OZONE-INDUCED DNA STRAND BREAKS IN GUINEA PIG TRACHEOBRONCHIAL EPITHELIAL CELLS

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Ozone (O₃), the major oxidant of photochemical smog, is thought to be genotoxic and a potential respiratory carcinogen or promoter of carcinogenic processes. Because of oxidative reactions with the mucus in the upper airway, O_3 reaction products are able to penetrate into the tracheobronchial epithelial (TE) cells. The carcinogenic effects of O_3 on the TE cells are especially of interest since most previous studies have focused on the morphology or permeability changes of tracheas only. Therefore, the objective of this study was to examine the potential O_3 genotoxicity in TE cells after an in vivo exposure, using DNA strand breaks as an index. Two-month-old male Dunkin-Hartley guinea pigs, specific pathogen free, 4 in each group, were exposed to 1.0 ppm O_3 for 0, 12, 24, 48, 72, or 96 h. Animals exposed to filtered air without O_3 exposure were used as controls. After O_3

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exposure, the trachea with two main bronchi was removed from each animal, and TE cells were isolated and employed for determination of DNA strand breaks by fluorometric analysis of DNA unwinding (FADU). The statistical significance level was set at $\alpha = .05$. Compared with controls, ozone exposure did not alter the TE cell yield or viability, but caused an increase in protein content in tracheal lavage and an increase in DNA strand breaks. The amount of DNA left in the alkali lysate of TE cells found at 72 h exposure was significantly decreased from controls for 3 different alkali incubation times. An increase of the double-stranded DNA left in the alkali lysate of TE cells was observed at 96 h of exposure and approached the value of 24 h of exposure. The same pattern was seen with all 3 different alkali incubation times at 15°C. One Q_d unit was estimated to correspond to 100 strand breaks per cell. The Q_d was also used as an indicator for O_3 damage. Compared to controls, the Q_d increases significantly after 1 ppm O_3 exposure for 72 h, regardless of the alkali incubation time at 15°C.

Ozone exposure can cause injuries to lung tissues and cells, including DNA. Such injuries are attributable to the oxidative properties of the O_3 molecule and its ability to generate a variety of free radicals (Mustafa, 1990). Ozone is also thought to be a respiratory carcinogen or promoter of carcinogenic processes (Mustafa et al., 1988; Witschi, 1988) that might be associated with DNA damage. Free radicals can cause damage to DNA, possibly by a scission of the sugar–phosphate backbone, producing strand breaks (Lesko et al., 1980; Brown & Fridovich, 1981; Simic & Jovanovic, 1986). Thus, O_3 has been shown to cause DNA single-strand breaks and/or double-strand breaks in L929 fibroblasts (Ven der Zee et al., 1987a), wild-type *Escherichia coli* (Hamelin et al., 1978), calf thymus DNA (Ven Der Zee et al., 1987b), and alveolar macrophages (Bermúdez et al. 1991). Chromosome aberration and/or sister chromatid exchanges have been observed after an in vivo O_3 exposure in the peripheral lymphocytes of animals (Zelac et al., 1971; Mckenzie et al., 1977) and humans (Merz et al., 1975; Guerrero et al., 1979).

Because of oxidative reactions with the mucus in the upper airway, O_3 reaction products are able to penetrate into the tracheobronchial epithelial (TE) cells (Miller et al., 1985; Pryor & Church, 1991; Pryor et al., 1991). The effects of O_3 on the TE cells are of interest, even though it has been documented that the primary anatomical site for cellular damage by O_3 is the alveoli in the central region of the pulmonary acini and adjacent bronchiolus (Stephens et al., 1972; Evans, 1984). Several approaches have been made in testing the toxic effects on TE cells, such as TE cells grown in culture or full-length tracheas in organ culture (Rasmussen & Bhalla, 1989; Alpert et al., 1990). However, due to the existence of enzymatic or non-enzymatic detoxification mechanisms, which can eliminate the toxic intermediates generated during O_3 exposure (Elsayed & Mustafa, 1982; Burton et al., 1983; Elsayed et al., 1989, 1990), it is necessary to carry out the study after in vivo exposure and examine the effects on the isolated TE cells. This process will prevent an under- or over-

estimation of O_3 toxicity, and will reflect the effects occurring on the TE cells in situ. Previous studies on toxic effects of O_3 on TE cells focused on the morphology (Nikula et al., 1988) or permeability (Bhalla & Crocker, 1987) changes of the tracheas. Genotoxic effects on TE cells have seldom been investigated. Therefore, the objective of this study was to estimate the potential genotoxicity of O_3 to TE cells after an in vivo exposure.

MATERIALS AND METHODS

Animals, Housing, and O_3 Exposure

Two-month-old male guinea pigs (Dunkin-Hartley strain, specific pathogen free) with a body weight of 457–490 g were used in this experiment. Animals were housed in standard cages (one per cage) in a laminar-flow isolation unit receiving air passed through a coarse pre-filter, and then a 4-in-bed charcoal filter to remove any possible contaminants. Animals received free access to water and food, had a 12-h light–dark cycle in the room, and had a 7-d acclimatization before being used in the experiment.

For O_3 exposure, animals were randomly divided into control and various exposure groups, transferred to stainless-steel open-mesh cages (1 per cage), and then placed in 300-L stainless steel inhalation exposure chambers. Animals (4 in each group) were exposed to 1.0 ± 0.05 ppm (1960 ± 98 µg/m³) O_3 for 0, 12, 24, 48, 72, or 96 h continuously. Animals exposed to filtered air were used as controls.

The O_3 atmosphere in the chamber was generated by passing 100% medical-grade oxygen through an ozonizer at a rate of 0.5 L/min, then diluting with filtered room air. The O_3 concentration was monitored continuously by a Dasibi 1003-PC ozone analyzer, which was calibrated periodically by the potassium iodide wet chemical method (APHA Intersociety Committee, 1972).

TE Cell Isolation and Characterization

For TE cell isolation, the method developed by Johnson et al. (1987) was adapted. In brief, the animals were sacrificed by an intraperitoneal injection of pentobarbital solution (Nembutal, 100 mg/kg body weight). The chest was sprayed with 70% ethanol and the chest cavity was opened. A vascular perfusion of the lungs was performed using physiological saline (0.9% NaCl). The trachea was exposed and rinsed with saline to remove the blood. A tracheal cannula (a number 15 gauge blunt stainless steel needle) was inserted at the lower end of the larynx. The trachea with two main bronchi (henceforth called tracheobronchi) was excised, removed from the body, placed on a custom-designed trachea holder, and flushed with 10 ml of physiologi-

cal saline (37°C). After the flushing, the tracheobronchi was clamped at the ends with microclips. The tracheobronchi was then filled with hyaluronidase solution (0.2% in Joklik modified minimum essential medium, MEM), immersed in Hanks balanced salt solution (Ca^{2+} and Mg^{2+} free), and incubated in a water bath at 37°C for 30 min. After the incubation, the tracheobronchi was flushed again with a 10 ml of flushing medium containing Joklik modified MEM, 10% fetal calf serum, and 0.5 mM ethylenediamine tetraacetic acid (EDTA, pH 7.2–7.4). The lumen was refilled with cytochalasin B solution (0.5 µg/ml in Joklik modified MEM), and incubated for 60 min at 37°C. The lumen was flushed once more as before. Finally, the lumen was filled with pronase E solution (15 mg/ml in Joklik modified MEM) and incubated for 90 min followed by another flushing. All the flushes (except the first one) were collected and kept on ice. Ten milliliters of the collected fluid was used further to flush the lumen seven additional times. The total fluid was centrifuged (200 × g, 10 min), the supernatant was discarded, and the pellet, upon resuspension in 10 ml of the flushing medium, was recentrifuged (200 × g, 10 min). After removing the supernatant, the pellet was finally resuspended in 5 ml of an isotonic buffer solution (0.25 M meso-inositol, 10 mM sodium phosphate, and 1 mM $MgCl_2$, pH 7.2–7.4). The cell count and viability test were carried out by using the trypan blue dye exclusion technique (a suspension of cells was diluted 1:3 with 0.4% trypan blue solution and counted on a hemocytometer).

Protein Assay

The protein assay was performed in the first tracheobronchial flush after removing the cellular debris by a centrifugation at 27,000 × g for 20 min. Total soluble protein content in the flush supernatant was measured according to Bradford (1976). A standard curve containing 2–10 µg/ml of bovine serum albumin was prepared each time when this assay was done. The optical density was measured in a spectrophotometer at 595 nm. The total protein was determined by extrapolation against standard curve and presented as micrograms protein per tracheobronchi.

Assay of DNA Strand Breaks

The fluorometric analysis of DNA unwinding (FADU) method (Birnboim, 1990) was used to determine DNA strand breaks in isolated TE cells. The principle is that when exposed to alkali (pH 12.8), the hydrogen bonds of double-stranded DNA will break and the two strands unwind. The FADU method assumes that each strand break serves as a strand-unwinding point during alkali denaturation. The more breakage points in a DNA, the less double-stranded DNA is left

after the alkali treatment. This is a rapid and sensitive method used for monitoring DNA strand breaks in homogenous suspension of mammalian cells.

In brief, 0.2 ml of a TE cell suspension (1.5–2.6×10^6 cells/ml) corresponding to 15–26 µg DNA/ml was distributed in disposable polyethylene test tubes (set of 4 for each sample), labeled as T (total double-stranded DNA), P (double-stranded DNA left in the cell lysate after alkali incubation), and B (blank). Then, solution I (9 M urea, 10 mM NH$_4$OH, 2.5 mM cyclohexane diamine tetraacetate, and 0.1% sodium dodecyl sulfate, pH 7.2) was added to each tube and incubated at 0°C for 10 min. The cell lysis and chromatin disruption occurred during this time period. After this process all steps were conducted in a subdued light and in a covered ice bath during incubations. Solution III (1 M glucose, 14 mM 2-mercaptoethanol) was added to tubes T prior to solution IIa (0.55 volumes of solution I in 0.20 N NaOH) and IIb (0.45 volumes of Solution I in 0.20 N NaOH) to neutralize the lysate, so that the DNA in tubes T was never incubated at a denaturing pH. Otherwise, 0.1 ml of solution IIa and 0.1 ml of solution IIb were added gently into each tube against the wall without mixing and incubated at 0°C for 30 min. During this incubation time the alkali diffused into the viscous lysate to bring up the pH to approximately 12.8 (measured at 23°C), with the occurrence of some DNA unwinding. Then the lysate of the tubes B was sonicated for 30 s at 70 W to denature the DNA rapidly in the alkali solution. Subsequently, all tubes were incubated for 0, 30, or 60 min at 15°C to allow unwinding to proceed. Tubes P were removed from the incubator at an interval of 30 min. To stop the denaturation, the tubes were chilled to 0°C and solution III (0.4 ml) was added and mixed to lower the pH to about 11.0 (measured at 23°C). The lysates were then sonicated for 2 s at a low power (40 W) to render them homogeneous. Finally, after adding solution IV (ethidium bromide, 3.3 µg/ml, and 13.3 mM NaOH, 1.5 ml) to each tube, the tubes were vortexed and the fluorescence was measured at room temperature in a fluorescence spectrophotometer (excitation at 520 nm, emission at 590 nm). The scale was set between 0 with air and 120 (arbitrary) units with one of the tubes T.

The readings from quadruplicate tubes for each set were averaged to give a single T, P, or B value for each experimental sample at each alkali exposure time. The percent double-stranded DNA left after partial unwinding for different incubation time at 15°C was calculated by $D_i = 100(P_i - B)/(T - B)$, where B (blank) was the fluorescence used to estimate the components other than double-stranded DNA (including free dye), T (total) was the fluorescence used to estimate the total amount of double-stranded DNA plus contaminants, and P_i was the

fluorescence used to estimate the amount of double-stranded DNA remaining in the alkali cell lysate after incubation at 15°C for different time periods, i = 0, 30, or 60 min.

The quantity of 100 DNA strand breaks per TE cell (Q_d) induced by a given treatment was $100[\log(D_c) - \log(D_{ei})]$, where D_c was the mean of the double-stranded DNA left in the cell lysate of controls, and D_{ei} was the mean of the double-stranded DNA left in the cell lysate of exposed animals under different ozone exposure protocols and alkali incubation conditions, e = 12, 24, 48, 72, or 96 h, i = 0, 30, or 60 min. A linear dose-response curve was obtained when Q_d was plotted against ^{60}Co radiation in the range of 0–4 Gy (0–400 rad). One Q_d unit was estimated to correspond to 100 strand breaks (frank breaks plus alkali-labile sites) per cell (McWilliams et al., 1983).

Statistical Analysis

To test the equality of means of variables between groups under various exposure protocols, the Bonferroni multiple comparison test was used, and the statistical significance level was set at .05 (Neter et al., 1985). Due to the rebound at the 96-h exposure, examination of the linearity between double-stranded DNA left in the cell lysate and the O_3 exposure time was done by using 0–72 h exposure data only. To test the linearity between ozone exposure time and double-stranded DNA left in the alkali lysate (or DNA strand breaks per TE cell) and parallelism among three regression lines of different alkali incubation time periods simultaneously, a multiple linear regression model with indicator variables for the incubation times was created (Afifi & Clark, 1996). To explore the optimal condition for the detection of DNA strand breaks in TE cells by the FADU method, a coefficient of determination (r^2) was used to evaluate the strength of linear relationship between DNA strand breaks and O_3 exposure times (Kleinbaum & Kupper, 1978).

The assumption was made that the maximum difference and variation of D_i between O_3-exposed and unexposed TE cells is the same as the difference between ^{60}Co γ-rays irradiated and unirradiated white blood cells, which were 24% or 4%, respectively (Birnboim & Jevcak, 1981). Setting the significance level of the test at α = .05 with 4 animals in each group, the power (1 – β) of the statistical test was more than 95% (Dixon & Massey, 1983).

RESULTS

TE Cells Yield and Viability

In preliminary experiments, results with various enzyme solutions were compared until the ideal conditions were achieved permitting a maximal cell yield and viability. The yield and viability of TE cells

after exposure to 1.0 ppm O_3 for 0–96 h are presented in Figures 1 and 2, respectively. The average TE cells yield and viability for the tracheobronchi of unexposed guinea pigs were $10.6 \pm 6.1 \times 10^6$ and $88.0 \pm 8.4\%$, respectively. The data showed that neither the yield nor the viability of the TE cells from various O_3 treatment groups was significantly different from that of controls.

Protein Content in the Tracheal Lavage Fluid

The protein content in the lavage fluid of the trachea was used as an index of altered TE cell membrane permeability after O_3 exposure. The protein content per tracheobronchi was independent of the total TE cells yield ($r = .39, p > .05$). As shown in Figure 3, the protein content per tracheobronchi generally increased with O_3 exposure time. The protein content per tracheobronchi was 2.1-fold and 3.3-fold higher than controls after 24 h and 72 h of exposure, respectively. After 96 h of exposure, the protein content decreased approaching the value seen at 48 h of exposure. Compared with controls, only 72 h of O_3 exposure showed a significant increase in protein content from the tracheobronchial lavage fluid.

Double-Stranded DNA Left in the Alkali Cell Lysate

The amount of double-stranded DNA left in the alkali lysate is presented in Figure 4. The double-stranded DNA left in the alkali
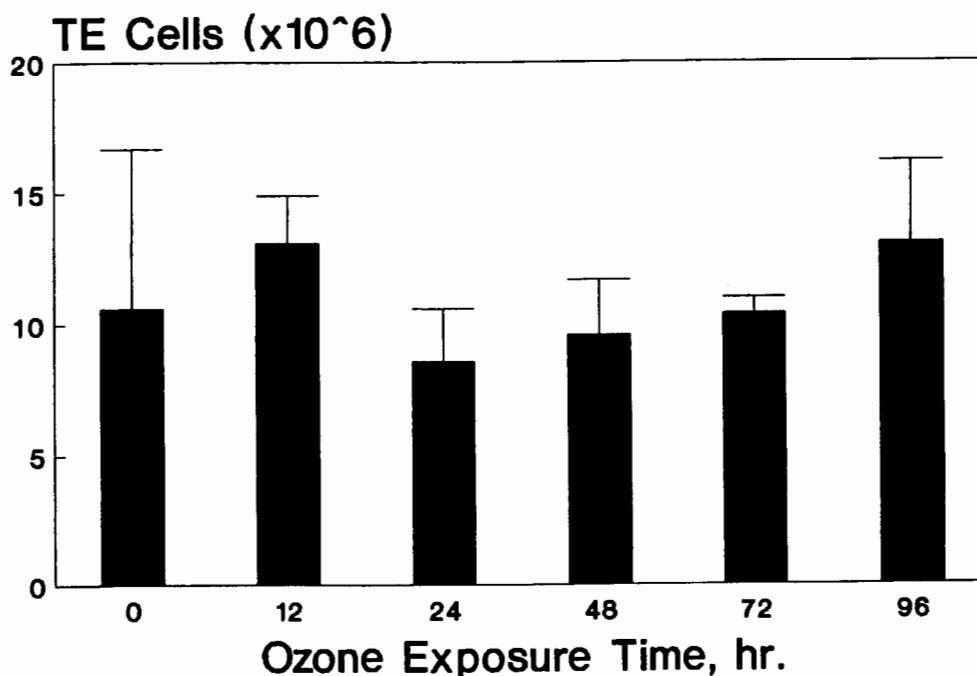

FIGURE 1. Yield of TE cells after exposure to 1.00 ppm O_3 for 0 to 96 h. Mean ± SD, $n = 4$ for each group.
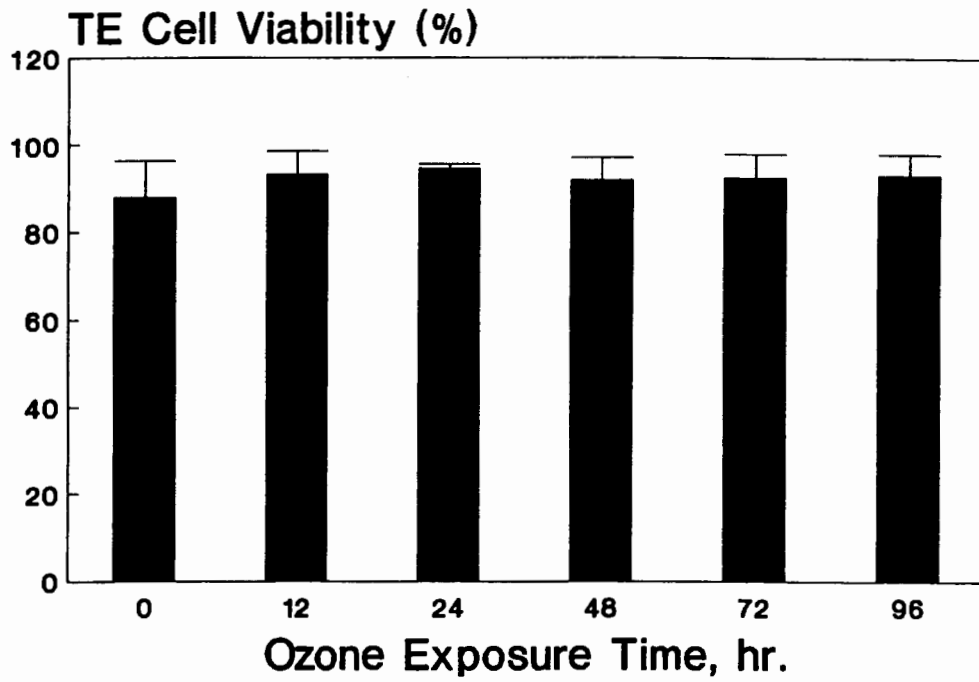
FIGURE 2. Viability of isolated TE cells after exposure to 1.00 ppm O_3 for 0–96 h. Mean ± SD, n = 4 for each group.
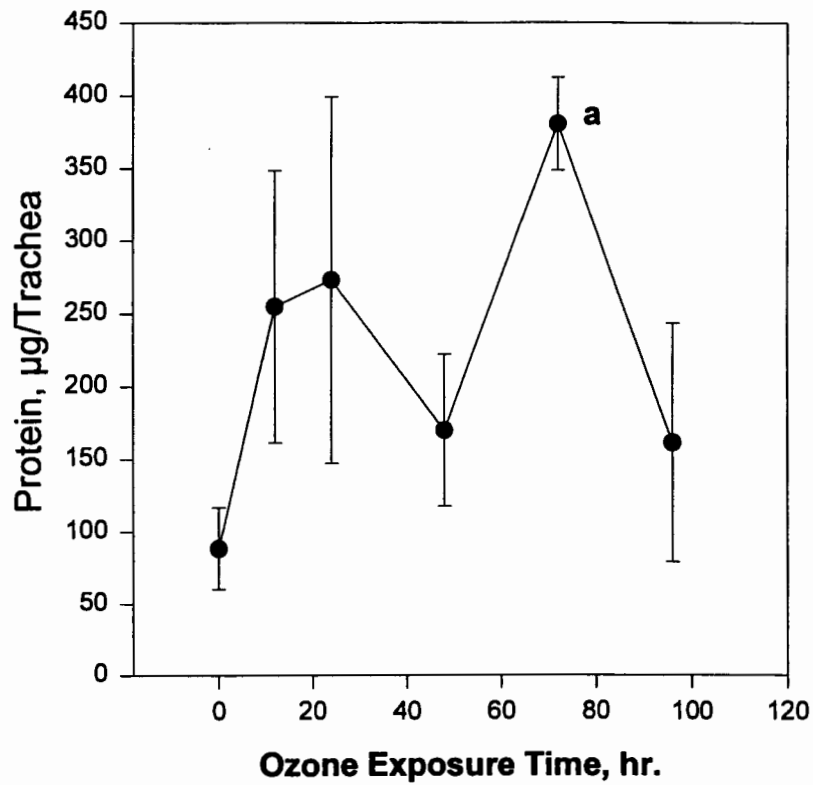

FIGURE 3. Protein content in the lavage fluid of the tracheobronchi after exposure to 1.00 ppm O_3 for 0–96 h. Mean ± SD, n = 4 for each group. a: Statistically significant, $p < .05$.
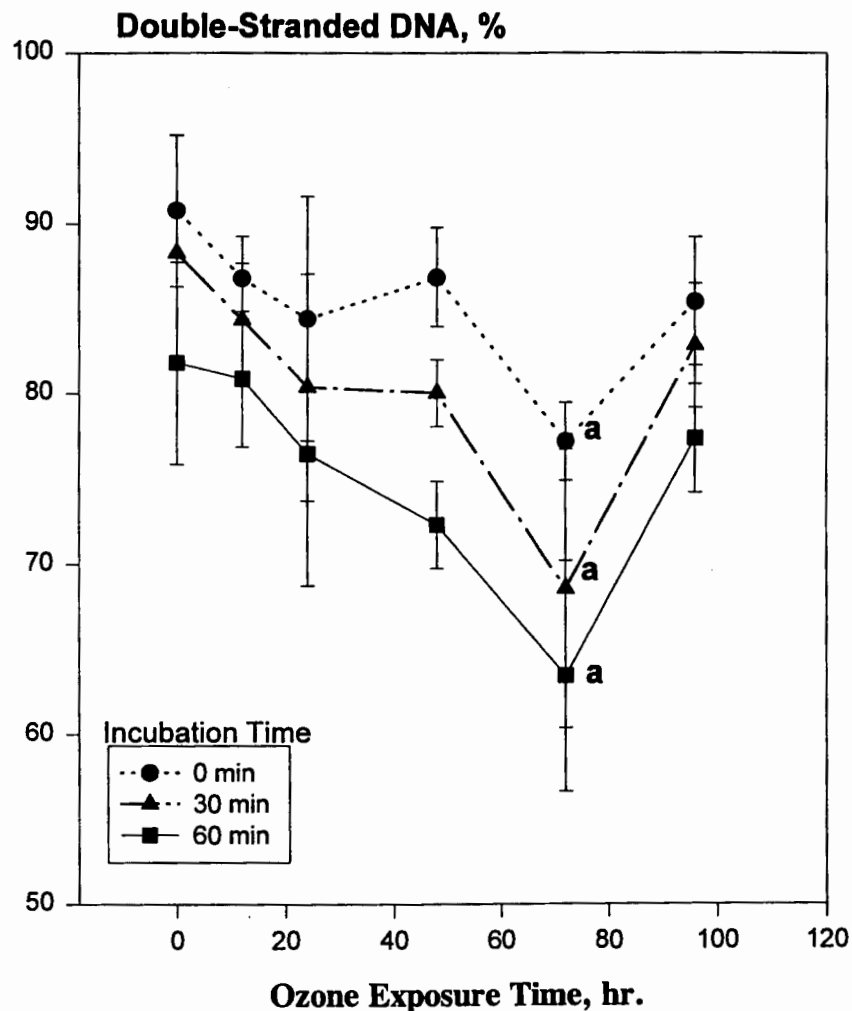
FIGURE 4. Double-stranded DNA of TE cells left in the alkali cell lysate after animals were exposed to 1.00 ppm O$_3$ for 0–96 h. The cell lysate was incubated in alkali for 0–60 min at 15°C. Mean ± SD, n = 4 for each group. a: Statistically significant, p < .05.

lysate of TE cells from controls was 90.8 ± 4.5%, 88.3 ± 6.9%, and 81.8 ± 5.9% for 0, 30, and 60 min of alkali incubation, respectively. In comparison with controls, the amount of double-stranded DNA left in the alkali lysate decreased for all O$_3$ exposure periods. A significant decrease was found at 72 h of exposure for each of the 3 different alkali incubation times. The double-stranded DNA left in the alkali lysate of TE cells from after 72 h of O$_3$ exposure was 77.2 ± 2.3%, 68.6 ± 8.2%, and 63.4 ± 6.8% for 0, 30, and 60 min of alkali incubation, respectively. An increase was observed at 96 h of exposure that approached the value for 24 h of exposure. The same pattern was seen with all 3 different alkali incubation times at 15°C.

Regression coefficients of simple regression models for the relation between double-stranded DNA left in the cell lysate and O$_3$ exposure

are presented in Table 1. The results show that despite the alkali incubation times, there is significant linear relationship between double-stranded DNA left in the cell lysate and O_3 exposure. The 60-min alkali incubations demonstrated the best linear relationship between DNA strand breaks and O_3 exposure period, with a determination coefficient of 63%.

The results of the complete multiple regression model showed that the regression coefficients of the interaction terms between O_3 exposure times and alkali incubation times were not significantly different from zero. Therefore, the three regression lines (double-stranded DNA left in the cell lysate as a function of O_3 exposure time at three different alkali incubation times) were considered parallel (data are not presented).

The number of 100 DNA strand breaks per TE cell (Q_d) after O_3 exposure is presented in Figure 5. Compared to the controls, an exposure to 1.0 ppm O_3 for 72 h resulted in a significant amount of DNA strand breaks in TE cells, regardless of the alkali incubation time at 15°C. The Q_d value of the 1 ppm O_3 exposure for 72 h was 7.1 ± 1.3, 11.2 ± 5.5, and 11.3 ± 4.5 for 0, 30, and 60 min of alkali incubation, respectively.

DISCUSSION AND CONCLUSIONS

In the present study, the modified tracheobronchial cell isolation method allowed harvesting of TE cells with a good yield and viability. Ozone exposure did not affect the TE cell yield or viability, but caused a decrease in the amount of double-stranded DNA left in the alkali cell lysate with a corresponding increase in DNA strand breaks.

The increase of protein content in the tracheobronchial lavage fluid was used as an indicator for the possible ozone-initiated inflammatory reaction. The increased protein contained in the excised tracheobronchi could be the result of increased mucus production in the trachea or

TABLE 1. Summary of regression coefficients of the simple regression model for the double-stranded DNA of TE cells left in the alkali lysate as a function of O_3 exposure time

Incubation times[a]	Intercepts	Regression coefficients	Coefficients of determination (r^2)
D0	89.9	−.149[b]	.44
D30	87.9	−.243[b]	.56
D60	83.0	−.256[b]	.63

Note. The cell lysate was incubated for 0–60 min at 15°C.

[a]D0, D30, or D60 is the double-stranded DNA left in the alkali cell lysate after 0, 30, or 60 min, respectively, of incubation at 15°C.

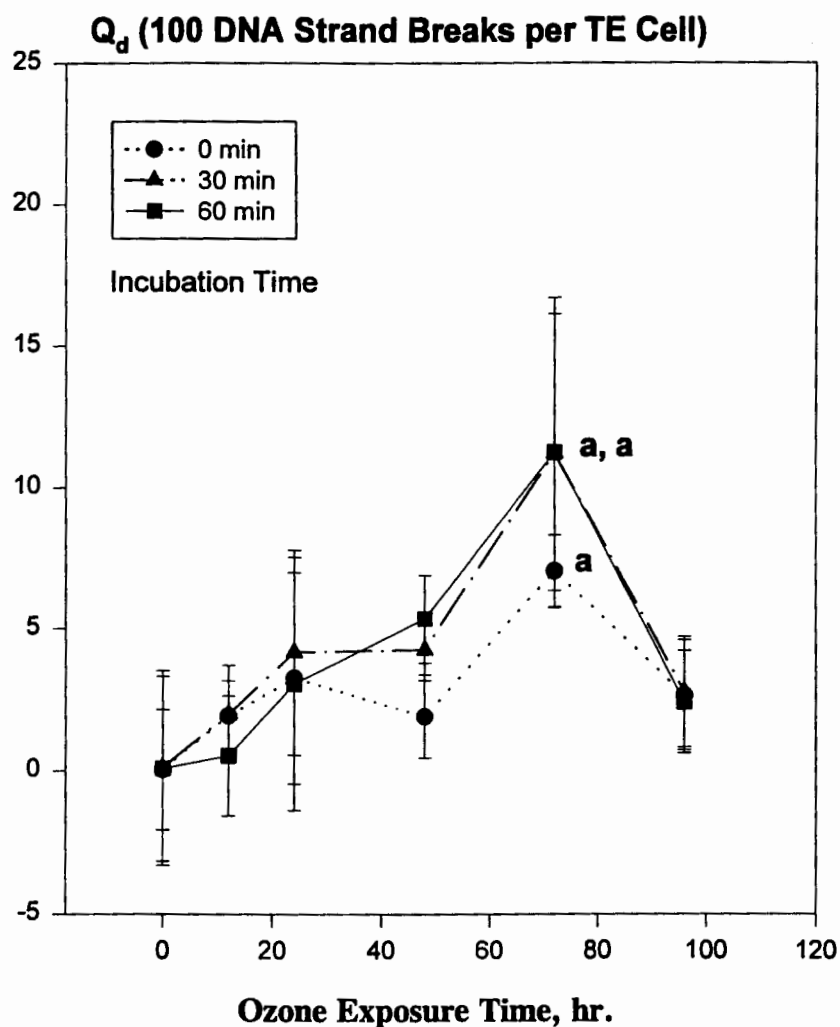[b]Statistically significant, $p < .05$.

Q$_d$ (100 DNA Strand Breaks per TE Cell)

FIGURE 5. Number of 100 DNA strand breaks per TE cells (Q_d) after animals were exposed to 1.0 ppm O₃ for 0–96 h. The cell lysate was incubated in alkali for 0–60 min at 15°C. Mean ± SD, n = 4 for each group. a: Statistically significant, p < .05.

lower in the respiratory tract, and carried to the tracheobronchi, or could even result from the cytolysis of some of the epithelial cells. Therefore, more direct measurements or morphologic information will be needed as evidence.

Cell preparation artifacts might be the sources of variation in determining the original amount of double-stranded DNA. Any undesirable cell disruption would produce artificial strand breaks, elevating the background number of strand breaks (Morris & Shertzer, 1985). In this study, however, the isolated TE cells from unexposed guinea pigs presented a similar amount of double-stranded DNA (82%) as observed for human white blood cells (80%; Birnboim & Jevcak, 1981) or cultured L929 fibroblasts (85%; Van der Zee et al., 1987a). The variations in quadruplicate samples were small and consistent

throughout the experiment (with a coefficient of variation ranging between 1% and 5%). Thus, the cell isolation artifact, if present, appears to be constant across the published studies.

In this time-course study for DNA strand breaks of isolated TE cells, a peak increase was observed at 72 h of continuous 1 ppm O_3 exposure. The pattern was similar to the biochemical and morphological changes in the lungs, which reached a plateau after 3 d of continuous O_3 exposure (Stephens et al., 1974; Mustafa & Lee, 1976). A balance of cell injury and repair in the lungs that occurs as a state of adaptation after the first few days of O_3 exposure might explain the decrease in DNA strand breaks of TE cells at 96 h following the peak increase at 72 h of O_3 exposure. The number of 100 DNA strand breaks per TE cell ($Q_d = 11.0 \pm 4.9$) caused by a 72-h exposure to 1.0 ppm O_3 was similar to the strand breaks of cultured human peripheral blood lymphocytes induced by 1 Gy (100 rad) of ^{60}Co γ-rays (McWilliams et al., 1983) or cultured murine L929 fibroblasts induced by 550 rad of x-rays (Van der Zee et al., 1987a).

The high reactivity of ozone makes it unlikely that it can penetrate far into the tissue–air boundary in the lung (Pryor, 1992). It has been suggested that the reaction of ozone with the lung lining fluids will produced aldehydes and hydrogen peroxide and organic radicals, which in turn may be the mediators of ozone toxicity (Pryor & Church, 1991; Pryor et al., 1991). The toxicity of O_3 in TE cells might be similar to ionizing radiation (Pryor et al., 1983; Van der Zee et al., 1987b; Kennedy et al., 1991; Mustafa, 1990; Pryor, 1994). Therefore, among the possible sequelae of O_3 damage to TE cells is that ozone reacts with the mucus in the tracheobronchi and ozonation products are formed. These products can then reach TE cells, causing alterations of membrane permeability, DNA strand breaks, and ultimately cell death. Alterations of DNA and/or cell death can trigger cellular proliferation, dysplasia (via similar mechanisms that occur in proliferation, and/or irreparable base alteration or substitutions), and the possible development of cancers (Steinberg et al., 1990). The cancer potential of DNA strand breaks caused by ozone under the conditions of this study is not known. However, the evidence that ozone caused lung tumors in mice (Hasset et al., 1985) leaves open the possibility that DNA damage can lead to biochemical alterations that may subsequently lead to the malignant transformation of cells or cancer.

In conclusion, the results of this study have demonstrated that continuous exposure to 1 ppm O_3 for 72 h could result in a significant increase in DNA strand breaks in TE cells. However, the O_3 concentration used in this study is higher than that generally encountered in ambient air (smog). Moreover, it has been postulated that the toxic effects of ozone are due, at least in part, to a cascade of lipid ozonation products rather than to ozone itself (Pryor et al., 1995). To

what degree ozone itself or the ozonation products contribute to the DNA damage observed needs to be determined. Therefore, it is recommended that further investigation in the genotoxicity effects and mechanisms of ambient or lower levels of O_3 and the lipid ozonation products in TE cells be conducted. The results can then be applied to finding the no observable effect level (NOEL) of O_3 on TE cells and to judging whether the current O_3 standard (0.12 ppm or 235 µg/m³) is adequate for protecting public health.

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