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SHORT COMMUNICATION

Ozone-Induced Micronuclei Frequency in Rat Alveolar Type II Cells

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Summary

The effect of ozone, a ubiquitous air pollutant, was tested on cultured pulmonary epithelial type II cells isolated from rats. After 40-hour culture, the cells were exposed for 6 h to 400 ppb of ozone or air. The number of micronucleated cells was counted after the exposure. In each group, 17 000 cells were evaluated. The number of micronucleated cells was significantly increased in the ozone-exposed group (12.24 per 1000 cells) compared to the control group (5.00 per 1000 cells). The results showed the mutagenic effect of ozone exposure on alveolar type II cells, manifested in the increased frequency of their micronuclei.

Key words

Micronuclei • Alveolar epithelial type II cells • Ozone • In vitro

Ozone is a ubiquitous secondary air pollutant and is able, as a consequence of its high redox potential, to react with all hydrocarbon molecules. The biological effects of ozone have been studied extensively in plants, animals and humans. Both epidemiological and experimental studies proved that exposure to ozone caused changes in pulmonary function (Beckett 1991), tissue injury (Ibrahim *et al.* 1980) and lung inflammation (Kleeberger *et al.* 1993).

The WHO European air quality one-hour guideline is 150-200 g/m^3 (0.076-0.1 ppm) and that for 8 h is 0.05-0.06 ppm (WHO 1987).

Many studies have suggested that ozone is genotoxic, possessing both mutagenic and carcinogenic effects in mammalian systems. However, the results from the *in vivo* cytogenetic studies with laboratory animals after inhalation exposure are contradictory, while those from *in vitro* experiments demonstrated its genotoxic effects. Ozone was shown to react with DNA and RNA, to induce chromatid type aberrations, sister chromatid exchanges and neoplastic transformation in cell cultures (Victorin 1996).

The primary target cells in the lung are thought to be type I and type II epithelial cells, where oxidative damage may initiate an inflammatory response (Stevens *et al.* 1974). In the present study, we focused on type II pneumocytes because of their importance in metabolic

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pathways, surfactant metabolism and as stem cells of type I pneumocytes.

As they have mitotic activity, we considered them to be a suitable *in vitro* model for the detection of genetic damage. The utility of these cells as a new *in vitro* model for testing the mutagenic effect of cigarette smoke by micronucleus assay was demonstrated in our previous study Kováčiková *et al.* (1998). The present study on the genotoxic effect of ozone on alveolar epithelial type II pneumocytes *in vitro* was conducted within the frame of a European Research Network.

Animals. Male Wistar rats weighing 274±17 g were used in the experiments. The animals were kept in a conventional animal house, with 12 h dark/light cycles, in wired bottom cages. The animals had free access to water and laboratory food.

Reagents and media. Waymouth's 752/1 medium, CO_2 independent medium, fetal calf serum (FCS), Hepes, penicillin-streptomycin solution (10 000 U/ml and 10 000 μg/ml, respectively), fungizone (250 μg/ml) and L-glutamine (200 mM) were obtained from GIBCO (Merelbeke, Belgium). All other chemicals were supplied by Sigma (Bornem, Belgium).

Cell isolation and culture. Rat alveolar type II cells were isolated according to the method of Richards *et al.* (1987) and Hoet *et al.* (1994). Briefly, after intraperitoneal sodium pentobarbital (60 mg/kg) anesthesia, the lung was perfused *via* the pulmonary artery with sterile saline and was mechanically ventilated. The lung with the trachea were removed and bronchoalveolar lavage was performed. The lung was partially trypsinized and the cell mixture was purified by centrifugation on a Percoll gradient and differential attachment. The purity of isolated cells was about 80 %. The cells were cultured on 96-well plastic microplates (Corning; International Medical, Belgium) in Waymouth's medium supplemented with 10 % FCS at 37 °C in an atmosphere of 95 % air/5 % CO_2 at the density of 100 000 cells in 200 μl per well. After 40 h of culture, when the cells had still all markers of type II cells (Kováčiková *et al.* 1999) the medium was replaced by 40 μl of CO_2 independent medium and the cells were exposed for 6 h to ozone or air (control cells).

Ozone and air exposure apparatus. Ozone was electrically generated (Fisher OZ 500 M, Germany) using pure O_2 (99.999 %, Praxair, Oevel, Belgium). An air flow of 1 l per minute was continuously blown into each (control and ozone) plexiglass box (7 l content). The generated ozone was fed into this flow using feedback regulation (Flow-Therm, Brussels, Belgium). Both the incoming and outgoing ozone concentration was continuously monitored (EIT 4500 Sensor sticks, Flow-Therm, Brussels, Belgium). The average concentration of ozone was 400 ppb.

Micronucleus test. The micronucleus assay was performed according to Liu *et al.* (1996) in our modification. After the ozone exposure, the cells were washed twice with phosphate buffered saline, trypsinized, rewashed and cytospined. The slides were fixed by methanol and stained with Giemsa. Micronuclei (MN) were analyzed under a Jenaval microscope (Zeiss, Germany) with 1000x magnification using oil immersion. The criteria used to score MN followed those reported by Schmid (1975): staining intensity equal to that of the nucleus, diameter less than one-fifth that of the nucleus, no contact with the nucleus and location in the cytoplasm. The results were expressed as the average number of cells with MN per 1000 alveolar type II cells ± S.D. The frequency of MN was based on 17 000 cells scored per group.

Statistical evaluation. Mann-Whitney-Wilcoxon test was used to compare the experimental and control data in the micronucleus test.

The effect of ozone exposure on micronuclei frequency in alveolar type II cells is shown in Table 1. The 6-hour ozone (400 ppb) exposure induced a significant increase in the frequency of micronuclei compared to the control group. The result of the present experiment confirmed the mutagenic effect of ozone *in vitro* in alveolar type II cells.

Table 1. Effect of ozone exposure on the frequency of micronuclei in alveolar epithelial type II cells

Group	Number of analyzed type II cells	Type II cells with MN/1000 cells (mean ± S.D.)
Control	17 000	5.0±1.57
Ozone-exposed	17 000	12.24±2.46*

** Significantly higher than control group ($P<0.002$)*

Type II epithelial cells, the primary target cells for toxic agents, begin to proliferate following and during continued exposure, and their proliferation is a hallmark of ozone injury, regardless of species (Stevens *et al.* 1974). While type II cells proliferated in the ozone-

exposed lung, complete maturation of type II cells to type I cells did not occur. Continued ozone exposure was shown to inhibit both ciliagenesis and type II cell maturation (Moore and Schwartz 1981). Upon inspiration, the first physical interface encountered by ozone is a thin layer of aqueous material, the epithelium lining fluid (ELF), which overlays and is partially derived from the underlying pulmonary epithelium. Toxic effects of ozone have been shown to be mediated not through the action of ozone itself but rather through the reaction products formed by its reaction with biomolecules as it is passing through the ELF, and thus its toxicity was transmitted to the pulmonary epithelium by secondary and tertiary reaction products (Kelly and Mudway 1997).

The exposure of biological material to ozone leads to generation of reactive species (such as hydroxyl radicals), which form the basis for the reaction of ozone with biological molecules, particularly with lipids, proteins and nucleic acids. Such alterations of important biological molecules interfere with biochemical processes in cells, and therefore lead to an altered cell phenotype, cytotoxicity and genotoxicity. A consensus of opinion now exists that the reaction of ozone with organic molecules in aqueous solutions of near neutral or alkaline pH is a combined effect of the reactivity of ozone itself and the reactivity of a number of other reactive oxygen metabolites, particularly that of the hydroxyl radical (Cotgreave 1996). The hydroxyl radical is highly reactive and is regarded to be the most important with respect to the biological consequences (Yasukawa *et al.* 1989).

Ozone is supposed to be genotoxic and a potential respiratory carcinogen or promoter of carcinogenic processes that might be associated with DNA damage. Ozone or reactive oxygen metabolites can cause a variety of alterations in the structure of DNA, including double strand breaks, sugar peroxidation, base hydroxylation, single-strand breaks and protein-base cross-links (Cotgreave 1996). DNA damage proceeds both directly, *via* ozone itself, and indirectly *via* hydroxyl radicals, when solutions of nucleotides or DNA are treated with ozone, DNA adducts can also be formed (van der Zee *et al.* 1987).

The results from *in vivo* cytogenetic studies on laboratory animals after inhalation ozone exposure are contradictory. As ozone is a highly reactive compound, which is probably not taken up unreacted from the lung, the positive finding of chromatid type aberrations in lung macrophages in rats (0.43 mg/m^{-3} for 6 h) (Rithidech *et al.* 1990) might be important for the risk assessment. Ozone was shown to be genotoxic *in vitro*. Mutations and DNA strand breaks occurred in bacteria and yeast cells, mainly in experiments in which ozone was bubbled through suspension cultures of cells, in which case hydroxyl radicals and hydrogen peroxide were also formed. In cell cultures, chromatid-type chromosomal aberrations, sister chromatid exchanges and neoplasmic transformation were demonstrated (Victorin 1996).

Due to their mitotic activity, type II cells can be considered a suitable model for the detection of genetic damage by micronucleus assay. The micronucleus assay (Schmid 1975) is a widely used short-term screening test for determining the induction of spindle or chromosome damage by estimating the frequency of micronuclei. Micronuclei are formed spontaneously or in response to the genotoxic exposure from acentric chromosomal fragments or whole chromosomes that are left outside the daughter nuclei during cell division.

The results confirmed the mutagenic effects of ozone exposure on alveolar type II cells manifested by a significant increase in the frequency of their micronuclei. Our data suggest the suitability of the experimental system for studying induction of micronuclei and the effect of ozone *in vitro*, respectively.

In summarizing the results of this study and of our previous experiments (Kováčiková *et al.* 1998), we conclude that alveolar epithelial type II cells are useful for the assessment of mutagenicity and may serve as an appropriate model for *in vitro* genotoxic testing of toxic agents.

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