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In vivo and in vitro effect of ozone and formaldehyde on human nasal mucociliary transport system*

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SUMMARY

The effect of ozone and formaldehyde on the nasal mucociliary transport system after short-term exposure was comparatively evaluated in human by using in vivo and in vitro test systems. Concentrations of ozone used were 10, 100, 500 and 1000 μg/m³ of ozone and of formaldehyde 100, 500 and 5000 μg/m³. The in vivo effect of ozone was monitored by measuring the saccharin transport time before and two hours after exposure to ozone. The in vitro effect of ozone and formaldehyde was evaluated by quantifying the ciliary beat frequency (CBF) of isolated respiratory epithelial cells before and after one, two, and three hours of exposure. Control experiments were performed using synthetic air.

Ozone had no effect on the human nasal mucociliary transport system under the conditions tested here. Neither in vivo nor in vitro any significant changes of saccharin transport time nor CBF were measured. In contrast, formaldehyde significantly reduced (67.1%) CBF at the highest dosis (2 hours, 5000 μg/m³). These results will be discussed according to the environmental impact of ozone and formaldehyde in air pollutants and compared to sulphur dioxide and nitric oxide, which were tested under similar conditions, and to results revealed from animal experiments.

Key words: ozone, formaldehyde, human nasal epithelium, CBF

INTRODUCTION

The upper respiratory tract is the primary contact organ of inhaled bacterial, viral, chemical, and other air pollutants. The mucociliary transport system belongs to the physical barrier of the upper respiratory tract and is part of the non-immunological defense system by adsorbing, neutralising and eliminating inhaled pollutants (Deitmer, 1989; Hee and Guilerm, 1977; Procter, 1983; Toremalm, 1985).

Inhaled volatile and partially volatile environmental chemicals can damage the respiratory tract after resorption. Local toxic effects on the mucosal epithelia are implicated in the pathophysiologic disturbance of the mucociliary transport system, causing bronchial obstruction and inflammation (Brooks et al., 1985; French et al., 1973; Magnussen et al., 1989; McManus et al., 1989; Wagner, 1994).

The effectiveness of nasal mucociliary clearance depends on the co-ordinated ciliary beat of the respiratory epithelial cells, the mucus level, which adsorbs and transports inhaled irritants, and the composition of the mucus. Disturbances of the mucociliar clearance are caused by change of the CBF and/or co-ordination of the ciliary beat (Riechelmann et al. 1992; Robson et al., 1992). Furthermore the change of viscoelastic properties in the mucus was shown to influence the nasal mucosal clearance (King, 1980).

The aim of this study was to evaluate the effect of variable doses of ozone and formaldehyde on the mucociliary transport system in vivo and in vitro. The combination of examination techniques such as the saccharin transport time and measuring of the CBF, can be used to more exactly evaluate possible harm of the mucociliary transport system (Karnitzki, 1993).

Therefore, we measured the nasal mucociliar clearance by performing the saccharin test after exposing healthy volunteers with ozone in variable concentrations. The results were compared to the CBF of ciliated human respiratory epithelial cells exposed in vitro to the same dose of ozone. Furthermore, the in vitro effect of formaldehyde on CBF was analysed.

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MATERIALS AND METHODS

Volunteers

In vivo exposure to ozone was performed with eight volunteers, two women and six men, aged 42 (14 years. All of them were healthy non-smokers without a nasal allergy, chronic or acute respiratory diseases. Ciliated nasal epithelial cells for in vitro exposure were collected from twelve volunteers, five women and seven men, aged 38 (15 years. All of these volunteers were healthy non-smokers without a nasal allergy, chronic or acute respiratory disease. The experiments were done in accordance to the local ethic committee. The volunteers gave their written informed consent before participating in the experiments.

Saccharin test

To realise possible effects of ozone in vivo the saccharin test was performed as published recently (Riechelmann et al., 1992). Briefly, a saccharin particle with a diameter of 0.5 mm was placed on the bottom of the nasal cavity, below the head of the inferior turbinate. The volunteers were instructed to swallow all 30 seconds, then the time was noted until the volunteer noticed a sweet taste (Anderson et al., 1974; Riechelmann et al., 1992).

Collecting respiratory cells and measuring of ciliary beat frequency

For in vitro exposure ciliated epithelial cells of the human nasal mucosa were collected from the bottom of the nasal cavity using a house curette with a head diameter of 3 mm (Riechelmann et al., 1992). With this technique the three main surface cell types of the respiratory tissue, goblet cells, ciliated cells and brush cells were gained. The cells gathered by this were freshly taken for measuring the CBF and for exposure.

For visualisation and measurement of the CBF the cells were placed on a cover glass and suspended with 100 µl of RPMI-1640. Thereafter the cover glass was placed upside down on a slide with a hollow grinding. The slide was placed on a heated microscopetable (37°C). Before CBF was measured, cells were conditioned for 15 min to the medium and to temperature. Then the ciliated cells were visualised using an interference contrast microscope (Leitz Orthoplan ICTL, Leitz, Wetzlar) connected to the AVC-D1 CCD video camera and monitor (Sony Deutschland GmbH, Köln), CBF was documented with a video recorder AG-6720 (Matsushita Deutschland GmbH, Germany) connected to the AV-TC 15 time code generator (Alpermann & Velte GmbH, Remscheid, Germany). Each sample was recorded for ten seconds at ten locations (s. figure 2). CBF was calculated as published recently using the slow motion of the video recorder (Hafner et al. 1997; Riechelmann et al., 1994, 1992; Kienast et al., 1994, 1993). This procedure was carried out before and after one and two hours of exposure.

Exposure to ozone in vitro and in vivo

For the in vitro experiments ciliated respiratory epithelial cells were first placed on a cover glass to quantify the CBF before exposure, as described before. Thereafter, cells were transferred on inserts with semipermeable porous polycarbonate-membranes (Falcon, Becton-Dickinson, Heidelberg, Germany) which were placed in 6 well cluster plates (Nunc, Wiesbaden, Germany). Thus the primary respiratory epithelial cells were cultivated on monolayer like conditions. Beneath the insert bottom RPMI-1640 was used as culture medium (Dulbeccos, Eggenstein, Germany). The plates were located in a borosilicat glass chamber which was heated up to 37°C and humidified up to 99% by warm water. Temperature and humidity were checked continuously with a testoterm 601 (Testo, Lenzkirch, Germany) (s. schematic drawing of figure 2a). To gain steady-state

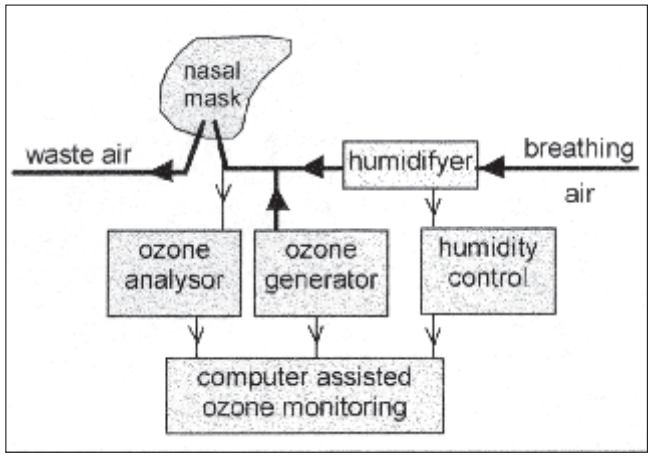

Figure 1: Schematic drawing of the in vivo ozone exposure system.

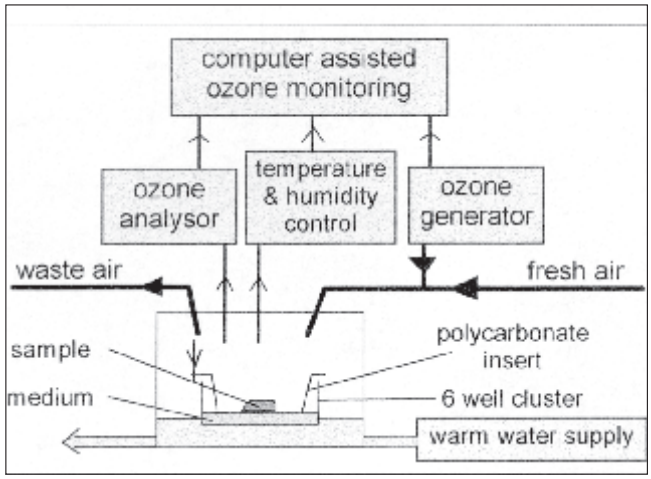

Figure 2a: Schematic drawing of the in vitro ozone exposure system.

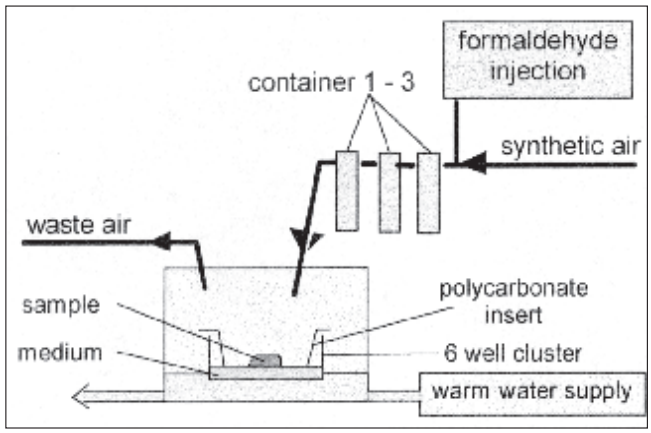

Figure 2b: Schematic drawing of the in vitro formaldehyde exposure system.

conditions, the chamber was put in operation two hours before starting the exposure. The cells were exposed on an air liquid interface to ozone (synthetic air, 10, 100, 500 and 1000 μg/m^3). After one, two and three hours, cells again were transferred to the cover glass on the microscopetable to determine CBF.
The in vivo exposure to ozone was performed using an inhalation mask, covering only the nose. The ozone was injected in a breathing gas current of 40 l/min. The upstream flow was monitored with an rotameter (Dräger, Lübeck, Germany). The concentration of ozone was tested directly in front of the nasal inhalation mask (s. schematic drawing of figure 1). To prevent ozone depletion, all non biological materials were made of inert materials like teflon or glass.

Ozone was generated with an Ozone-Generator-1030 (UPM, Langöns, Germany) using the principle of silent electric arc discharge. The current ozone concentration was monitored with an ozone analyser, type O3 (EKU, Leiningen, Germany). Because of the high reactivity of ozone and short half-life of a few minutes, gas samples were taken directly above the cell cultures by suction through glass and teflon tubing. The ozone concentration we measured had a variance of +/– 10%. The concentration of ozone was automatically quantified all seven minutes. The exposure chamber was put in operation two hours before starting the actual exposure of ciliated respiratory cells.

Exposure to formaldehyde in vitro
For exposure to formaldehyde (synthetic air, 100, 500 and 5000 μg/m^3) the same exposure condition technique was used as described for Ozone. To achieve equilibrium of formaldehyde the exposure chamber was put in operation two hours before starting the exposure of the ciliated respiratory cells. A constant concentration in the chamber was measured after 1 hour. Exposure was carried out for 1 hour and 2 hours. To rule out effects of formaldehyde which might had been dissolved in the culture medium during the time of exposure, a 6- well plate was exposed to 5000 μg/m^3 for two hours. In this culture medium a concentration of 0.6 μg/ml was analysed. The determination of formaldehyde was done photometrically by a selective colour reaction of formaldehyde with chromotropic acid (Lange & Véjdeleck 1980). To exclude effects from this formaldehyde concentration in the culture medium, ciliated cells were exposed for two hours with a medium containing a formaldehyde concentration of 1μg/ml and a formaldhyde-free medium. The cells were cultivated on an air-liquid-interface as described before. One group of ciliated cells was cultivated with formaldehyde enriched (1μg/ml) medium at the beginning of exposure with synthetic air. The other group of ciliated cells was continuously perfused with formaldehyde enriched medium (1μg/ml) to achieve a constant formaldehyde concentration over a period of two hours. Compared to cells exposed to for-

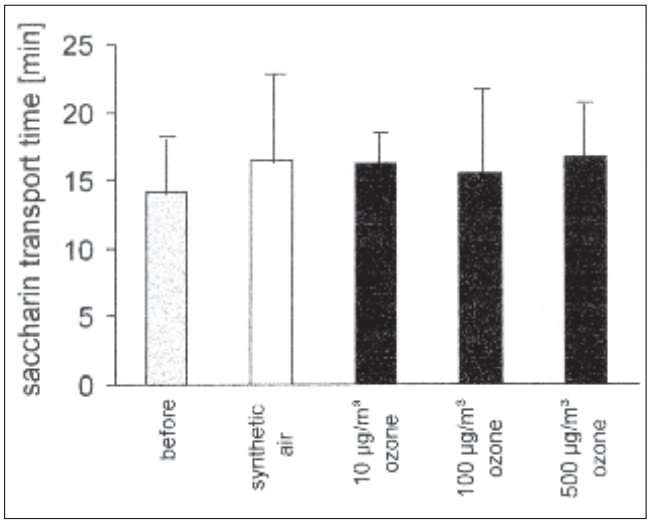

Figure 3: Saccharin transport time after ozone exposure
The in vivo effect of ozone on the saccharin transport time was measured using variable concentrations of ozone. Ozone was exposed for two hours. Results are given as mean of 8 healthy volunteers, bar indicating SEM.

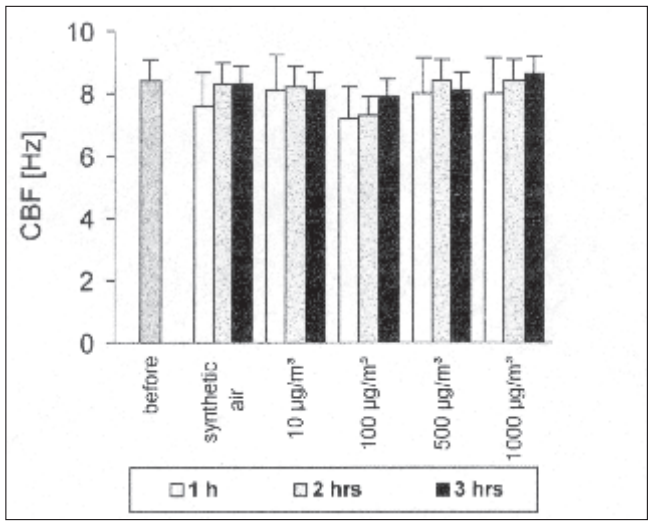

Figure 4: Ciliary beat frequency after ozone exposure
The in vitro effect of ozone on CBF was documented using variable concentrations of ozone and variable times of exposure. CBF of samples from 12 healthy volunteers were measured. Results are given as mean, bar indicating SEM.

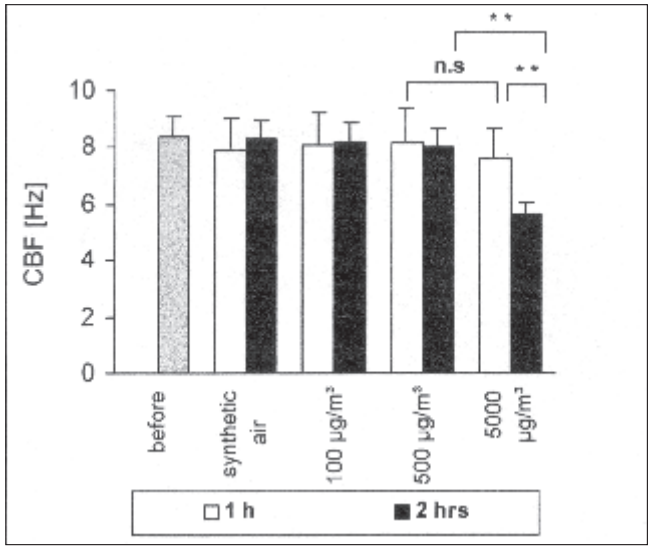

Figure 5: Ciliary beat frequency after formaldehyde exposure
The in vitro effect of formaldehyde on CBF was documented using variable concentrations of ozone and variable times of exposure. CBF of samples from 12 healthy volunteers were measured. Results are given as mean, bar indicating SEM.

maldehyde-free medium, there were no significant changes of CBF, neither in the group exposed with the enriched medium at the beginning of exposure nor in the group exposed with enriched medium by continuous perfusion.

Formaldehyde concentrations were achieved using the technique of the continuously injection of calibrating gas according to the guidelines of the VDI (VDI-Richtlinien, 1991). In brief, the solution of formaldehyde (Merck, Darmstadt, Germany) was continuously injected into a synthetic air gas flow. The formaldehyde gas was guided through three gas containers achieving a homogenous mixture of formaldehyde gas before cultivated cells were exposed (s. schematic drawing of figure 2b). The concentrations of formaldehyde were calculated by the formula:

$$C\ [\mu g/m^3] = (FA[g/l] \times FFA[ml/h] \times 10^6) / (60 \times FSA[ml/min],$$

(C = concentration of formaldehyde in $\mu g/m^3$, FA = formaldehyde, FFA = flow of injected formaldehyde solution and FSA = flow of synthetic air)

Formaldehyde concentrations were monitored using a Multigasmonitor-1302 (Bruel & Kjær, Naerum, Danmark).

RESULTS

In vivo effect of ozone on saccharin transport

Eight healthy volunteers were exposed to ozone with concentrations of 10, 100, and 500 $\mu g/m^3$ for the time of two hours. None of the chosen concentrations of ozone caused any significant effect on saccharin transport time. The saccharin transport time was 14.2±4.8 before and 16.5±5.8 minutes after exposure to synthetic air. Exposure to ozone with 10, 100, and 500 $\mu g/m^3$ revealed a saccharin transport time of 16.2±2.5, 15.5±6.4, and 16.8±3.7 minutes, respectively (Figure 3). Focusing on nasal mucociliary clearance no in vivo effect of ozone was measured.

In vitro effect of ozone and formaldehyde on CBF

Isolated ciliated nasal epithelial cells were exposed in vitro to variable concentrations of ozone (10, 100 500, and 500 $\mu g/m^3$) and for variable times of exposure (1, 2, and 3 hours). These in vitro experiments also showed no significant effects of ozone at any dose of ozone. CBF before exposure to synthetic air was 8.2±0.8 Hz, after exposure for 1, 2, and 3 hours CBF were 7.6±1.1, 8.3±0.6, and 8.3±0.6 Hz.

Exposure to 10 $\mu g/m3$ ozone for 1, 2, and 3 hours resulted in CBF of 8.1±0.1, 8.2±0.6, and 8.1±0.7 Hz, respectively. After exposure to 100 $\mu g/m^3$ ozone for same time intervals CBF were determined with 7.2±0.8, 7.3±0.6, and 7.9±0.6 Hz. Exposure to 500 $\mu g/m^3$ ozone for the times mentioned above CBF were measured with 8.0±0.8, 8.4±1.1, and 8.1±0.9 Hz. The CBF after exposure to 1000 $\mu g/m^3$ of ozone for indicated times were 8.0 ±1.6, 8.4±1.1, and 8.6±0.9 Hz. Figure 4 summerizes these data.

Concentrations used for exposure with formaldehyde were 100, 500 and 5000 $\mu g/m^3$. Exposure times were 1 hour and 2 hours. Only the exposure for 2 hours to 5000 $\mu g/m^3$ formaldehyde decreased highly significant ($p > 0.01$) (5.3±2.5Hz) the CBF of isolated ciliated nasal epithelial cells whereas after 1 hour of exposure no significant decrease of CBF (7.6±1.1 Hz) was measured. The exposure for 1 or 2 hours to 100 $\mu g/m^3$ formaldehyde (8.1±0.7 and 8.3±0.7 Hz, respectively) or 500 $\mu g/m^3$ formaldehyde (8.2±0.6 and 8.0±0.8 Hz, respectively) had no effect on CBF. The data are presented in figure 5.

DISCUSSION

The effect of ozone on the nasal mucociliary transport system was evaluated in vivo and in vitro. All results were compared to exposure with synthetic air. For none of the chosen concentrations of ozone (10, 100, 500, and 1000 $\mu g/m^3$) any effect was measured, neither in vivo nor in vitro, by investigating the saccharin transport time or CBF, respectively. Lower doses of formaldehyde had no effect on CBF whereas the highest doses (5000 $\mu g/m^3$ for 2 hours) reduced CBF for 67.1%. These data give evidence that ozone does not effect the CBF of isolated ciliated human nasal epithelial cells from healthy volunteers and that formaldehyde has an effect only at the highest doses tested.

The velocity of CBF measured before the exposure to ozone or formaldehyde was in accordance to former studies of our group (Hafner et al. 1997; Riechelmann et al., 1994, 1992; Kienast et al., 1994, 1993) and other studies (Deitmer et al., 1993). Differences of CBF compared to other studies are caused by methodical differences like temperature, variable culture mediums, and of course the method of counting like using strobe lights, photodiodes and different video techniques.

The concentrations of ozone were closely related to naturally environmental occurrence as published for indoor and outdoor concentrations (Fischbein and Hemminki, 1993; Krause et al. , 1991; ZIMEN, 1995; Wallace, 1991). Low concentrations of ozone were measured in the rural areas whereas peak concentrations were maintained in metropolitan areas with differences in the USA and Germany. Ozone concentrations in Los Angeles exceeded 120 $\mu g/m^3$ on 141 day and 400 $\mu g/m^3$ on 68 days of the year, whereas in german metropolitan areas the concentration of ozone was between 120 $\mu g/m^3$ and 300 $\mu g/m^3$ only during the summertime (Wagner, 1994).

Our results of exposure to ozone correlate with other studies exposing animals under similar conditions. In these studies sheep (Abraham et al., 1980) and rats (Groset et al., 1991) were exposed to high concentrations of ozone (1000 $\mu g/m^3$ for 2 hours and 196 $\mu g/m^3$ for 3 hours, respectively) with no effect on CBF. In other experiments exposing sheep (Allegra et al. 1991) and rats (Phalen et al. 1980) to ozone (2000 $\mu g/m^3$ 2 hours and 1600 $\mu g/m^3$ for 4 hours, respectively) a decrease of CBF was measured in sheep. Summarising these findings in comparison with our data this might give evidence for interspecies differences and shows the difficulty when data of animal experiments are transferred to the relevance in human health.

Healthy volunteers, who were exposed in vivo to formaldehyde showed nasal eosinophilia (Pazdrak et al. 1993), chronic inflammation and metaplasia (Edling et al., 1993; Falk et al., 1994). A reduction of mucociliar transport time was found when humans were exposed for 1 - 6 hours to 300 - 2000 $\mu g/m^3$ of formaldehyde (Anderson and Molhave, 1993). Our results, at least partly, underline the findings of these studies even though using a

different technique. Animal experiments were performed using much higher doses of formaldehyde with up to three weeks of exposure for more than six hours at 620 up to 18,700 $\mu g/m^3$ of formaldehyde (Mannix et al., 1983; Morgan et al., 1988). Further studies have to be done to evaluate the in vitro technique using samples of sensitive individuals.

Former findings of our group focusing on the effects of nitric oxide or sulphur dioxide, showed dose dependent changes of CBF (Anderson et al., 1974; Kienast et al., 1994). Taking in consideration the former results and the data presented here, the measurement of CBF of isolated respiratory epithelial cells is an appropriate in vitro method for the direct evaluation of short term effects of volatile air pollutants on the mucociliary transport system. The relevance of the technique used is underlined by comparable results when ozone was exposed to animals. Therefore, the in vitro technique presented is an appropriate method to evaluate effects of volatile air pollutants on the human mucociliary system avoiding animal experiments with known species differences of pollutant sensitivities. Further studies are necessary to evaluate the long term effect of ozone on CBF of human nasal epithelial cells.

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