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### Authors

Kleinman, MT  
Mc Clure, TR  
Mautz, WJ  
[et al.](#)

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Peer reviewed

**PART 1**  
**INHALED PARTICLES SYMPOSIUM**  
**SECTION 4**  
**BIOLOGICAL REACTIONS TO DUST**



## EFFECTS OF INHALED OXIDANT AND ACIDIC AIR POLLUTANT COMBINATIONS ON NASAL AND TRACHEAL TISSUES IN EXERCISING RATS

MICHAEL T. KLEINMAN, THOMAS R. MCCLURE, WILLIAM J. MAUTZ,  
ROBERT F. PHALEN and T. TIMOTHY CROCKER

Department of Community and Environmental Medicine, University of California, Irvine CA 92717

**Abstract**—Groups of rats inhaled mixtures of O<sub>3</sub> and acid particles for three hours while exercising. One group received purified air, a second group breathed 0.6 ppm O<sub>3</sub>, a third group breathed a mixture: SO<sub>2</sub> (5 ppm) plus sulphuric acid aerosol containing iron and manganese ions (1 mg/m<sup>3</sup>, 0.5 μm mass median aerodynamic diameter). The fourth and fifth groups breathed the above mixture plus 0.6 ppm O<sub>3</sub>; group 4 was exposed while exercising and group 5 was exposed at rest. Tritiated thymidine (<sup>3</sup>HT) was injected into the rats 17 hours post-exposure; lungs, trachea, and nasal airways were fixed and sectioned. Focal lesion areas were measured in the parenchyma and <sup>3</sup>HT uptake by replicating cells in the upper airways was determined by autoradiography. Significant amounts of label were incorporated by squamous and respiratory epithelia of the nose, but little uptake was observed in the olfactory or tracheal tissue. In the squamous tissue none of the exposures resulted in labelling rates in excess of those observed in rats exposed to purified air. In respiratory epithelium, however, the acid-containing multicomponent atmospheres significantly increased labelling rates above background in animals exposed while exercising. O<sub>3</sub> alone did not affect <sup>3</sup>HT uptake, nor did the presence or absence of O<sub>3</sub> in the multicomponent atmosphere alter the observed uptake rates.

### INTRODUCTION

INHALED TOXIC AGENTS may damage respiratory tract epithelium, but the degree of injury depends upon the chemical nature of the pollutant, its dose at the site of injury, and the relative sensitivity of the tissue. Under normal conditions, damaged tissue is repaired by replacement of injured or killed cells. This cellular proliferation represents an acceleration of normal cell regeneration processes (OEHLERT, 1973). During proliferation DNA precursors are taken up by cells. By introducing labelled nucleotides it is possible to radioactively 'tag' these cells and subsequently map their locations in the airways by autoradiography (EVANS and BILS, 1969).

In distal bronchii and bronchiolar airways, the ciliated cells are among the most easily damage. In the alveoli the cells of greatest vulnerability are often the Type I epithelial cells, but there may also be damage to vascular endothelial and other cells. At ambient episodic concentrations of O<sub>3</sub> (0.4 to 0.8 ppm), for example, the lung parenchyma of rats exposed for 4 hr exhibits focal lesions; walls of alveoli are thickened and there are cellular debris and infiltrating cells in the alveoli, alveolar ducts and the alveolar interstices (PLOPPER *et al.*, 1973). Pollutant mixtures do not in general act in simple additive fashions, and atmospheres containing O<sub>3</sub> in combination with acidic gases and particles produce more damage in lung parenchyma than the sum due to each component alone (KLEINMAN *et al.*, 1985a). These findings have stimulated us to examine the effects of acid-containing pollutant mixtures on the trachea and upper respiratory tract nasal tissues.

The atmospheres tested included purified air, O<sub>3</sub> alone (0.6 ppm), and a mixture containing 0.6 ppm O<sub>3</sub>, 5.0 ppm sulphur dioxide (SO<sub>2</sub>), and 1 mg/m<sup>3</sup> of sulphuric acid aerosol (SAM) to which iron and manganese ions were added to catalyse reactions between the gases and particles (KLEINMAN *et al.*, 1985b). The animals were exposed at rest or while exercising for 3 hr.

#### METHODS AND MATERIALS

*Atmosphere Generation and Characterisation.* Atmospheric mixtures were introduced into an enclosed 10 channel treadmill (MAUTZ *et al.*, 1985). Ten rats were exposed simultaneously under well-controlled environmental conditions while running at a predetermined speed and grade level. Mixed expired respiratory gases were analysed for O<sub>2</sub> uptake and CO<sub>2</sub> production; these data provided objective confirmation of the metabolic work load.

SO<sub>2</sub> was metered into highly purified air, which was maintained at a relative humidity of 85%, to achieve a concentration of 5 ppm. O<sub>3</sub> (0.6 ppm) was generated from medical grade oxygen using a corona-discharge unit. Aerosols were generated from solutions containing the desired constituents using a Collison nebulizer. The droplets were brought to equilibrium with 85% relative humidity (RH) and passed through a <sup>85</sup>Kr static discharger.

Gas concentrations were monitored throughout the exposures, SO<sub>2</sub> with a pulsed fluorescence detector (ThermoElectron Corp. Model 43) and O<sub>3</sub> by ultraviolet light absorption (Dasibi Environmental Corp., Model 1003-AH). Aerosol samples were collected and weighed on acid-washed, distilled water rinsed, quartz fibre filters. Size-fractionated aerosol samples were obtained using an eight-stage Sierra cascade impactor. Filter and impactor samples were ultrasonically extracted in aqueous media. Aliquots were analysed for sulphate and other anions by ion chromatography, for Fe and Mn by atomic absorption spectrophotometry, and for hydrogen ion concentration by potentiometry. An electrical aerosol size analyser (TSI Model 3030) provided real-time particle size and concentration data to assess the stability of the aerosol atmospheres during each study.

*Exposure Protocol.* Six-week old barrier-reared male Sprague-Dawley rats (Hilltop Lab Animals, Inc.) were delivered to the laboratory in filtered shipping containers. The rats were housed in a laminar-flow air barrier system for one week prior to exposure. Microbiological assays supplied by the breeder and histopathological examinations performed at our laboratory indicated that the rats were generally free of major respiratory infections. Of the 50 animals studied, the lungs of 4 exhibited signs of intercurrent infection; data from these 4 rats were not included in the analyses.

Rats were randomly assigned to one of five groups (10/group); the first 4 groups were exposed while exercising to a) purified air, b) O<sub>3</sub> alone, c) SAM alone, and d) O<sub>3</sub> + SAM. The last group was exposed at rest to e) the O<sub>3</sub> + SAM mixture.

*Histopathological Examination.* Animals were injected intraperitoneally with <sup>3</sup>HT 17 hr post-exposure. After 1 hr, the rats were anaesthetised and killed by exsanguination. The lungs were excised and fixed by airways perfusion with 10% neutral-buffered formalin for 72 hours at 30 cm fluid pressure. A portion of trachea with attached larynx and the intact nasal cavity were fixed by immersion in formalin under a slight vacuum.

Lung tissues were embedded in paraffin, sectioned at 6 μm, and stained with haematoxylin and eosin. The proportion of lung area with focal lesions typical of O<sub>3</sub>

damage was evaluated (ELIAS and HYDE, 1980). Lesions were classified into two types. Type 1 lesions were characterised by free cells in alveolar spaces but with no change in septal walls. Type 2 lesions were characterised by thickening of alveolar walls and evidence of interstitial cellular infiltration with or without free cells in alveolar spaces. In our assay, there is generally a low background level of Type 1 lesion, even in rats exposed only to purified air. By contrast, Type 2 lesions are never seen in rats exposed to purified air, but are present in rats exposed to pollutant atmospheres.

Trachea was split longitudinally, embedded in glycol methacrylate, and sectioned at 2  $\mu\text{m}$ . Specimens of decalcified nasal cavity, 2 to 3 mm thick, were cut vertically through the hard palate and nasal septa. This block, which had a dorsal-ventral orientation, was embedded anterior face down in glycol methacrylate and sectioned at 2  $\mu\text{m}$ . Rates of cell turnover in the trachea and head sections were evaluated using autoradiography (Kodak NTB-2 nuclear track emulsion). The slides were developed and stained with toluidine blue. Four distinct cell types were differentiated in this plane of the nasal sections examined, squamous epithelium (S), olfactory epithelium (OL), and two types of respiratory epithelium. Of the last-mentioned, one, designated R, is a high pseudostratified columnar epithelium with ciliated and goblet cells and is located over the medial surfaces of the turbinates. The second, designated R1, is simple columnar with ciliated, but without goblet, cells and is found in the maxillary fossae lateral to the turbinates.

The total numbers of cells were estimated by aligning a microscope eyepiece grid with the basement membrane and counting all cells within one of ten randomly selected grid spaces. This process was repeated 10 times and an average number of cells per unit area computed. The numbers of cells labelled with  $^3\text{HT}$  in 100 such unit areas were determined and the labelling index computed as the percent of the labelled to total cells, for each type of epithelium.

*Statistical Analysis.* Group mean parenchymal lesion areas and cell labelling indices for upper respiratory epithelium were contrasted between exposure conditions using analysis of variance. Tukey's multiple comparison test was used to test whether differences in effects on tissues between the experimental condition on a pairwise basis were significant at the  $\alpha \leq 0.05$  level.

## RESULTS

The group means of Type 1 and Type 2 parenchymal lung lesion areas in each of the five groups of exposed rats are shown in Figure 1. The relative standard errors are not shown; the statistical significance of observed differences between the means was evaluated using analysis of variance and Tukey's multiple comparison test. All of the pollutants tested yielded greater areas of Type 1 lesion than observed in the clean air (control) group; however, the differences were significant ( $\alpha \leq 0.050$ ) only for the groups exposed while exercising to  $\text{O}_3$  alone or to the complex mixture containing  $\text{O}_3$ , sulphuric acid aerosol, catalytic metal ions, and sulphur dioxide ( $\text{O}_3 + \text{SAM}$ ). The  $\text{O}_3 + \text{SAM}$  mixture exposure yielded significantly ( $\alpha \leq 0.05$ ) greater involvement of the lung parenchyma in Type 1 and Type 2 lesions. The lungs of rats exposed to the mixture, at rest, or to the mixture without  $\text{O}_3$  were not significantly different from those of the control group.

The rates of cell turnover in nasal and tracheal epithelial tissue as determined from

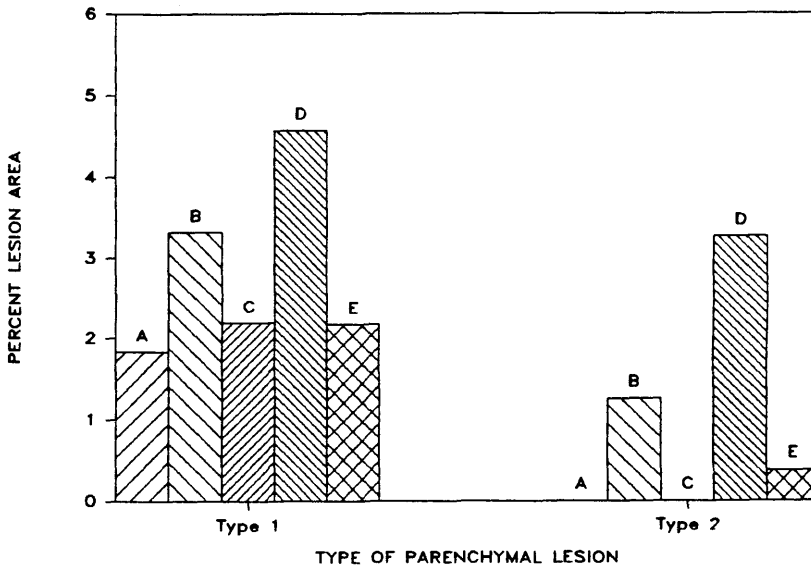


FIG. 1. Lesion areas (as percent) in lung parenchymal tissue in rats exposed while exercising to: A) purified air; B) 0.6 ppm O<sub>3</sub>; C) 5 ppm SO<sub>2</sub> + 1 mg/m<sup>3</sup> sulphuric acid aerosol with Fe and Mn ions (SAM); D) 5 ppm SO<sub>2</sub> + SAM + 0.6 ppm O<sub>3</sub>. Rats in group (E) were exposed at rest to the same atmosphere as in (D).

autoradiographic evaluation of the cell labelling index are shown in Figure 2. The analyses of variance showed that there were no significant differences in cell labelling index values attributable to exposure atmospheres in the R, O or tracheal epithelium. The group mean labelling index for squamous tissue showed that there was a slight but

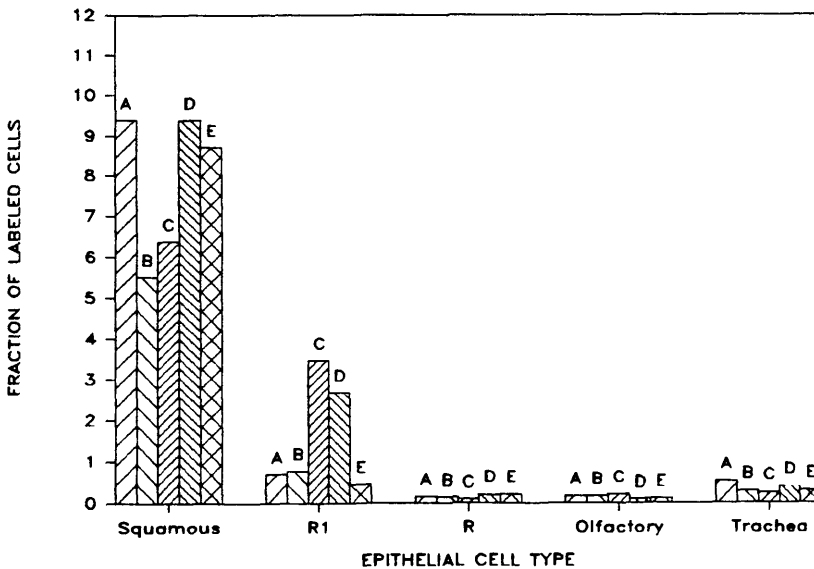


FIG. 2. Cell turnover rates (as fraction of epithelial cells labelled with <sup>3</sup>HT) in nose and trachea in rats exposed while exercising to: A) purified air; B) 0.6 ppm O<sub>3</sub>; C) 5 ppm SO<sub>2</sub> + 1 mg/m<sup>3</sup> sulphuric acid aerosol with Fe and Mn ions (SAM); D) 5 ppm SO<sub>2</sub> + SAM + 0.6 ppm O<sub>3</sub>. Rats in group (E) were exposed at rest to the same atmosphere as in (D).

statistically significant ( $\alpha \leq 0.05$ ) reduction in cell turnover in pollutant-exposed animals relative to the controls. The Tukey multiple comparison test was applied to all possible pairs of exposure conditions and showed, however, that none of the treatments, when considered individually, were different from the controls or from any of the other treatments. Striking and significant differences were observed in the R1 tissue. The animals exposed while exercising to the acid/SO<sub>2</sub>-containing atmospheres (SAM and SAM + O<sub>3</sub>) exhibited significantly greater rates of cell turnover than rats exposed to either O<sub>3</sub> alone, clean air, or to the acid/SO<sub>2</sub> mixture at rest.

## DISCUSSION

At breathing frequencies and tidal volumes typical of the resting state, the human nose collects 50% or more of inhaled particles with aerodynamic median diameters greater than about 4  $\mu\text{m}$  (RAABE, 1982). The nose is also an efficient scrubber of water soluble gases such as SO<sub>2</sub> and formaldehyde from inspired air. At the breathing frequencies and rates of minute ventilation typical of the exercising state the particle collection efficiency of the upper respiratory airways is increased; however, larger fractions of gaseous pollutants can penetrate deeper into the respiratory tract (YOKOYAMA and FRANK, 1972; KLEINMAN, 1984). It is likely that these generalisations apply to rats as well as humans.

Exposure of exercising rats to an atmospheric mixture containing sulphuric acid aerosol and SO<sub>2</sub> did not result in increased area of parenchymal lesions relative to controls. When O<sub>3</sub> was added to the mixture, larger lesions were formed, reaching about 2.5 times the size observed in rats exposed to O<sub>3</sub> alone and at the same exercise level. Exercise played an important role; rats exposed to the acid/O<sub>3</sub> mixture at rest did not exhibit differences in size of lesions. In nasal tissue, we observed a somewhat different pattern. Ozone, which has a relatively low solubility in water and which is not efficiently absorbed in the upper respiratory tract, did not cause any significant increase in cell turnover rates, as measured by cell labelling with <sup>3</sup>HT. The type R1 respiratory epithelium in the noses of rats exposed while exercising was markedly affected by the atmospheres which contained the sulphuric acid/SO<sub>2</sub> mixture. When O<sub>3</sub> was added to the mixture, the labelling index was not significantly different. The mixture had no significant effect on rats exposed at rest. It is uncertain whether the observed effects were attributable to the sulphuric acid, to the SO<sub>2</sub>, or to some synergism between these components. The available data do not directly address this question, but our laboratory has in the past exposed rats at rest to up to 40 ppm of SO<sub>2</sub> with no significant increase in the labelling index of R1 epithelium. The exercise level in our present study represented about a two-fold increase in ventilation above the resting rate. Hence, if the nose were a perfect collector of SO<sub>2</sub>, the deposited amount in an exercising rat might be twice that in a resting rat or, in the case of the present 5 ppm exposure concentration, equivalent to an exposure to 10 ppm at rest. Since 40 ppm SO<sub>2</sub> had no effect at rest, it appears unlikely that 5 ppm in exercise could be responsible for the increase in cell turnover observed in the rats exposed to acid/SO<sub>2</sub>.

ALARIE (1973) reported that inhalation of sensory irritants caused reflex-driven changes in breathing patterns, that animals which inhaled substances such as sulphuric acid or SO<sub>2</sub> shifted to a pattern of slow deep breathing. This type of pattern might result in improved collection of gases such as SO<sub>2</sub> in the upper respiratory tract, but could



allow greater numbers of small particles as well as gases of low water solubility such as O<sub>3</sub> to penetrate to the parenchyma more readily. The observations of damage to lung parenchyma in this study are consistent with the above suppositions. At the concentrations and exercise level employed, exposure to O<sub>3</sub> alone had a significant effect, but that effect was substantially augmented when both O<sub>3</sub> and the acid/SO<sub>2</sub> were present. The acid/SO<sub>2</sub> without O<sub>3</sub> had no effect, suggesting that the augmentation is due to increased penetration and deposition of O<sub>3</sub> in the parenchyma when acidic pollutants are present, that the co-pollutants may affect tissue which has been initiated in some way by O<sub>3</sub>, or that an intermediate chemical product, such as sulphites or free radicals, might be more potent than O<sub>3</sub> alone.

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## DISCUSSION

M. LIPPMANN: Did upper respiratory regions, other than  $R_1$ , show any effects of pollutant exposure?

M. T. KLEINMAN: We saw cell turnover rates of significant numbers in the squamous cells, but none of the atmospheres were significantly different from control. None of the atmospheres caused any change in the trachea, the olfactory, or the type R. The only one that really showed a significant response, when you looked at specific exposure condition versus any others, was the type  $R_1$ .

J. F. HICKS: Some recent modelling studies have implied that acid sulphate may be neutralised by ammonia originating from bacteria in the mouth. Could this be a factor in your results?

M. T. KLEINMAN: In this particular type of study, rats are basically nose-breathers, so that ammonia neutralization of the inhaled materials is probably not an important factor in the rat exposure. I have done some work with exposure of humans to sulphuric acid, and in chamber studies we have actually seen significant amounts of neutralization, but the amount of ammonia generated by a human depends on a number of factors including what they had for breakfast. The number of teeth they have, in fact, plays an important role, so I think that would be a very variable sort of factor.

J. A. SIMMONS: Alveolar cells can repair fairly efficiently after an acute insult. Your results, taken at one fixed time after the induction of the damage, do not appear to take this into consideration. Are your conclusions really valid?

M. T. KLEINMAN: It is actually 18 hours after the exposure that we injected the thymidine, and then one hour later killed the animals. We have found that the peak of ozone-type damage occurs somewhere between 24 and 48 hours, so that the results we showed were possibly even less exaggerated than what we would have seen if we had waited for the damage to actually peak in the parenchyma. If we waited any longer we would have lost the effect on the upper airways, because repair mechanisms there seem to operate much more quickly.

F. F. HAHN: Did you look at the histopathology of the nasal cavity? How do you evaluate increased cell turnover as an injury, where does it fall on the scale of lesions, is it a more sensitive method of evaluating lesions?

M. T. KLEINMAN: We did look for any evidence of injury, using optical light microscopy. On that scale, we did not see any real evidence. Going to an electron microscopic evaluation in previous studies, where cell turnover was accelerated, we found a tremendous disorganisation of the ciliated cells. Cilia were just all snarled up, many were destroyed and sloughed off. I evaluate the cell turnover picture as being representative of the tissues response to killed or injured cells that are no longer viable, and these are being replaced by cells from neighbouring un-injured areas.

F. F. HAHN: Is this a relatively mild injury you are talking about then? Is this a more sensitive way to document injury? Is that what you are putting forward here?

M. T. KLEINMAN: I think it is a sensitive way to document and localize injury. I also think that continuous damage to these tissues could lead eventually to significant long-term chronic effects; certainly with exposure to certain materials you can get nasal cancers. It also seems that when you irritate the upper airways, you change the breathing pattern of many different animal species. This occurs in humans, it occurs in dogs, and we have observed it in rats and a number of other rodent species. When you change this breathing pattern, for example, in general these upper airways irritants tend to cause animals to breath more slowly and more deeply, and what we have actually seen is that this will in fact increase the amount of ozone that can penetrate to the deep lung. Damage to the upper airway can actually have some sort of effect on the dose and dose distribution to other parts of the respiratory tract.

A. G. HEPPLESTON: To which cell types do you attribute the thickening of alveolar walls in the type 2 lesion? In gauging 'replication' of cells or 'cell turnover' you relied on the 3HT labelling index, whereas cell population studies also require the stathmokinetic technique. Was this employed?

M. T. KLEINMAN: We had seen macrophages mobilised within the lung. Much of what we see in terms of the free cells can either be injured type 1 cells that have been sloughed-off, macrophages that have been mobilised into the lung or possibly some polymorphs that appear there.