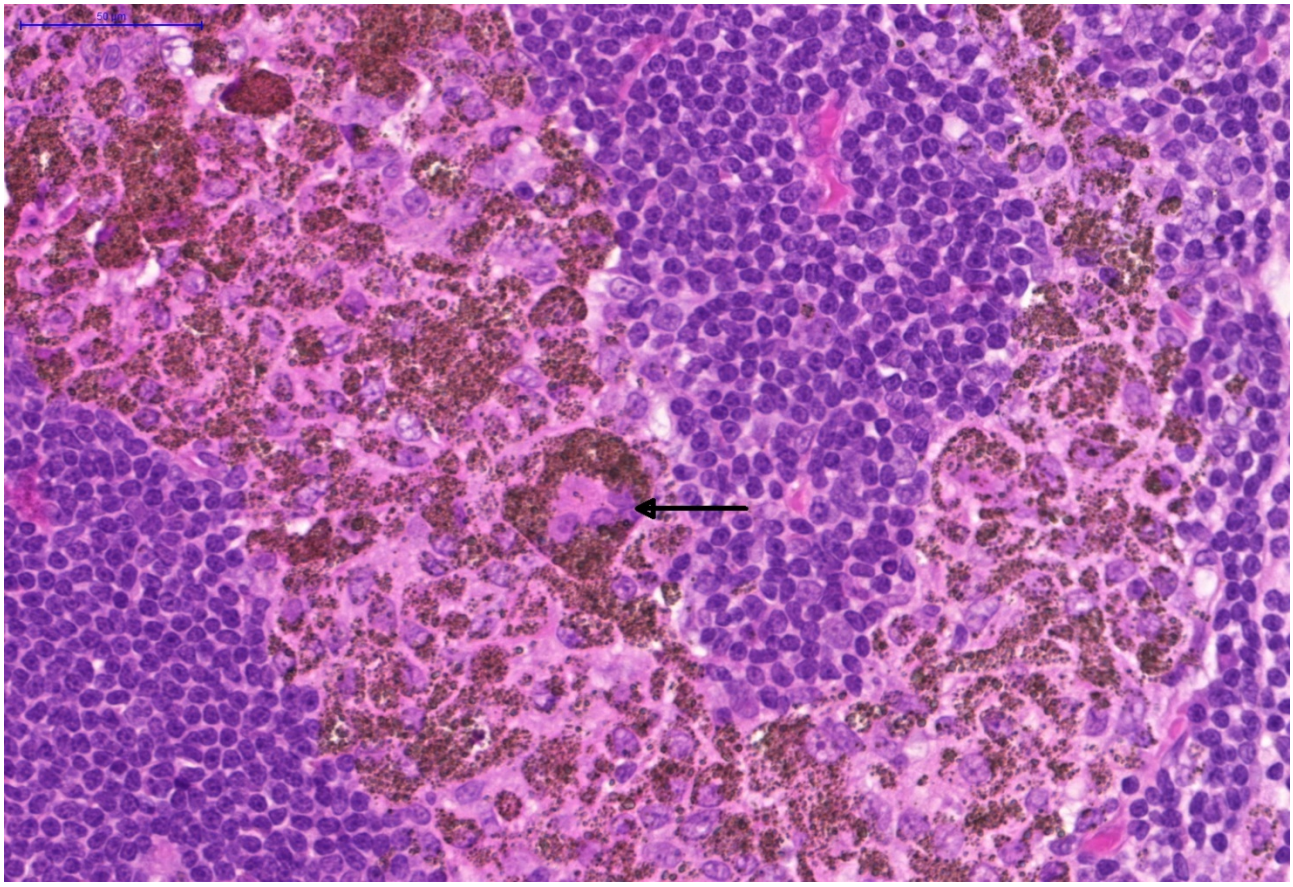
Hematoxylin and eosin stained tissue slide of the tracheobronchial lymph node of a rat exposed for 24 months with clean air with the same magnification as figure 8.

Figure 8: Tracheobronchial lymph node of a high dose cerium dioxide exposure group



Hematoxylin and eosin stained tissue slide of the tracheobronchial lymph node of a rat exposed for 24 months with 3.0 mg/m^3 CeO_2 with the same magnification as figure 7.

Figure 9: Higher magnification of the tracheobronchial lymph node of a high dose cerium dioxide exposure group



Hematoxylin and eosin stained tissue slide of the tracheobronchial lymph node of a rat exposed for 24 months with 3.0 mg/m^3 CeO_2 with particle-laden macrophages. Arrow depicts a particle-laden multinucleated syncytial giant cell.

3.4.2.5 Histopathology of the remaining organs of the respiratory tact

Within the remaining organ of the respiratory tract, the nasopharynx, no lesions were detected in any investigated group. The lungs of the animals were investigated within another project.

3.4.3 Histopathology of the other organs (carcinogenicity, 24 months)

In addition to the respiratory tract, all remaining organs required by the OECD test guideline no. 453 (see table 6) were also investigated. Special emphasis was laid on the organs, in which elevated cerium content was detected by the German Federal Institute for Risk Assessment, for example: liver, spleen, kidney, heart, brain and olfactory bulb (Laux et al., 2017).

However, no exposure-related lesions were detected within the other organs investigated. In addition, no particles were visible microscopically in any other organs but the tracheobronchial and mediastinal lymph nodes of the treatment groups.

Within the organs investigated many incidental findings were detected, which were in similar incidence and grade between the clean air control and the cerium dioxide exposure groups. The organ affected most commonly by tumors was the pituitary gland, more specifically the distal part, with tumor incidences of 18/50, 30/50, 24/50, 25/50 and 16/50 in the test group 00, 01, 02, 03 and 04, respectively. Within the mammary gland, tumors were detected in 5/50, 9/50, 9/50, 8/50 and 4/50 animals of the test group 00, 01, 02, 03 and 04, respectively. Malignant tumors of the uterus, which

have sometimes metastasized into the lung, were found in 2/50, 2/50, 2/50, 0/50 and 0/50 rats of test group 00, 01, 02, 03 and 04, respectively. Within the liver, tumors were observed in 3/50, 2/50, 1/50, 4/50 and 2/50 animals of test group 00, 01, 02, 03 and 04, respectively. All these tumors represent commonly found tumors in this rat strain. Within the other organs investigated only incidental tumors were noticed. All observed tumors were interpreted to be unrelated to the exposure.

Many basophilic foci of cellular alterations were detected within the liver. The incidences were 36/50, 47/50, 40/50, 48/50 and 37/50 in rats of test group 00, 01, 02, 03 and 04, respectively. The kidney of 27/50, 19/50, 27/50, 23/50 and 24/50 animals of test group 00, 01, 02, 03 and 04, respectively, showed a (multi)focal chronic nephropathy. As a further age-related change, very slight to slight degenerative to fibrotic (multi)focal changes were seen in the heart in 17/50, 18/50, 20/50, 22/50, 20/50 rats of test group 00, 01, 02, 03 and 04, respectively. Furthermore, in many organs incidental lesions were overserved, which were all interpreted to be unrelated to the exposure.

3.4.4 Histopathology of the respiratory tract (carcinogenicity, 30 months)

Exposure-related microscopic changes were observed in the nasal cavity, larynx, trachea, tracheobronchial and mediastinal lymph nodes.

3.4.4.1 Nasal cavity

Exposure-related finding such as (multi)focal very slight **accumulation of particle-laden macrophages within the NALT** (nasal mucosa-associated lymphoid tissue) were diagnosed in 5/48, 12/49, 21/49 and 35/50 animals of test group 01, 02, 03 and 04, respectively.

In contrast to the investigation after 12 months of exposure, no exposure-related increase of **eosinophilic globules** was observed. There were (multi)focal intracytoplasmic **eosinophilic globules** within the olfactory epithelium in the 3 mg/m^3 CeO_2 exposure group (test group 54: 23/50; 9/50 very slight, 5/50 slight, 8/50 moderate, 1/50 severe), in 1 mg/m^3 CeO_2 exposure group (test group 53: 49/49; 21/49 very slight, 18/49 slight, 7/49 moderate, 3/49 severe), the 0.3 mg/m^3 CeO_2 exposure group (test group 52: 30/49; 12/49 very slight, 8/49 slight, 8/49 moderate, 2/49 severe), the 0.1 mg/m^3 CeO_2 exposure group (test group 51: 35/50; 18/48 very slight, 5/48 slight, 8/48 moderate, 4/48 severe) and the clean air control (test group 50: 34/49; 18/49 very slight, 6/49 slight, 8/49 moderate, 2/49 severe).

Similarly, the eosinophilic globules in the respiratory epithelium were neither dose- nor exposure-dependent resulting in the incidence in test group 50 of 7/49 (6/49 very slight, 1/49 slight), in test group 51 5/48 (4/48 very slight, 1/48 slight), in test group 52 7/49 (7/49), in test group 53 7/49 (7/49 very slight) and in test group 54 5/50 (5/50 very slight).

Similarly, (multi)focal **hyperplasia/metaplasia of mucous cells** were observed in 36/49 (27/49 very slight, 7/49 slight, 2/49 moderate), in 37/48 (28/48 very slight, 9/48 slight), in 45/49 (33/49 very slight, 12/49 slight), in 44/49 (34/49 very slight, 10/49 slight) and in 47/50 (35/50 very slight, 12/50 slight) of test group 50, 51, 52, 53 and 54, respectively.

Incidental findings in the nasal cavity which were considered to be unrelated to particle exposure included corpora amylacea, dilatation of submucosal glands, subepithelial infiltration of mixed inflammatory cells, acute inflammation, chronic active inflammation, subepithelial mononuclear cell infiltration, squamous cell metaplasia and were seen in single up to 5/50 animals in the different test groups.

Single animals of different groups showed an infiltration of the nasal cavity by lymphoma cells.

3.4.4.2 Larynx

In 1/50 animals of the 3 mg/m^3 CeO_2 exposure test group, a moderate (multi)focal subepithelial **accumulation of particle-laden macrophages** was observed as exposure-related finding.

Spontaneous findings included very slight to moderate (multi)focal subepithelial mononuclear cell infiltration in up to 15 animals, very slight to moderate dilatation of submucosal glands in up to 18 animals and very slight to slight focal epithelial alteration in up to 5 females of the different test groups as well as multifocal very slight mixed inflammatory cell infiltration in up to 2 animals per test group.

One animal showed a metastasis from a primary tumor in the salivary glands and single animals exhibited an infiltration by lymphoma cells.

3.4.4.3 Trachea

As an exposure-related finding very slight (multi)focal subepithelial **accumulation of particle-laden macrophages** was detected at the bifurcation in 4/48, 5/49, 12/49 and 11/50 animals of the test group 51, 52, 53 and 54, respectively. Furthermore, (multi)focal subepithelial accumulation of particle-laden macrophages was seen in 4/50 (2/50 very slight, 2/50 slight) animals of test group 54 at another location in the trachea.

Spontaneous findings included a focal subepithelial infiltration of mononuclear cells at the bifurcation in 10/49 (9/49 very slight, 1/49 slight), 8/48 (8/48 very slight), 9/49 (9/49 very slight), 14/49 (14/49 very slight) and 13/50 (13/50 very slight) in rats of test group 50, 51, 52, 53 and 54, respectively.

3.4.4.4 Tracheobronchial and Mediastinal lymph nodes

As correlate to the macroscopic findings ‘enlargement’ and ‘discoloration’, the lymph nodes at both sites showed a (multi)focal very slight to very severe **accumulation of particle-laden macrophages**. Regarding the tracheobronchial lymph node, the incidences were 32/48 (24/48 very slight, 8/48 slight), 41/49 (2/49 very slight, 14/49 slight, 19/49 moderate, 6/49 severe), 44/49 (1/49 slight, 22/49 moderate, 19/49 severe, 2/49 very severe), 48/50 (6/50 slight, 8/50 moderate, 22/50 severe, 12/50 very severe) of test groups 51, 52, 53 and 54, respectively. **Syncytial (multinucleated) giant cells** were detected in 1/48 (1/48 very slight), 20/49 (17/49 very slight, 3/49 slight), 29/49 (24/49 very slight, 5/49 slight), 32/50 (31/50 very slight, 1/50 slight) of test groups 51, 52, 53 and 54, respectively.

The incidence of (multi)focal accumulation of particle-laden macrophages in the mediastinal lymph nodes were 9/48 (6/48 very slight, 2/48 slight, 1/48 moderate), 15/49 (3/49 very slight, 4/49 slight, 5/49 moderate, 3/49 severe), 29/49 (4/49 slight, 8/49 moderate, 16/49 severe, 1/49 very severe), 41/50 (1/50 slight, 10/50 moderate, 22/50 severe, 8/50 very severe) of test groups 51, 52, 53 and 54, respectively. **Syncytial (multinucleated) giant cells** were detected in 1/48 (1/48 very slight), 9/49 (6/49 very slight, 3/49 slight), 18/49 (16/49 very slight, 2/49 slight), 24/50 (22/50 very slight, 2/50 slight) of test groups 51, 52, 53 and 54, respectively.

Metastasis from malignant tumors such as lymphoma, uterine carcinomas or thyroid gland carcinomas were detected within the lymph nodes in few animals without any correlation to the exposure.

3.4.4.5 Histopathology of the remaining organs of the respiratory tact

Within the remaining organ of the respiratory tract, the nasopharynx, no lesions were detected in any investigated group. The lungs of the animals were investigated within another project.

3.4.5 Histopathology of the other organs (carcinogenicity, 30 months)

In addition to the respiratory tract, all remaining organs required by the OECD test guideline no. 453 (see table 6) were also investigated. Special emphasis was laid on the organs, in which elevated cerium content was detected by the German Federal Institute for Risk Assessment, for example: liver, spleen, kidney, heart, brain and olfactory bulb (Laux et al., 2017).

However, no exposure-related lesions were detected within the other organs investigated. In addition, no particles were visible microscopically in any other organs but the tracheobronchial and mediastinal lymph nodes of the treatment groups.

Within the organs investigated many incidental findings were detected, which were in similar incidence and grade between the clean air control and the cerium dioxide exposure groups. The organ affected most commonly by tumors was the pituitary gland, more specifically the distal part, with tumor incidences of 26/49, 28/48, 32/49, 28/49 and 35/50 in the test group 50, 51, 52, 53 and 54, respectively. Within the mammary gland, tumors were detected in 16/49, 16/48, 20/49, 18/49 and 19/50 animals of the test group 50, 51, 52, 53 and 54, respectively. Malignant tumors of the uterus, which have sometimes metastasized into the lung, were found in 4/49, 6/48, 3/49, 8/49 and 2/50 rats of test group 50, 51, 52, 53 and 54, respectively. Within the liver, tumors were observed in 3/49, 6/48, 5/49, 7/49 and 4/50 animals of test group 50, 51, 52, 53 and 54, respectively. All these tumors represent commonly found tumors in this rat strain. Within the other organs investigated only incidental tumors were noticed. All observed tumors were interpreted to be unrelated to the exposure.

Many basophilic foci of cellular alterations were detected within the liver. The incidences were 41/49, 28/48, 31/49, 34/49 and 31/50 in rats of test group 50, 51, 52, 53 and 54, respectively. The kidney of 23/49, 20/48, 19/49, 22/49 and 20/50 animals of test group 50, 51, 52, 53 and 54, respectively, showed a (multi)focal chronic nephropathy. As a further age-related change, very slight to slight degenerative to fibrotic (multi)focal changes were seen in the heart in 19/49, 20/48, 16/49, 16/49, 20/50 rats of test group 50, 51, 52, 53 and 54, respectively. Furthermore, in many organs incidental and age-related lesions were overserved, which were all interpreted to be unrelated to the exposure.

4 Summary and Conclusion

CeO_2 exposure-related findings were exclusively observed in the respiratory tract. These included reactive/adaptive changes such as accumulation of particle-laden macrophages in the nasal cavity, larynx, trachea, tracheobronchial and mediastinal lymph nodes. Furthermore, particle-laden syncytial (multinucleated) giant cells were observed dose-dependent in the in the tracheobronchial and mediastinal lymph nodes.

Accumulation of particle-laden macrophages were also observed in the lungs after 12 months of inhalation. The lungs of the animals after 24 months and 30 months were examined in another research project funded by the German Environment Agency.

Adverse effects in the lungs after 12 months of inhalation included dose-dependent alveolar/interstitial inflammatory cell infiltration, alveolar/interstitial granulomatous inflammation and interstitial fibrosis. Alveolar lipoproteinosis was observed in the 3 mg/m^3 high-dose CeO_2 exposure group only and cholesterol granulomas occurred in a single female each of the 1 and 3 mg/m^3 CeO_2 exposure groups.

Non-adverse findings in the lungs after 12 months of inhalation consisted of accumulation of particle-laden macrophages in the alveolar/interstitial areas and in the BALT as well as particle-laden syncytial giant cells in the BALT. In addition, bronchiolo-alveolar hyperplasia of the bronchiolar type graded no more than “very slight” (grade 1) or “slight” (grade 2) was considered as a non-adverse finding. After 12 months of inhalation exposure neither neoplastic nor pre-neoplastic treatment-related findings were seen in the lungs of CeO_2-exposed animals.

After 12 months of inhalation the nasal cavity showed an increase of the incidence of age-related intra-epithelial eosinophilic globules in the 3 mg/m^3 high-dose CeO_2 exposure group as compared to the control group and associated with minimal inflammatory cell infiltration. However, this difference was no longer apparent after 24 months of inhalation. At this time point the incidence of intra-epithelial eosinophilic globules was comparable in the clean air control to the CeO_2 exposure groups like 3 mg/m^3 high-dose CeO_2 exposure group. Eosinophilic inclusions are observed occasionally in otherwise normal epithelium of untreated rats, more frequently in aged animals (Boorman et al. 1990; Monticello et al. 1990). Increases in the incidence and severity of eosinophilic globules in respiratory and olfactory epithelia are frequently observed in inhalation studies (Renne et al., 2009). However, in this study it seems that after 24 months of inhalation the age-relation has a higher influence on the extent of this change obscuring the exposure-relation.

In other organs than the organs of the respiratory tract, no exposure-related findings were detected after 12, 24 and 30 months. In addition, the amount of tumors was not increased within the other organs. The organs in which elevated cerium content was detected by the German Federal Institute for Risk Assessment, for example: liver, spleen, kidney, heart, brain and olfactory bulb (Laux et al., 2017), did not reveal any exposure-related morphological changes.

The current results show that CeO2 induced a specific granulomatous inflammation in the lungs after 12 months inhalation characterized by the formation of microgranulomas and the presence of syncytial giant cells. These findings were also overserved after 24 months inhalation of CeO_2 with the same doses (Ernst et al., 2018). Many of these microgranulomas appeared as immune granulomas characterized by a rim of lymphocytes. Such granulomas are not seen with other poorly soluble particle with low toxicity (PSP) (Nikula, 2000). In addition, within the BALT and the lung-associated lymph nodes (tracheobronchial and mediastinal lymph node) particle-laden macrophages and particle-laden syncytial giant cells were observed. Syncytial giant cells are cells formed by fusion of two or more activated macrophages into one large cell with two or more nuclei (Ackermann, 2017). However, in addition to the granulomatous inflammation, the majority of the dose-related significant findings in the cerium groups after 12 months of inhalation are also typical for other PSP. These findings were increase of (particle-laden) macrophages (alveolar/interstitial, bronchus-associated lymphoid tissue [BALT]), inflammatory cell infiltration (mainly mononuclear cells, few granulocytes), alveolar bronchiolization (bronchiolo-alveolar hyperplasia, bronchiolar type), interstitial and pleural fibrosis, alveolar lipoproteinosis, and cholesterol granulomas. These changes were overserved in an increased extent after 24 months inhalation (Ernst et al., 2018).

Chronic exposure of rats to high (overload) concentrations of PSP causes progressive histopathologic alterations in the lungs. Incidence and severity of the alterations and their rate of progression are influenced by the inherent toxicity of the particle and dose factors such as exposure concentration or lung burden (Nikula, 2000). The PSP induced inflammation in the lung usually starts with an initial alveolar increase of particle-laden macrophages, which tend to aggregate in the area of the bronchiolo-alveolar junction. Depending on time, exposure concentration and particle characteristics, the aggregated macrophages may degenerate with subsequent release of various inflammatory mediators and alveolar accumulation of cellular debris and particles. In these areas, a progressive inflammation, formation of cholesterol crystals, cholesterol granulomas, bronchiolo-alveolar hyperplasias including alveolar bronchiolization, squamous cell metaplasias, alveolar lipoproteinosis and interstitial fibrosis may then develop as secondary alterations. These events and their dependency on the used exposure concentration have been described for PSP at high overload doses such as TiO_2 (Wahrheit et al., 1997; Lee et al., 1986;) and carbon black (Driscoll et al., 1996; Elder et al., 2005).

A surprising and unexpected result of the current study was that CeO_2 already in the lowest dose (0.1 mg/m^3) produced inflammatory lung lesions including microgranulomas after 12 months of inhalation. These findings are in compliance with the changes in gene expression of inflammatory mediators in pneumocytes type II after subchronic low dose CeO_2 exposure (Schwotzer et al., 2017). This highly sensitive method predicted the upcoming inflammation at an early stage of exposure and showed that pneumocytes type II contribute to the inflammatory reaction (Schwotzer et al., 2018).

The CeO_2-induced (micro)granulomatous inflammation was somewhat different to the granulomatous inflammation observed in rat lungs after repeated intratracheal instillations of non-biopersistent amorphous SiO_2 (Ernst et al., 2002; Kolling et al., 2008). To compensate the high clearance rate, up to 30 intratracheal instillations of 0.5 mg amorphous SiO_2 (Aerosil® 150) were applied in this study to rats at intervals of 14 days in order to produce a persistent inflammation. The histopathological findings revealed a multifocal granulomatous inflammation, which was characterized by a lack of intraalveolar macrophages and alveolar lipoproteinosis. The granulomas induced with amorphous SiO_2 had a much larger size than the CeO_2-induced (micro)granulomas in the present study and seemed to result from acute epithelial damage at the site of particle deposition with subsequent production of granulation tissue. Due to the fast removal rate of the SiO_2 particles from the deposition site, those granulomas became progressively ‘interstitialized’ and resolved by leaving focal fibrotic scar tissue (Ernst et al., 2002). However, some reversibility can be assumed during the 6-month post-exposure recovery period for the majority of the CeO_2-induced inflammatory changes in the present study. The CeO_2-induced microgranulomas showed also signs of “scarring” with a diminished proportion of inflammatory cells (mononuclear cells and syncytial giant cells) and appeared as small spots of collagen surrounding CeO_2 particle deposits (aggregations). It has to be emphasized, however, that the observed interstitial and pleural fibrosis in the present study was clearly not a “stand-alone” finding, but always associated with inflammatory changes and macrophage aggregations.

Particles were only observed microscopically in the respiratory tract including the lymph nodes draining the lymph fluid from the lung. The lung lymph nodes represent also the organs, in which the highest cerium content outside the lung was detected by Laux and coworkers (2018). This is in compliance with other inhalation studies, in which particle-laden macrophages were detected by microscopy in the lung-associated lymph nodes (e.g. Eydner et al., 2012; Creutzenberg, 2013).

In the other organs including the organs, in which elevated cerium content was measured by Laux and coworkers (2017), no particles and no exposure-related changes were seen microscopically.

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