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RIVM Report 650010 016

Toxicity of Ambient Particulate Matter
IV. Acute toxicity study in pulmonary hypertensive rats following 3-day exposure to ultrafine and fine model compounds for the secondary aerosol fraction of PM_{10}.

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37. - 52 Bureau Rapportenbeheer
53. - 58 Reserve

Contents

Preface

Toxicity studies on components of ambient particulate matter (PM_{10}) are conducted within the framework of a research program on adverse health effects of ambient particulate air pollution. These studies are part of the Dutch "Wintertime Smog and Traffic Program ", performed by order and at the account of The Ministry of Housing, Spatial Planning and Environment. The first part of this program included epidemiological studies on health effects of ambient PM_{10} and the possible role of traffic emissions. The second part of this program includes toxicity studies on ambient PM_{10} and has been designed in accordance with the simplified strategy of the health risk assessment model for ambient PM_{10}, i.e. the "Pentagon" model for possibly main critical fractions with respect to particle size and chemical composition.

This research will be a meaningful help to prove causality and biological plausibility of the acute effects of PM_{10} in healthy people and, in particular, people with cardiovascular diseases as a possible susceptible group.

Aerosols that are formed out of gaseous components in the atmosphere are generally referred to as secondary aerosols. In this fourth report on acute inhalation toxicity studies on model components of ambient PM_{10} results are presented of studies with secondary aerosols (ammonium nitrate, bisulfate and ferrosulfate) in healthy and pulmonary hypertensive rats. The results of these and subsequent studies performed in this framework will be integrated later on in a review report on the toxicity of carbonaceous and secondary aerosols.

Samenvatting

Ammoniumnitraat is de belangrijkste component van het secundair gevormde fijnstof in Nederland. Een tweede belangrijke fractie wordt, afhankelijk van de neutraliserende componenten, gevormd door bisulfaat of sulfaat. De acute inhalatoire effecten van deze aerosolen zijn onderzocht in gezonde ratten en ratten met pulmonaire hypertensie. In dit type onderzoek is het gebruik van diermodellen die een bepaalde humane cardiopulmonaire aandoening representeren essentieel, omdat epidemiologisch onderzoek juist deze groep mensen heeft aangeduid als een groep die gevoelig is voor expositie aan fijnstof. Voor de studies die in dit rapport worden beschreven is een model voor pulmonaire hypertensie (gepaard gaande met een inflammatie en een hypertrofie van het rechter ventrikel) toegepast. Dit ziektebeeld wordt chemisch geïnduceerd met monocrotaline (MCT).

Uitgaande van de wetenschap dat de effecten van fijnstof zeer acuut kunnen zijn, zijn in deze studies dieren 4 uur per dag gedurende drie dagen blootgesteld aan ultrafijn (count median diameter (CMD) ≈ 40-70 nm; maximaal 4 x 10^5 deeltjes per cm^3) en fijn (CMD ≈ 300-350 nm; maximaal 9 x 10^3 deeltjes per cm^3) ammonium aerosolen. De studie met ammoniumbisulfaat is twee maal uitgevoerd, waarbij de massaconcentraties in de tweede studie hoger waren dan in de eerste. De gemiddelde massa concentratie in deze studies lagen tussen 70 en 420 $\mu g/m^3$ voor ultrafijn ammoniumbisulfaat, ammoniumnitraat, en ammoniumferrosulfaat en tussen 275 en 410 $\mu g/m^3$ voor de fijne aerolen. Eén dag na de laatste blootstelling werden de effecten vastgesteld middels longspoelvloeistof (BALF) analyse en histopathologie.

De behandeling met MCT resulteerde in de verwachte pathologische symptomen: mediale hypertrofie van spieren van pulmonare slagaders en neomuscularisatie van kleine bloedvaten. Er zijn geen aanwijzingen gevonden voor het optreden van cytotoxiciteit (gemeten met de indicatoren lactaat dehydrogenase (LDH), eiwit- en albumine gehaltes in BALF) als gevolg van de blootstelling aan (ultra)fijn ammonium aerosol. Macrofagen lijken in alle met MCT behandelde groepen geactiveerd, bepaald middels de aktiviteit van N-acetylglucosaminidase (NAG), maar een effect van de test atmosfeer is niet opgetreden. Het celdifferentiatiebeeld laat geen consistent beeld zien ten aanzien van de MCT behandeling. Een effect van ammonium aerosolen kan mede door de latent aanwezige Haemophilus sp infectie, niet worden vastgesteld. Middels pathologisch onderzoek kon geen effect van de blootstelling aan aerosolen worden vastgesteld.
De hier gepresenteerde gegevens suggereren dat bij de gehanteerde aerosol concentraties, die vergelijkbaar waren met concentraties in eerdere studies in astmatische muizen geen pulmonaire effecten in gezonde en pulmonair hypertensieve ratten optreden.

Summary

In this (4^{th}) report on acute inhalation studies with model compounds for particulate matter (PM) in ambient air results are presented of a study with ammonium bisulfate, ammonium ferrosulfate and ammonium nitrate in healthy rats and rats with monocrotaline induced pulmonary hypertension (PH). Sulfates and nitrates are main components of the secondary aerosol fraction of PM_{10} in the Netherlands. The objective of this study was to test the hypothesis that secondary aerosols exert pulmonary effects in rats and that rats with PH are more sensitive to these aerosols than normal healthy animals. This aim was based on the epidemiological indications that people with cardiopulmonary diseases are distinguished as a sensitive part of the general population for acute exposure to PM and that ambient mass concentrations of sulfate and nitrate appear associated with health effects in many studies.

Healthy and PH rats were exposed to ultrafine (count median diameter (CMD) ≈ 40-70 nm; 4 x 10^5 particles per cm^3) and fine (CMD ≈ 300-350 nm; 9 x 10^3 particles per cm^3) ammonium aerosols during 4 hr a day for three consecutive days. Two independent studies with ammonium bisulfate were performed, differing only in the mass concentrations of the aerosol test atmospheres. The mean mass concentrations ranged from 70 to 420 $\mu g/m^3$, respectively for ultrafine ammonium bisulfate, nitrate and ferrosulfate and 275 to 410 $\mu g/m^3$ for fine aerosols. The animals were sacrificed 1 day after the last exposure to establish effects on the airways using bronchoalveolar lavage fluid (BALF) analysis, airway reactivity measurements and histopathological microscopical examination.

The MCT treatment (to induce pulmonary hypertension accompanied by inflammation and ultimately heart failure) was successful as seen by medial hypertrophy of muscular pulmonary arteries and neomuscularization of small blood vessels.
Histopathological examination did not reveal test atmosphere related abnormalities.There were no signs of cytotoxicity due to the aerosol exposures (as measured with lactate dehydrogenase (LDH), protein- and albumin contents in BALF). Macrophages were not activated due to the MCT treatment or the test atmospheres, since no changes were observed in N-acetyl glucosaminidase (NAG). Cell differentiation profiles were inconsistent, partly caused by an unavoidable infection with Haemophilus sp. However, we believe that despite this phenomenon, the test atmospheres did not affect cell differentiation or total cell counts..
The presented results suggest that at exposure levels comparable with previous studies in asthmatic mice ammoniun salt aerosols do not exert marked adverse health effects in both healthy and PH rats.

1. Introduction

The PM_{10} toxicity studies are conducted within the framework of a toxicity research program on health effects of relevant components of ambient particulate matter (PM). This program is part of the Dutch "Wintertime Smog and Traffic Program". This is the 4th report in a series on acute inhalation studies with model components of ambient particulate matter. The previous reports describe the effects of ammonium salt aerosols in health and asthmatic mice. The overall conclusion of those studies was that no significant differences were observed in responses to the test atmospheres between healthy and asthmatic mice.

The studies described in this report were aimed to identify adverse effects of ammonium bisulfate, nitrate, and ferrosulfate aerosol in rats. We hypothesized that fine (~ 200-1000 nm) and ultrafine (<100 nm) ammonium particles as model compounds for the secondary aerosol fraction of PM_{10} enhances inflammation in a rat airway disease model that mimics pulmonary hypertension and that these effects were dependent on particle size, mass concentrations and chemical composition of the aerosols.
We have exposed rats during 4 hr/day for 3 consecutive days and investigated the adverse effects of fine and ultrafine particles on the lung. In this study we have focused on biochemical, histopathological, cytological and immunological parameters indicative for cytotoxicity and inflammatory symptoms. This exposure protocol will roughly result in a 10 times higher deposition per unit surface area in the lungs compared to humans, who may be exposed to a ten times lower concentration during a whole 3-day period (24 hr).

2. Experimental

2.1 Testing facilities

The studies described in this report were conducted in 1997 at the department of Toxic Effects of the Laboratory of Health Effects Research, RIVM, the Netherlands.

2.2 Test material

Crystalline ammonium bisulfate, ammonium ferrosulfate and ammonium nitrate were supplied by Aldrich-Chemie, Steinheim, Germany. Monocrotaline was obtained from Sigma Chemical Co.(St. Louis, MO).

2.3 Test animals

Specified pathogen free male Sprague Dawley rats (6-8 weeks old) were obtained from the breeding colony of Harlan, UK and USA. The rats were housed in macrolon cages and food and water was provided *ad libitum* during non-exposure periods. The acclimatization period was at least 7 days. The room temperature was maintained at 22 ± 2°C and the relative humidity at 40-70% with a 12 hr light/dark cycle. The rats were fed a cereal-based rodent diet (SMR-A; Hope Farms, Woerden, the Netherlands). Animals were subcutaneously injected with 0 or 60 mg/kg monocrotaline at the beginning of a study, i.e. 21 days prior to exposure to the test atmospheres

2.4 Conduct of the studies

Each of the three studies was performed with 36 rats. Twenty-one days before exposure to the test material, half the number of rats were treated with monocrotaline (MCT). The animals were exposed to the test material during 4-hr/day for 3 consecutive days. Immediately after exposure, the animals were returned to their living cages, 4-6 animals per cage. All animals were sacrificed one day after the last exposure. Rats were used for bronchoalveolar lavage and histopathology.

2.5 Exposure system

Rats were individually and nose-only exposed in a small exposure chamber of the Institute's design. During exposure the animals were restrained in animal holders (modified Battelle tubes) which closely fitted to the exposure chamber. The animals were allowed to breathe freely from the stream of fresh test atmosphere. During exposure animals were deprived from food and water. Animals were trained in retraining tubes for 1-2 hr a day during the three days prior to the first exposure. The rats were daily visually inspected for reactions to treatments.

2.6 Generation of the atmosphere[1]

The aerosol was generated by ultrasonic nebulization (Omron NE-U12, Omron Corporation, Tokyo, Japan) of an aqueous solution of ammonium nitrate. The molarity of this solution determined the size of the final aerosol. Large droplets were removed by impaction. The main aerosol stream was led through a heating tube to evaporate the water. Water vapor was removed in a diffusion-drying chamber filled with silica beads. This resulted in solid

[1] A detailed generation report is added to the file of this study.

ammonium salt particles. The final concentration was reached by diluting the aerosol stream with purified air.

2.7 Analysis of the test atmosphere[2]

The mass concentrations of the test atmospheres were determined wet-chemically by analyzing sodium carbonate coated filters on which particles were collected. The mass concentrations of ammonium were determined by a photometric determination of a colored complex of ammonium in an auto analyzer, based on Continuous Flow Analysis (CFA). Nitrate and sulfate concentrations were determined by ion-chromatography
Particle size distributions and numbers were determined with a scanning mobility particle sizer (SMPS) consisting of an electrostatic classifier (TSI 3071, TSI Inc., St. Paul, Min., USA) and a condensation nucleus counter (TSI 3022A, TSI Inc., St. Paul, Min., USA).
Relative humidity and temperature of the test atmosphere were measured conventionally.
Freshly generated particles were sampled on a Teflon filter and processed for SEM.

2.8 Bronchoalveolar lavage and measurements

Rats were i.p. anaesthetized with sodium pentobarbital, the abdominal aortas were severed, and the tracheas were exposed. A catheter was inserted into the trachea and tied in place. The right lungs were lavaged three times with a volume saline corresponding with 27 ml/kg body weight at 37 °C. The lavage was repeated three times per animal and the recovered fluid from each lavage was placed on ice. The collected fluid (BALF) from each animal was centrifuged at 200 g, 4°C, for 10 minutes. The cell-free fluid from the first lavage was reserved for protein and biochemical assays. The pellet was resuspended in saline. Half of the pellet from the first lavage and the complete pellets of the second and third lavages were pooled and used for a macrophage test.
Lactate dehydrogenase (LDH), N-acetyl glucosaminidase (NAG) and albumin were determined using a commercially obtained reagent kit (Boehringer Mannheim (Mannheim, Germany).
Total protein was determined using a reagent kit obtained from Pierce (Oud-Beijerland, the Netherlands). LDH was measured as a marker for cytotoxicity, NAG was determined as an indicator for macrophage activation, and albumin and total protein levels in BALF were measured as indicators for increased permeability of the alveolar-capillary barrier.

Total number of cells and the viability were counted using a Bürker-Türk chamber. For differential cell counts cytospin preparations were made and stained with May-Grunwald Giemsa. Per cytospin preparation 400 cells were counted and the proportion of each cell type (macrophages, neutrophilic granulocytes, eosinophilic granulocytes and lymphocytes) was calculated based on total viable cells per BALF sample.

2.9 Histopathology

At autopsy the animals were anaesthetised and the left lung was cut at the end of the trachea. The left lung was weighted and a small canule was inserted into the bronchus. The lungs were fixed intratracheally with 2% glutaraldehyde in a 0.1 M phosphate buffer at a pressure of 20 cm for one hour. After immersion fixation in formalin the lungs were embedded in Paraplast and sliced in 5 µm sections. Sections were stained with haematoxylin-eosin and periodic acid Schiff-Alcian blue.

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[2] A detailed exposure report is added to the file of this study.

2.10 Phagocytose

The capacity of alveolar macrophages to phagocytose E-coli's was only tested in the second 4 substudy with ammonium bisulphate using a commercial available kit (Phagostest, Orpegen Pharma) modified for BALF.

2.11 Statistical analysis

All values are expressed as the mean ± SD. All statistical analysis were performed using STATA (version 2.05, CRC, Oasis, Nieuwegein the Netherlands). The (log-transformed) data were analyzed using multiple one-way analysis of variance. The assumption of homogeneity of variance was tested with the Bartlett test. For some parameters, analysis of the control animals showed differences between the three sub-studies. Therefore responses of dose were analyzed separately.

2.12 Retention of the records

Raw data, the master copy of the final report and all other information relevant to the quality and integrity of the study, will be retained in the archives of the Laboratory of Health Effects Research of the RIVM for a period of at least 5 years after the reporting of the study.

3. Results

3.1 Analytical results of the test atmosphere

3.1.1 Particle number concentration

The actual concentration was determined by measuring the number of particles per volume and the results are summarized in Table 1.

Table 1 Actual exposure concentrations (#/cm^3) of fine and ultrafine ammonium aerosol. Values are expressed as means, 5 percentile and 95 percentile.

Group	Study	Fine (#/cm^3) . 10^3	Ultrafine (#/cm^3) . 10^5
Bisulfate I	9701	4.1 (3.1- 5.4)	2.0 (1.8- 2.2)
Bisulfate II	9810	9.2 (8.2-9.8)	3.0 (2.9- 3.1)
Ferrosulfate	9701	2.5 (2.2 -2.9)	1.7 (1.3- 1.7)
Nitrate	9703	1.8 (1.0 -2.2)	4.0 (2.7- 6.5)

3.1.2 Temperature and Relative Humidity

The mean temperature was kept between 20 and 25 °C and the relative humidity was maintained between 1 and 20 % for the ultrafines and 30 and 60% for the fines.

3.1.3 Particle size and mass measurements

The results of the size and mass measurements are summarized in Table 2.

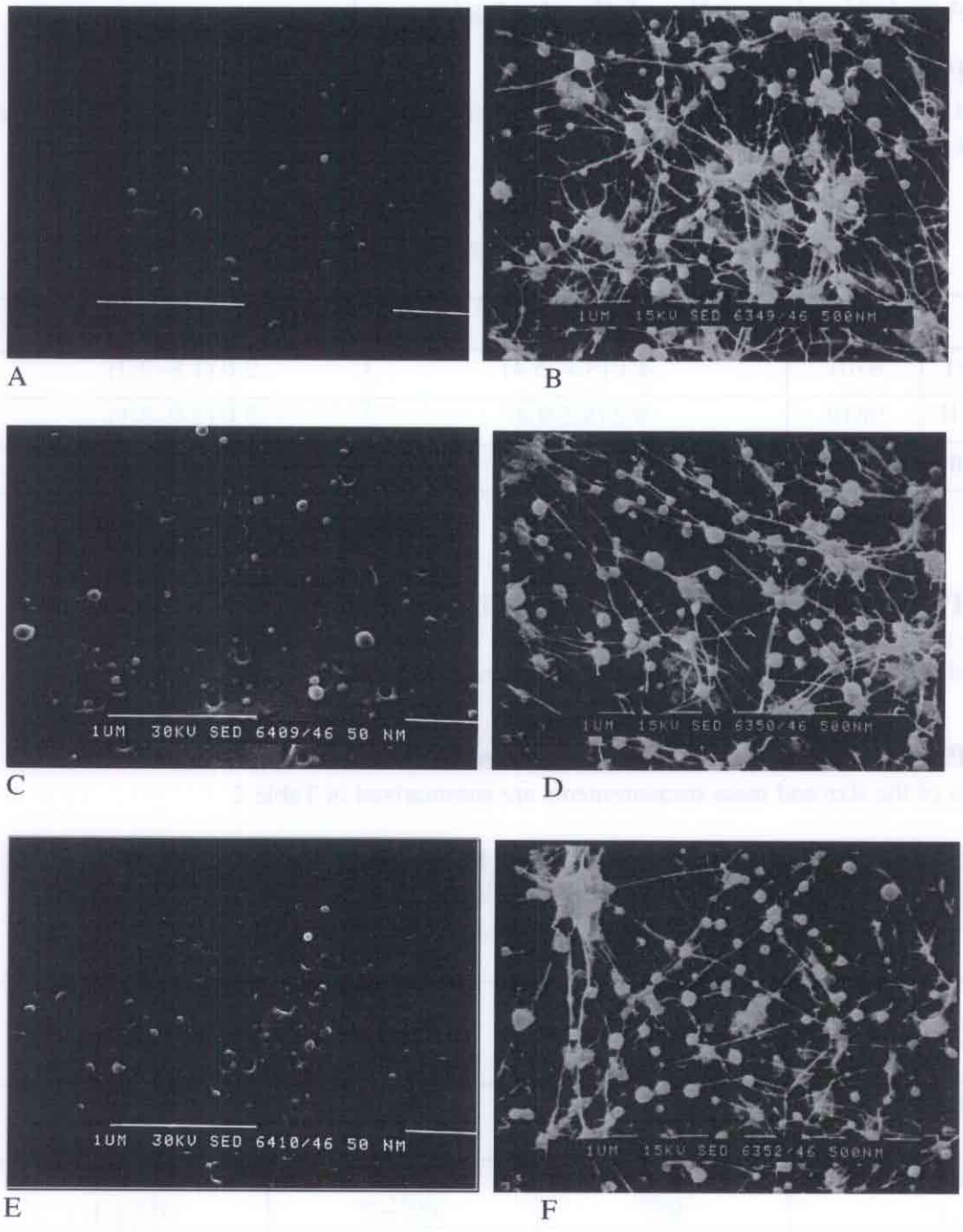
Table 2 Count Median Diameter (CMD) and Mass Median Diameter (MMD) as determined with SMPS of fine and ultrafine ammonium aerosol. Values are expressed as means ± geometric standard deviation.

Group	Mass concentration (µg/m^3)	Particle Diameter (nm)	
		CMD	MMD
Ammonium bisulfate I	70[a] 275[b]	49 ± 1 299 ± 2	70 ± 2 565 ± 1
Ammonium bisulfate II	344[c] 407[c]	69 ± 2 335 ± 2	107 ± 2 633 ± 1
Ammonium ferrosulfate	285[a] 340[a]	58 ± 1 353 ± 2	84 ± 2 617 ± 1
Ammonium nitrate	418[a] 361[a]	41 ± 2 343 ± 3	87 ± 2 643 ± 2

a. n =2
b. Based on one time-weighted measurement. A second measurement of 75 µg/m^3 was rejected.
c. n =3

3.2 SEM pictures of the ammonium aerosols

Ultrafine particles **Fine particles**



A B

C D

E F

Fig. 2: Scanning Electron Microscope photographs of ultrafine and fine particles collected on Millipore VMTP04700 and Millipore FALP04700 filters, respectively. (A, B) ammonium bisulfate, (C,D) ammonium ferrosulfate and (E,F) ammonium nitrate.

3.3 Clinical observations

No abnormalities were observed in rats that were treated with MCT or exposed to the test atmospheres, or both. All were infected by Haemophilus ssp.

3.4 Body weights

No ammonium aerosol related changes were seen in body and organ (lung, heart and liver) weight. Decreased body weights and increased lung weights were generally seen in groups of rats treated with MC. (Appendix A: Table 1).

3.5 Bronchoalveolar lavage

3.5.1 Biochemistry

The results of the BALF LDH, NAG, protein and albumin measurements are shown in Appendix B, Table 2. MCT treatment generally resulted in increased levels of these indicators of pulmonary toxicity. Exposure to ammonium aerosols did not further affect the levels of these parameters. The exception was formed by fine ammonium nitrate, that showed to reduce the LDH levels expressed per mg protein. LDH values expressed per liter BALF were not affected, but relatively high protein and albumin levels were seen in rats treated with MCT and exposed to fine ammonium nitrate. Moreover, ultrafine ammonium ferrosulfate seems to increase albumin (and thus protein) levels in healthy but not in PH rats.

3.5.2 Cytology

The results of the cell measurements in BALF are summarized in Appendix B, Table 3.
In general, MCT treatment resulted in increased numbers of neutrophils. No aerosol exposure related effects were identified.

3.5.3 Phagocytic activity

There was a consistent trend that MCT treated animals had a higher overall phagocytic capacity, but, despite the fact that exposure to ultrafine ammonium bisulfate seem to have resulted in increased mean fluorescence per cell and an increased percentage of phagocytising cell compared to the controls, the day-to-day differences and the use of two different assay kits of the phagotests do not allow to draw the conclusion that exposures to bisulfate had an effect on macrophages.

3.6 Pathology

3.6.1 Autopsy

Macroscopic examination of the rats at autopsy did not reveal treatment-related abnormalities.

3.6.2 Histopathology

All rats receiving MCT showed a slight to moderate medial hypertrophy of muscular pulmonary arteries and neomuscularization of small blood vessels. Only in these rats aggregates of foamy alveolar macrophages were frequently present. This histological picture may explain the significant increase in lung weights in these groups. In most of the livers of monocrotaline treated rats periportal glycogen rich areas were noted. This correlates well with the significant increases in liver weights in MCT treated animals. In all groups no histological changes were observed in heart tissue.

Exposure to fine or ultrafine aerosol of ammonium-iron-sulfate or of ammonium hydrogen sulfate did not induce any histological effect in lungs of control rats, nor an additional effect in lungs of MCT-treated rats. Only after exposure to an ammonium nitrate aerosol (fine and ultrafine) the histological lesions due to a background infection (vide infra) seemed to be more frequent and stronger compared to their respective controls.
In this study all Sprague-Dawley rats had a minimal to strong perivascular inflammation of mononuclear cells, alveolitis and perivascular edema. In areas with a moderate to strong alveolitis and perivascular inflammation often hypertrophy of the bronchiolar epithelium was present. Only a few rats showed interstitial pneumonitis or foci of fibrotic alveolar septa. This histopathological picture may be associated with a background infection with Haemophilus ssp.

4. Discussion and Conclusions

Particles inhaled by humans are segregated by size during deposition within the respiratory system. The major regions of the respiratory system differ widely in structure, size, function, sensitivity, or reactivity to deposited particles and in mechanism of particle clearance. Larger particles deposit preferentially in the upper respiratory tract, while smaller inhalable particulates travel deeper into the lungs and are retained for longer periods of time. Dosimetry models have shown that the relation between particle size and traveling distance in the airways before deposition is a non-linear relationship (Anjilvel and Asgharian, 1995). Toxicity of particles retained in the lungs also varies with chemical composition. Some chemicals such as sulfuric acid may react directly with the system inducing cytotoxicity, while others (insolubles) may act to retard clearance of other particles from the lungs, resulting in a more indirect toxicity.

This study was designed to test the hypothesis that the adverse effects of ammonium salts, if any, would be greater in animals with MCT induced pulmonary hypertension compared to healthy animals and that these effects were dependent on particle size, mass concentrations and chemical composition of the aerosols. This pulmonary hypertension animal model is nowadays frequently used in inhalation toxicity studies with particulate matter. Pulmonary hypertension and related inflammatory reactions and cardiac dysfunction is a common feature and complication in people suffering from chronic obstructive pulmonary disease (COPD) and these people are considered to be at increased risk for health effects of PM_{10}.

The results obtained in this study show that ammonium salts up to 400 $\mu g/m^3$ do not exert biologically relevant adverse effects, neither in normal healthy rats nor in compromised pulmonary hypertensive rats after a 4 hr/day, 3-day inhalatory exposure. The background infection with Haemophilus ssp might have overshadowed an adverse effect. Furthermore, the treatment of the rats with MCT resulted in relative large variations in the increase of the levels of the investigated parameters. Consequently, minimal to mild effects cannot be detected using this model for pulmonary hypertension accompanied by an emerging heart failure. Exposing animals to higher concentrations of these aerosols is a possibility to try to establish an adverse cardiopulmonary effect, though such levels will be far beyond reality. Current levels of ammonium aerosols in the Netherlands are around 2 $\mu g/m^3$.

Previous studies in healthy and 'asthmatic' mice (Cassee et al., 1998a-c) resulted in the same observations, namely that no serious adverse health effects were found using similar exposure protocols. Kleinman *et al.* (1998) have reported that ammonium bisulfate in a study in which 2-year old F344N rats were exposed for 4 hr/day, 3 consecutive days a week, for 4 weeks to 70 $\mu g/m^3$ (0.3 μM MMAD) resulted in increased cell labeling in epithelial and interstitial cells. Although this is a longer exposure duration compared with the short-term exposure studies reported here, the overall dose in that study was in the same range. In addition, a combination of carbon black (50 $\mu g/m^3$, 0.3 μm MMAD), ammonium bisulfate and ozone (0.2 ppm) increased the adverse effects produced by ozone, or vice versa. Last (1991) demonstrated that a synergistic interaction between ozone and sulfuric acid aerosol can occur at environmentally relevant concentrations (ozone 0.12 parts per million (ppm) and 5 to 20 $\mu g/m^3$ sulfuric acid aerosol) of both pollutants. From these studies it was concluded that the acidity of the aerosol is a condition for such a synergistic interaction between an oxidant gas and a respirable aerosol to occur. In our studies, we did not use acidic aerosols since most acids are (at least partly)

neutralised by ammonia resulting in ammonium salts. However, the results of for example (Bolarin *et al.* (1997) and Last (1991) suggest that a mixture of oxidant gasses with particles might be more effective in exerting adverse effects than particulates alone.

Dosimetry models for both human and rat show that particles in the range of 50 - 500 nm will only deposit with an efficiency of maximal 20% in the lungs. Particles larger than 100 (nm will mainly deposit in the upper respiratory tract (nose, mouth, larynx), whereas smaller particles are predominantly deposited in the lower airways. This means that only a fraction of the calculated dose (total inhaled volume during the exposure period times the exposure concentration) will deposit on the lung epithelium. To achieve the same dose per unit lung surface, humans have to be exposed to half the concentration for 24 hr or triple the concentration for the same exposure period, assuming no physical activity of both species. Assuming annual ambient concentrations of ammonium salt of 20 $\mu g/m^3$, these estimations illustrate that the concentrations used in these studies most probably have resulted in 10 times higher dose per unit surface area in the lung than can be expected in healthy humans exposed to realistic PM concentrations. People with compromised airways (asthma, COPD) will perceive up to a factor of 3-5 higher deposition of particulates in the airways Miller *et al.*, 1995). Similar increases can be calculated for exercising people or young children.
Summarizing, it may be said that the exposure regimes used in these studies resemble near-realistic concentrations and that these exposures do not exert an adverse effect in healthy and compromised rats. It should be emphasized that this study was only focused on acute effects rather than (sub) chronic effects of these secondary aerosols.

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Appendix A: Health Parameters

Table 1: Body, lung heart and liver weights of healthy and PH rats exposed to fine or ultrafine ammonium aerosols during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n= 5-6 for all groups). bw = body weight; n.s. = treatment does not result in statistically significantly differences from control groups

	Body weight (g)	Lung weight (g)	Heart weight (g)	Liver weight (g)	Lung/ bw	Heart/ bw
	Ammonium bisulfate (I)					
Sham	331 ± 13.4	566 ± 92	1023 ± 68	10.9 ± 0.5	1.7	3.1
Sham/PH	316 ± 15.4	583 ± 58	987 ± 72	12.1 ± 1.2	1.8	3.1
UF	334 ± 17.9	553 ± 88	1011 ± 100	11.0 ± 0.6	1.7	3.0
UF/PH	323 ± 24.4	754 ± 80 [1]	1003 ± 93	12.1 ± 0.9	2.3	3.1
F	340 ± 25.4	532 ± 27	1039 ± 70	11.7 ± 1.4	1.6	3.1
F/PH	321 ± 11.0	669 ± 87 [5]	1035 ± 60	12.1 ± 1.0	2.1	3.2
ANOVA p	<0.05	<0.001	n.s.	n.s.	N.D.	N.D.
	Ammonium bisulfate (II)					
Sham	325 ± 23.4	533 ± 82	944 ± 48	10.5 ± 1.1	1.6	2.9
Sham/PH	303 ± 10.2	641 ± 65	930 ± 64	11.0 ± 0.5	2.1	3.1
UF	326 ± 17.9	507 ± 42	980 ± 88	11.0 ± 1.2	1.6	3.0
UF/PH	312 ± 23.1	679 ± 111	1024 ± 151	11.7 ± 1.4	2.2	3.3
F	332 ± 22.2	531 ± 80	964 ± 82	10.7 ± 1.1	1.6	2.9
F/PH	317 ± 26.2	686 ± 104	1106 ± 218	11.7 ± 1.6	2.2	3.5
ANOVA p	n.s.	< 0.001	n.s.	n.s.	N.D.	N.D.
	Ammonium ferrosulfate					
Sham	366 ± 10.1	547 ± 73	1198 ± 66	11.9 ± 0.7	1.5	3.3
Sham/PH	348 ± 23.9	734 ± 135 [1]	1153 ± 82	12.4 ± 1.8	2.1	3.3
UF	378 ± 18.7	575 ± 87	1256 ± 94	13.1 ± 1.2	1.5	3.3
UF/PH	350 ± 5.5 [2,3]	739 ± 133 [1]	1191 ± 103	13.0 ± 0.7	2.1	3.4
F	367 ± 11.8	504 ± 35 [2]	1215 ± 93	12.2 ± 0.8	1.4	3.3
F/PH	350 ± 11.9	658 ± 119	1248 ± 79	13.1 ± 1.0	1.9	3.6
ANOVA p	< 0.01	<0.001	n.s.	n.s.	N.D.	N.D.
	Ammonium nitrate					
Sham	330 ± 14.3	515 ± 44	980 ± 43	12.5 ± 0.7	1.6	3.0
Sham/PH	302 ± 9.7 [1]	670 ± 95 [1]	976 ± 76	12.1 ± 0.9	2.2	3.2
UF	331 ± 20.0	530 ± 74	1009 ± 72	12.0 ± 1.1	1.6	3.1
UF/PH	310 ± 13.8	719 ± 102 [1,3]	1066 ± 75	12.5 ± 0.9	2.3	3.4
F	335 ± 13.1	550 ± 55	1025 ± 43	11.5 ± 0.8	1.6	3.1
F/PH	310 ± 19.2	715 ± 63 [1,3,5]	960 ± 51	11.8 ± 0.8	2.3	3.1
ANOVA p	<0.01	<0.001	n.s.	n.s.	N.D.	N.D.

Appendix B: Data BALF analysis

Table 2a. Biochemical determinations measured in BALF of healthy and PH rats exposed to fine or ultrafine ammonium bisulfate (I) aerosols during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n= 5-6 for all groups).

			LDH U/L	NAG U/L	Protein mg/L	Albumin mg/L	LDH U/g	NAG U/g	Alb/Prot %
Group	Exposure								
1	Sham	Mean	58	2.0	281	126	228	9.2	46%
		S.D.	23	0.4	147	62	63	5.5	6%
2	Sham/MCT	Mean	49	2,4	411	227	176	7.2	56%
		S.D.	19	0.7	337	179	52	2.2	5%
3	Ultrafine	Mean	57	2.5	215	114	279	13.3	55%
		S.D.	24	0.3	113	51	57	4.5	6%
4	Ultrafine/MCT	Mean	113 2	3.3	922 1,3	463 1,3	133 3	4.0 3	49%
		S.D.	45	1.0	483	279	30	1.3	10%
5	Fine	Mean	49 4	2.2	222 4	124 4	262 4	13.1 4	62%
		S.D.	26	0.5	204	87	78	5.7	10%
6	Fine/MCT	Mean	80	2.7	693 5	306	144 3,5	5.0 3,5	41%
		S.D.	22	0.7	407	247	65	2.5	11%

* 1t/m5 =significantly different from the group indicated by the figure next to the mean value of a parameter, Bonferroni P < 0.05

p values							
[L]ANOVA	<0.01	n.s.	<0.001	<0.01	n.s.	<0.001	n.s.
MCT	<0.05	<0.05	<0.001	<0.001	<0.001	<0.001	n.s.
EXPO	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
MCT*EXPO	<0.05	n.s.	n.s.	n.s.	n.s.	<0.05	<0.001

Table 2b. Biochemical determinations measured in BALF of healthy and PH rats exposed to fine or ultrafine ammonium bisulfate (II) aerosols during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n= 5-6 for all groups).

			LDH	NAG	Protein	Albumin	LDH	NAG	Alb/Prot
			U/L	U/L	mg/L	mg/L	U/g	U/g	%
Group	Exposure								
1	Sham	Mean	72	1.4	127	208	339	6.7	59%
		S.D.	34	0.7	68	87	28	1.1	8%
2	Sham/MCT	Mean	110	1.9	256	478	279	5.5	55%
		S.D.	42	0.5	170	352	107	4.0	7%
3	Ultrafine	Mean	61	1.2	89	159	368	7.5	56%
		S.D.	35	0.4	18	32	126	2.4	7%
4	Ultrafine/MCT	Mean	71	1.8	326	573	150	3.6	57%
		S.D.	28	0.8	211	361	59	1.5	4%
5	Fine	Mean	59	1.1	106	199	305	6.2	54%
		S.D.	21	0.1	37	85	68	1.8	4%
6	Fine/MCT	Mean	81	1.5	380	682	136	2.4	55%
		S.D.	37	0.4	204	333	68	0.8	3%
No differences were noted between the 6 different exposure groups that could be related to the exposure to ammonium bisulfate									

Table 2c. Biochemical determinations measured in BALF of healthy and PH rats exposed to fine or ultrafine ammonium ferrosulfate aerosols during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n= 5-6 for all groups).

			LDH U/L	NAG U/L	Protein mg/L	Albumin mg/L	LDH U/g	NAG U/g	Alb/Prot %
Group	**Exposure**								
1	**Sham**	**Mean**	58	1.7	196	73	282	8.3	36%
		S.D.	50	1.2	75	41	180	4.1	8%
2	**Sham/MCT**	**Mean**	146	2.2	1126	497	143	3.6	41%
		S.D.	132	1.3	1036	468	24	2.3	6%
3	**Ultrafine**	**Mean**	97	1.4	320	135	343	5.0	42%
		S.D.	53	0.5	162	72	169	2.4	4%
4	**Ultrafine/MCT**	**Mean**	139	2.4	966	439	150	3.8	39%
		S.D.	133	1.2	974	456	61	2.6	11%
5	**Fine**	**Mean**	34	1.4	132	48	253	10.6	36%
		S.D.	14	0.2	12	2	95	1.9	2%
6	**Fine/MCT**	**Mean**	111	2.3	672	309	173	4.6	43%
		S.D.	102	1.1	620	315	63	2.93	4%

* 1V/m5 =significantly different from the group indicated by the figure next to the mean value of a parameter, Bonferroni P < 0.05

	p values
ANOVA	
MCT	
EXPO	
MCT*EXPO	

Table 2d. Biochemical determinations measured in BALF of healthy and PH rats exposed to fine or ultrafine ammonium nitrate aerosols during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n= 5-6 for all groups).

			LDH U/L	NAG U/L	Protein mg/L	Albumin mg/L	LDH U/g	NAG U/g	Alb/Prot %
Group	**Exposure**								
1	**Sham**	**Mean**	57	2.0	242	116	255	9.6	47%
		S.D.	21	0.6	123	70	75	3.7	10%
2	**Sham/MCT**	**Mean**	138 1	3.0	616 1	281 1	233	6.0	50%
		S.D.	72	0.8	339	189	45	3.2	3%
3	**Ultrafine**	**Mean**	55 2	2.2	183 2	83 2	307	12.6 2	45%
		S.D.	23	0.5	75	39	65	3.3	10%
4	**Ultrafine/MCT**	**Mean**	81	2.2	546 1,3	282 1,3	154 1,3	4.2 1,3	52%
		S.D.	11	0.6	149	80	24	1.1	3%
5	**Fine**	**Mean**	52 2	2.0	246 2,4	132	223	8.9 4	53%
		S.D.	19	0.5	101	57	56	3.2	9%
6	**Fine/MCT**	**Mean**	123 1,3,5	2.7	907 1,3,5	463 1,3,5	137 1-3,5	3.1 1,3,5	51%
		S.D.	49	0.8	297	139	30	0.8	2%

* 1t/m5 =significantly different from the group indicated by the figure next to the mean value of a parameter, Bonferroni P < 0.05

p values						
ANOVA	<0.001	n.s.	<0.001	<0.001	<0.001	<0.001
MCT	<0.001	<0.05	<0.001	<0.001	<0.001	<0.001
EXPO	n.s.	n.s.	n.s.	<0.05	<0.01	n.s.
MCT*EXPO	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Table 3a. Cellular determinations measured in BALF of healthy and PH rats exposed to fine or ultrafine ammonium bisulfate (I) aerosols during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n= 5-6 for all groups).

Group	Exposure		Total nr. cells		Macro phages		Neutrophils	Lymphocytes
1	Sham	Mean	2487		1809		384	292
		S.D.	411		250		173	138
2	Sham/MCT	Mean	1128		900		105	135
		S.D.	267		274		59	68
3	Ultrafine	Mean	1798		1478		189	141
		S.D.	285		196		135	48
4	Ultrafine/MCT	Mean	3460	1	2678	1	473	327
		S.D.	772		563		132	123
5	Fine	Mean	1997		1827		153	47
		S.D.	407		426		93	22
6	Fine/MCT	Mean	2249		1837		148	283
		S.D.	671		588		43	109
	└ANOVA p value		0.038		0.041		0.313	0.071

* 1t/m5 =significantly different from the group indicated by the figure next to the mean value of a parameter, Bonferroni P < 0.05

Table 3b. Cellular determinations measured in BALF of healthy and PH rats exposed to fine or ultrafine ammonium bisulfate (II) aerosols during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n= 5-6 for all groups).

Group	Exposure		Total number (x 10e3)	Macro phages (x 10e3)	Neutrophils (x 10e3)	Lymphocytes+ eosinophils (x 10e3)
1	Sham	Mean	1242	1168	47	27
		S.D.	764	662	51	44
2	Sham/MCT	Mean	1438	1257	88	39
		S.D.	574	394	137	70
3	Ultrafine	Mean	954	897	12	3
		S.D.	244	217	11	3
4	Ultrafine/MCT	Mean	1352	1245	58	11
		S.D.	534	474	71	15
5	Fine	Mean	1117	941	107	17
		S.D.	425	266	223	36
6	Fine/MCT	Mean	1354	1222	103	29
		S.D.	387	293	95	46

* 1t/m5 =significantly different from the group indicated by the figure next to the mean value of a parameter Bonferroni P < 0.05

└ANOVA p value all > 0.05

Table 3c. Cellular determinations measured in BALF of healthy and PH rats exposed to fine or ultrafine ammonium ferrosulfate aerosols during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n= 5-6 for all groups).

Group	Exposure		Total number (x 10e3)	Macro phages (x 10e3)	Neutrophils (x 10e3)	Lymphocytes+ eosinophils (x 10e3)
1	**Sham**	**Mean**	1248	1211	33	4
		S.D.	237	225	29	2
2	**Sham/MCT**	**Mean**	1645	1045	455	154 1
		S.D.	441	200	227	68
3	**Ultrafine**	**Mean**	1264	928	286 1	45 1
		S.D.	304	195	117	21
4	**Ultrafine/MCT**	**Mean**	1878	1364	505	80 1
		S.D.	662	378	286	30
5	**Fine**	**Mean**	734	731	18	2
		S.D.	136	136	6	1
6	**Fine/MCT**	**Mean**	1664	1192	411 5	40 1,5
		S.D.	801	448	335	10

* 1t/m5 =significantly different from the group indicated by the figure next to the mean value of a parameter
Bonferroni P < 0.05

L ANOVA p value	0.476	0.659	0.0143	0.0001

Table 3d Cellular determination measured in BALF of healthy and PH rats exposed to fine or ultrafine amonium nitrate aerosols during 4 hr/dag for 3 consecutive days. Data are expressed as means ± SD (n= 5-6 for all groups)

			Total	Macro	Neutrophils	Lymphocytes		
			nr. cells	phages				
Group	**Exposure**							
1	**Sham**	**Mean**	1075	ND	ND	ND		
		S.D.	611					
2	**Sham/MCT**	**Mean**	1333	ND	ND	ND		
		S.D.	540					
3	**Ultrafine**	**Mean**	1697	369.0	15	13		
		S.D.	950	59.0	32	26		
4	**Ultrafine/MCT**	**Mean**	2032	332.0	29	33		
		S.D.	430	31.0	14	21		
5	**Fine**	**Mean**	1832	331.0	37	23		
		S.D.	999	53.0	53	16		
6	**Fine/MCT**	**Mean**	1358	263.0	63	70		
		S.D.	471	60.0	47	63		
* 1t/m5	=significantly different from the group indicated by the figure next to the mean value of a parameter							
	Bonferroni P < 0.05							
	└ANOVA	p value	0.132	0.191	0.096	0.0069		
	ND= not determined							