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Ozone Inhalation Leads to a Dose-Dependent Increase of Cytogenetic Damage in Human Lymphocytes

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Abstract

Ozone is an important constituent of ambient air pollution and represents a major public health concern. Oxidative injury due to ozone inhalation causes the generation of reactive oxygen species and can be genotoxic. To determine whether ozone exposure causes genetic damage in peripheral blood lymphocytes, we employed a well-validated cytokinesis-block micronucleus Cytome assay. Frequencies of micronuclei (MN) and nucleoplasmic bridges (NB) were used as indicators of cytogenetic damage. Samples were obtained from 22 non-smoking healthy subjects immediately before and 24-hr after controlled 4-hr exposures to filtered air, 100 ppb, and 200 ppb ozone while exercising in a repeated-measure study design. Inhalation of ozone at different exposure levels was associated with a significant dose-dependent increase in MN frequency ($P < 0.0001$) and in the number of cells with more than 1 MN per cell ($P < 0.0005$). Inhalation of ozone also caused an increase in the number of apoptotic cells ($P = 0.002$). Airway neutrophilia was associated with an increase in MN frequency ($P = 0.033$) independent of the direct effects of ozone exposure ($P < 0.0001$). We also observed significant increases in both MN and NB frequencies after exercise in filtered air, suggesting that physical activity is also an important inducer of oxidative stress. These results corroborate our previous findings that cytogenetic damage is associated with ozone exposure, and show that damage is dose-dependent. Further study of ozone-induced cytogenetic damage in airway epithelial cells could provide evidence for the role of oxidative injury in lung carcinogenesis, and help to address the potential public health implications of exposures to oxidant environments.

Keywords

air pollution; Cytome assay; oxidative stress; genotoxicity; physical exercise

Introduction

Ozone (O_3), a major component of air pollution, is a potent oxidant of biomolecules and causes airway injury in human lungs [Mudway et al., 2000]. Ozone reacts with respiratory tract lining fluid constituents and cellular membrane components to generate lipid ozonation products and reactive oxygen species (ROS), which in turn can cause oxidative damage to other biological molecules [Koren et al., 1989, Pryor et al., 1995, Schelegle et al., 1991]. Acute exposure to ambient levels of O_3 can induce short-term lung function abnormalities and airway inflammation, while chronic exposure may lead to remodeling of the small airways where deposition is the greatest [Aris et al., 1993, Arjomandi et al., 2005, Blomberg et al., 2003, Devlin et al., 1997, Frank et al., 2001, Seltzer et al., 1986].

To minimize the potential for oxidative injury, the human lung has an integrated system of antioxidant enzymes and expendable soluble molecules. ROS may overwhelm the antioxidant system leading to a state of "oxidative stress," which is thought to contribute to the pathogenesis of a number of respiratory diseases [Christian et al., 1998, Hackney et al., 1977, Jorres et al., 2000]. In addition, the inflammatory response of macrophages and neutrophils leads to production of more ROS [Grommes et al., 2011, Gwinn et al., 2006, Moraes et al., 2006].

Several studies have reported increased levels of genetic damage related to air pollution and specifically O_3 using different methodologies as follows. An increased level of chromosome aberrations in human lymphocytes was found in controlled experiments and natural settings [Merz et al., 1975, P. Rossner, Jr. et al., 2013]. Most of the studies involving exposed children and adults observed elevated frequencies of MN in nasal epithelial cells and lymphocytes [Gonseblatt et al., 1997, Lahiri et al., 2000, Neri et al., 2006, van Leeuwen et al., 2008]. Additionally, a number of studies employed analyses of DNA fragmentation using comet assay to demonstrate the genotoxicity of O_3 and other air pollutants [Calderon-Garciduenas et al., 1996, Pacini et al., 2003].

We utilized the cytokinesis-block micronucleus Cytome assay [Fenech, 2000], which, in addition to biomarkers of cytogenetic damage (micronucleus and nucleoplasmic bridges frequencies), also assesses endpoints for cytotoxicity (apoptotic and necrotic cells) and endoreduplication (nuclear buds) [Fenech, 2000]. This approach is well validated, and has been used to demonstrate genetic damage associated with exposure to radiation and environmental chemicals in human blood and exfoliated cells [Bolognesi et al., 2011, Fenech et al., 1999, Vral et al., 2011]. The mechanistic predictive value of the MN frequency assessment for cancer risk has been supported not only by theoretical considerations but also by a large range of experimental findings [Bonassi et al., 2011]. In a large prospective cohort study, the Human MicroNucleus (HUMN) international collaborative project found a significant increased incidence of all cancers for subjects in the groups with medium (RR = 1.84; 95% CI: 1.28–2.66) and high (RR = 1.53; 1.04–2.25) MN frequency levels [Bonassi et al., 2007]. The same groups also showed decreased cancer-free survival.

Previously, we reported that regional O_3 levels, which are highly correlated with season, were strongly associated with MN frequencies in lymphocytes and buccal cells of African-American children and their mothers from Oakland, CA [Huen et al., 2006]. Using a longitudinal study approach, we also reported elevated cytogenetic damage by the MN assay in healthy University of California, Berkeley students who were exposed to high ambient O_3 levels (with maximum 1-hr and 8-hr levels of 160 to 180 and 112 to 131 ppb, respectively) during summer breaks between the years 2000 and 2002 in Los Angeles or were acutely exposed to 200 ppb O_3 for 4-hr in a controlled chamber environment comparable to high pollution days in the urban environment [Chen et al., 2006].

The goal of the current study was to confirm the genotoxic effect of O_3 using an experimental design wherein the same individuals were exposed to clean filtered air (0 ppb), 100 ppb, and 200 ppb O_3, and to expand the number of biomarkers using the state-of-the-art Cytome approach. The 4-hr controlled exposure concentrations of 100 and 200 ppb were chosen to represent real world low and high ambient levels of O_3 in many metropolitan areas in the United States.

Materials and Methods

Study Description

This study used a repeated measures design in which subjects performed intermittent moderate-intensity exercise for 4-hr in a climate-controlled chamber with clean filtered air or ozone at 100 ppb or 200 ppb (Fig. 1). The endpoints were measured immediately before (0-hr) and on the following morning after exposure (24-hr). Each subject participated in three exposure experiments (one for each ozone concentration), with three-week recovery periods in between. Assignment of the order of rotation between filtered air, 100 ppb, and 200 ppb ozone was randomized to avoid any bias. Except for bronchoscopy, all endpoints were measured at the 0-hr and 24-hr time points. Bronchoscopy was performed at the 24-hr time point only.

Twenty-two subjects were recruited using the following inclusion criteria: (1) age between 18–50 years; (2) ability to perform moderate-intensity exercise; (3) healthy with no history of cardiovascular, hematologic, or pulmonary diseases other than mild asthma; (4) non-smoker as defined by having a history of less than ½ pack-year lifetime tobacco use and no history of any tobacco use in the past 6 months; and (5) no history of illicit drug use. Asthma was defined as self-report of physician-diagnosed asthma and airway hyper-responsiveness to inhaled methacholine (provocative concentration of methacholine resulting in a 20% decrease in forced expiratory volume in 1 sec (FEV1) compared with baseline [PC20] <8.0 mg/ml) measured in our laboratory according to a standard protocol [Arjomandi et al., 2005]. Among 22 subjects, females and males were represented equally, with an average age of 33.0 ± 7.4 years (Table I). Some of the participants (40%) were previously diagnosed with mild asthma but were in remission during their participation in this study.

The subjects were informed of the risks of the experimental protocol and signed a consent form that had been approved by the UCSF and UCB Institutional Review Boards (protocol #10-01078).

Experimental Protocol

After a telephone interview, subjects were scheduled for an initial visit to the laboratory, where a medical history questionnaire was completed. A 30-min exercise test designed to determine a workload that generated the target ventilatory rate (20 L/min/m^2 body surface area) was also completed on the initial visit. During a separate visit, the subjects underwent the exercise protocol. The exercise sessions lasted 4-hr and included alternating 30-min exercise and rest periods. The exercise consisted of running on a treadmill or pedaling a cycle ergometer. The exercise intensity was adjusted for each subject to achieve the target expired minute ventilation (V_E) of 20 L/min/m^2 body surface area (BSA), following a previously published protocol [Arjomandi et al., 2005]. During exercise, V_E was calculated (LabView 6.1; National Instruments, Austin, TX) from tidal volume and breathing frequency measured using a pneumotachograph for 1 min at 10-min and 20-min time-points during each 30-min exercise period. Subjects remained inside the chamber for the entire 4-hr exercise period. Each subject underwent blood pressure, heart rate measurement, and electrocardiographic (ECG) monitoring for heart-rate variability measurement at 0-h, 4-h, and 24-h time-points. Bronchoscopies were performed 20 ± 1-hr after the end of each exposure according to our laboratory's established bronchoscopy and bronchoalveolar lavage (BAL) procedures [Arjomandi et al., 2005]. Briefly, the bronchoscope was advanced into the right middle lobe bronchus and after obtaining a "wedge," BAL was performed with instillation of two 50-ml aliquots of 0.9% sterile saline warmed to 37°C and then application of gentle suction. The BAL was collected in a polyethylene tube and placed on ice transiently during transport to laboratory. A small aliquot (1 ml) of the BAL was separated for performance of cell counting, and the remainder was immediately fractionated into cells and fluid using centrifugation at 180g for 15 minutes at 4°C. The supernatant (BAL fluid) and a portion of the BAL cell pellet (BAL cells) were separated and frozen at −80°C.

Climate-Controlled Chamber and Atmospheric Monitoring

The exposures took place in a climate-controlled chamber ventilated with clean filtered air at 20°C and 50% relative humidity. The climate-controlled chamber is a stainless steel and glass room of 2.5×2.5×2.4 m (Model W00327-3R; Nor-Lake, Hudson, WI) that was custom-built and designed to maintain temperature and relative humidity within 2.0°C and 4% from the set points, respectively (WebCtrl Software; Automated Logic Corporation, Kennesaw, GA). The chamber make-up air was passed through both HEPA and purified charcoal filters. Temperature and relative humidity were recorded every 30s and displayed in real-time (LabView 6.1; National Instruments, Austin, TX).

Blood Draw and Cell Cultures

A sample of peripheral venous blood (25–30 ml) was collected in heparin vacutainers from the subject's arm at 0-hr and 24-hr time-points for each of the three exposure cycles that were separated by three weeks recovery periods (Fig. 1). Blood was transferred from the UCSF Human Exposure Laboratory at San Francisco General Hospital (JB, MA) to the Children's Environmental Health Laboratory (NH) at the University of California, Berkeley in a cooler no later than 4 to 6-hr after collection.

Methods of cell collection, processing, and scoring criteria for peripheral lymphocytes have been described previously [Titenko-Holland et al., 1998]. Briefly, whole blood was cultured for 72 hours at 5% CO_2 at 37°C. Cytochalasin B (Sigma, St.Louis, MO) was added at a concentration of 6 μg/mL of culture 44-hr post-initiation to arrest cytokinesis. Isolated lymphocytes were spun directly onto slides using a Shandon CytoSpin 3 (Thermo Scientific, Wilmington, DE), fixed in methanol, and stained with May-Grunwald-Giemsa.

Cytogenetic Analysis

Methods of cytogenetic analyses and scoring criteria for peripheral lymphocytes have been described previously [Fenech, 2000]. Briefly, one-thousand binucleated (BN) lymphocytes were scored under Zeiss microscope with immersion from two slides prepared from cultures established from peripheral blood collected before and 24-hr after controlled O_3 exposure. Frequencies of MN and NB were used to assess cytogenetic damage, and replicative index (RI) was calculated to measure cell proliferation in cell cultures. Replicative index was calculated as follows, and as described previously [Holland et al., 2002]:

$$RI = \frac{(1 \times \% mononuclear\ cells) + (2 \times \% binuclear\ cells) + (3 \times \% tri) + (4 \times \% tetra)}{100}$$

Prior to scoring, slides were mixed and coded so the scorer was not aware of the exposure status and time of collection. All "questionable" MN and other endpoints were double-checked by one of the authors (NH). Approximately 10% of the slides were re-scored to assure reproducibility. Variability of results did not exceed 20%, which is consistent with criteria of the Human International Micronucleus Project (HUMN) [Fenech et al., 2003, Fenech, 2006].

Scoring Criteria

Cytome assay criteria were based on the recommendations of Fenech [Fenech, 2000] and the HUMN Project [Fenech et al., 1999] as follows:

Criteria for scoring micronuclei (MN)—MN are morphologically identical to but smaller than the main nuclei. They also have the following characteristics: (1) The diameter of MN in human lymphocytes usually varies between 1/16 and 1/3 of the mean diameter of the main nuclei which corresponds to 1/256 and 1/9 of the area of one of the main nuclei in a BN cell, respectively. (2) MN are round or oval in shape. (3) MN are non-refractile and they can therefore be readily distinguished from artifacts such as staining particles. (4) MN are not linked or connected to the main nuclei. (5) MN may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary. (6) MN usually have the same staining intensity as the main nuclei but occasionally staining may be more intense.

Criteria for scoring nucleoplasmic bridges (NB)—NB are sometimes observed in binucleated cells following exposure to clastogens and are thought to originate from rearranged chromosomes with more than one centromere, i.e., dicentric chromosomes. They have the following characteristics: (1) NB are a continuous nucleoplasmic link between the

nuclei in a binucleated cell. (2) The width of an NB may vary considerably but usually does not exceed one-fourth of the diameter of the nuclei within the cell. (3) NB should have the same staining characteristics as the main nuclei. (4) On rare occasions more than one NB may be observed within one binucleated cell. (5) A binucleated cell with an NB may or may not contain one or more micronuclei.

Criteria for scoring apoptotic cells—(1) Early apoptotic cells can be identified by the presence of chromatin condensation within the nucleus and intact cytoplasmic and nuclear boundaries. (2) Late apoptotic cells exhibit nuclear fragmentation into smaller nuclear bodies within an intact cytoplasm/cytoplasmic membrane. (3) Staining intensity in the nucleus, nuclear fragments and cytoplasm is usually greater than in viable cells.

Criteria for necrotic cells—(1) Early necrotic cells can be identified by the presence of a pale cytoplasm with numerous vacuoles (mainly in the cytoplasm and some in the nucleus) and damaged cytoplasmic membrane with a fairly intact nucleus. (2) Late necrotic cells exhibit loss of cytoplasm and damaged/irregular nuclear membrane with only a partially intact nuclear structure and often with nuclear material leaking from the nuclear boundary. (3) Staining intensity of the nucleus and cytoplasm is usually less than that observed in viable cells.

Criteria for buds—The process of nuclear budding occurs during S-phase and the buds are characterized by having the same morphology as a micronucleus with the exception that they are linked to the nucleus by a narrow or wide stalk of nucleoplasmic material depending on the stage of the budding process [Fenech, 2006].

Sample Size and Power Calculations

Human inhalation O_3 exposure studies have reported significant changes in blood inflammatory and coagulatory biomarkers with a sample size of 30 subjects [Frampton et al., 1997, Frampton et al., 2004, Mills et al., 2005]. In addition, statistically significant changes in MN frequency in buccal cells have been reported with a sample size of 15 subjects with exposure to high (200 ppb) levels of O_3 [Chen et al., 2006]. We selected our sample size to detect a 10% change in blood lymphocyte MN frequency with presumed standard deviations twice the magnitude of the effect size. Given the above, sample sizes of 20 and 25 provided statistical powers of 81% and 86%, respectively, to observe a minimal change of 10% with a type I error of 5%.

Statistical Methods

This longitudinal study design allowed by-pair comparison of the data from each individual who served as his/her own control. This approach eliminated a significant part of the variability by excluding 1) inter-individual variability and 2) a part of intra-individual variability unrelated to O_3 exposure. Non-parametric Wilcoxon's sign-rank test was used to assess the significance of the change in the frequency of MN and other parameters due to O_3. All data were expressed as an average ± sd throughout the text and in the tables and graphs since sd is an index of the inter-individual differences. The change in the frequencies of MN and chromosome bridges may depend on baseline and level of O_3 exposure. Age and

sex were also considered as possible covariates. These associations were measured by non-parametric Spearman rank correlation coefficients. Linear correlation coefficients were also assessed. *P*-values were calculated based on the Spearman rank test.

All associations were examined using a generalized estimating equation (GEE) approach to account for the potential correlation of outcomes measured more than once on the same subject. Robust standard errors were used for all statistical inferences. For the non-count outcomes (RI and percent degenerate), GEE linear regression was used, whereas GEE Poisson regression was used for all other outcomes. Statistical significance was defined as *P* 0.05.

Results

Effects of Ozone Exposure on Cytogenetic Endpoints

We scored cytogenetic damage in blood lymphocytes analyzed by the Cytome assay before and 24-hr after exposure of 22 volunteers to filtered air (control) or either 100 or 200 ppb of O_3 during 4-hr exposure periods (Table II). Each of the participants was assigned randomly to a treatment group and rotated through all three exposure regimens with 3 week recovery periods, resulting in 66 individual experiments with two blood cell cultures for each experiment (before and 24-hr post-exposure), for a total of 132 subject-time points. Inhalation of O_3 was associated with a significant dose-dependent increase in the MN frequency (9.3 ± 1.5, 13.0 ± 2.6, and 28.7 ± 3.9 MN/1000 binucleated cells for FA, 100 ppb, and 200 ppb O_3, respectively; $P < 0.0001$). The difference in the MN frequency (calculated by subtracting the baseline MN frequency for each experiment from the post-treatment levels) doubled between FA and 100 ppb O_3, and was almost 7-fold larger at 200 ppb O_3 (Fig. 2).

A similar highly significant increase was observed in the number of micronucleated cells (average of 6.8 with FA to 16.6 MN cells/1000 binucleated cells after 200 ppb O_3, Table II). Quite a few of the cells, especially after ozone exposure, had multiple MN. In lymphocytes analyzed before the chamber treatment, most of the micronucleated cells had one MN (74.8%, Fig. 3). Only 33% of such cells had more than 1 MN after FA exposure, with an increase to 49.8% after 100 ppb, and to 61.9% following 200 ppb O_3 exposure ($P < 0.0005$). This clear trend is a further demonstration of the genotoxicity of ozone exposure by the MN test in human lymphocytes.

In addition to this evidence of the effect of O_3, we also observed a surprising 30% increase in the frequency of MN in subjects who were exposed to FA ($P < 0.001$; Table II, Fig. 2). This may be attributable to the effects of intermittent exercise or other factors during subjects' 4-hr in the chamber that could be associated with increased oxidative stress.

Nuclear buds are considered to be a biomarker of endoreduplication, and were also significantly more common after all treatments than at baseline ($P < 0.001$). However, the response to O_3 exposure was not statistically significant (Table II). While the absolute frequency at 200 ppb was slightly higher than at other exposure regimens, and overall values had a suggestive trend (4.6, 4.9 and 5.5% buds per 1000 cells, respectively, $P = 0.07$), the

changes were very similar after subtracting baseline levels (2.54, 2.87 and 2.91, respectively).

Nucleoplasmic bridges (NB), known to be a reliable biomarker of radiation damage and other genotoxic exposures [Fenech, 2006], responded to the chamber exposure in an unexpected way (Table II). Their frequency nearly doubled after all three exposure protocols in comparison to the baseline. However, there was no difference between post-exposure levels for FA, 100 ppb or 200 ppb O_3 (14.7, 14.8, 14.5 NB per 1000 binucleated cells, respectively).

We observed a slightly higher MN frequency (7%) in female study participants than in males, but it was not statistically significant ($P = 0.139$). No difference by sex was seen for bridges or other Cytome endpoints, and no difference was observed between healthy normal subjects and those with mild asthma in remission.

Effects of Ozone on Apoptosis, Necrosis and Cell Proliferation

Percent of apoptotic cells ranged from 2.2–2.4% at baseline to 3.2, 3.5 and 4.0% for FA, 100 and 200 ppb O_3, respectively. The post-exposure increase was dose-dependent and statistically significant ($P = 0.002$, Fig. 4). As previously noted for MN, NB and nuclear buds, there was a significant difference between baseline and FA levels, indicating that in addition to the effects of O_3, other factors during 4-hr chamber exposure contributed to the changes in the frequency of apoptotic cells.

In contrast, no difference in necrotic cells was observed in response to ozone exposure; the post-exposure levels were almost identical for all three O_3 levels (7.4–7.9%). However, there was again a noticeable difference between baseline and FA levels, with 16.2% increase post-exposure, comparable to the increase observed at 100 ppb O_3 (16.2%) and 200 ppb O_3 (13.4%).

Replicative index shows that cell proliferation was only mildly affected by the chamber exposure with no difference by O_3 level ($P = 0.783$). To confirm that altered proliferation was not responsible for the observed differences in cytogenetic response or apoptosis and necrosis, we conducted a sensitivity analysis with adjustment for replicative index. The results (not shown) were similar to the data reported above.

Neutrophilia and Cytogenetic Damage

To account for effects of inflammation, presence of neutrophils was measured in bronchoalveolar lavage fluid 20-hr after each chamber experiment (24-hr after the start of the exposure, as shown in Fig. 1). The concentration of neutrophils was higher at 100 ppb in comparison to FA ($P = 0.09$) and even higher at 200 ppb ($P = 0.05$) (Fig. 5). To consider whether the effects of neutrophilia and ozone exposure on cytogenetic damage were synergistic, we performed generalized logistic regression modeling that demonstrated that while both were statistically significantly associated with MN frequency ($P < 0.0001$ for ozone and $P = 0.033$ for neutrophilia), these two factors were independent (Fig. 6).

Discussion

This study, wherein repeated measures were used to assess the cytogenetic effects of ozone exposure in a controlled chamber environment, shows that inhalation of high ambient levels of ozone resulted in dose-dependent increases in MN frequency and apoptosis in peripheral blood lymphocytes. Additionally, neutrophilia measured in BAL had an independent effect on genotoxicity. A consistent increase in several Cytome biomarkers was observed after exposure to pure filtered air, indicating that other factors apart from ozone exposure, such as exercise, may also contribute to cytogenetic damage, inflammation, and cell apoptosis. To our knowledge, this is the first study with controlled exposure to filtered air and two doses of ozone that employed a repeated measures design and the Cytome assay.

These new data corroborate our previous observation of the relationship between cytogenetic effects and chronic and acute ozone exposure in humans [Arjomandi et al., 2005, Chen et al., 2006]. In a study of healthy students who were exposed to 200 ppb ozone for 4-hr with intermittent exercise, we also observed an almost two-fold increase in the frequency of micronuclei and nucleoplasmic bridges [Chen et al., 2006]. However, in that study we were not able to compare ozone to filtered air exposure, and only one exposure dose was used. Somewhat surprisingly, the increase we observed in the frequency of bridges in the current study cannot be attributed to ozone exposure, because this doubling of bridge frequency was observed in all three exposure protocols (filtered air, 100 and 200 ppb). Therefore, a more likely explanation of this increase may be related to the effects of exercise.

Several studies investigating the relationship between controlled exposure to ozone at different doses and outcomes of interest report inconsistent results regarding observed genotoxicity. For example, no increase in DNA single-strand breaks in human lymphocytes was observed after 2.5-hr exposure to 210 ppb ozone [Finkenwirth et al., 2014]. In contrast, in a study of subjects exposed to either filtered air or 200 ppb ozone, a significant increase in neutrophils was reported 6-hr later, with a positive correlation between the response in blood and bronchial biopsies [Bosson et al., 2013]. This observation corroborates our finding in the current study that neutrophilia was predictive of MN frequency, independent of ozone exposure.

The evidence from controlled ozone experiments is augmented by data from several studies that also report adverse effects of chronic exposure to air pollution and high oxidant environments on genotoxicity biomarkers. When seasonal changes in ozone levels were estimated based on local monitoring and geographic information system-based air pollution as a surrogate of traffic-related pollution, a statistically significant association was found between pollution levels and MN frequency in lymphocytes and buccal cells of African-American mothers and children in California [Huen et al., 2006]. Two studies from Mexico reported an increase in DNA damage (by comet assay) in lymphocytes and nasal cells (but not in buccal cells) in young adults in the polluted area of Mexico City in comparison to clean areas [Valverde et al., 1997]. Elevated levels of genotoxicity were also reported in nasal cells of children from the same region [Calderon-Garciduenas et al., 1996]. Similar relationships between air pollution and genotoxicity (measured by MN assay in buccal

epithelial cells) were observed in both Kolkata [Lahiri et al., 2000] and Turkey [Demircigil et al., 2014]. In the latter study, a significantly higher frequency of MN was found during the summer in urban children but not in the suburban group. The authors interpreted this seasonal variation, which has also been observed in students from California [Chen et al., 2006], as the result of the relatively high ozone levels and increased time spent outdoors during summer [Demircigil et al., 2014]. Overall, chronic and acute exposure to ozone appears to produce consistent increases in genetic damage in different tissues in both children and adults, with children likely to be more susceptible [Holland et al., 2011, Neri et al., 2006].

In a large prospective international consortium study, elevated human lymphocyte MN frequencies have been linked to an increased risk of cancer, consistent with earlier reports for the frequency of chromosome aberrations, another cytogenetic biomarker [Bonassi et al., 2008, Hagmar et al., 2004, P. Rossner et al., 2005]. This emphasizes the significance of the effects of O_3 and air pollution in general as a potential health risk including risk of cancer via a genotoxicity mechanism.

One of the most intriguing aspects of the current study is the finding that the significant cytogenetic damage observed in the filtered air exposure scenario can most likely be attributed to the effects of the intermittent exercise that subjects were performing over a 4-hr period while inside the chamber. The relationships between exercise-induced oxidative stress and molecular changes and health effects have been analyzed in both animal and human studies [Fisher-Wellman et al., 2014, Powers et al., 2011]. The association between muscular exercise and oxidative stress in humans was first reported more than 30 years ago [Dillard et al., 1978] when an attempt to ameliorate the adverse effects of ozone with the application of antioxidants proved to be unsuccessful. While it was shown that vitamin E did not protect from oxidative stress associated with physical activity, a number of later studies were focused on the potential molecular mechanisms of oxidative stress, including lipid peroxidation, changes in the mRNA expression of endogeneous oxidant systems, and the release of substantial amounts of ROS from phagocytic cells [Malech et al., 1987, Powers et al., 2011]. The definition of oxidative stress as "an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage" [Sies et al., 2007] helps to interpret the cytogenetic findings of the current study which show that not only ozone but also exercise and neutrophilia may contribute to higher levels of ROS in the blood stream, which may lead to chromosome damage.

Strengths of this study include its use of a repeated measures study design, an experimental protocol with use of filtered air as a control and two levels of ozone exposure, and the use of the state-of-the-art Cytome assay to assess multiple biomarkers of genetic damage and cytotoxicity. However, there were also some limitations. Only healthy participants or subjects with mild asthma in remission were included in this experiment due to ethical considerations. Patients with active asthma are likely to exhibit an even more pronounced response as they are known to be highly sensitive to air pollution and ozone. Additionally, the number of participants (n=22) was limited by the labor-intensive nature of this protocol (132 subject time points in total). This precluded an examination of the effects of genetic

polymorphisms that have been reported as potential modifiers of the response to air pollution, such as *GSTM1* and *GSTT1* gene deletions. Previously, large cohort studies demonstrated that individuals with homozygous deletions for these detoxification enzymes may be especially susceptible to cytogenetic damage [Kirsch-Volders et al., 2006].

In summary, the dose-dependent increase in cytogenetic damage that we observed after ozone exposure is particularly important when considered alongside the damage that can be attributed to the oxidative stress that results from moderate exercise. The experimental exposures used in this study are representative of urban environments with high seasonal levels of ozone, where outdoor fitness and occupational physical activity are common. Considering that cytogenetic damage is a prospective biomarker of cancer risk, our findings suggest oxidative injury as a possible mechanistic link between air pollution and cancer. Further, cytogenetic damage may also play a role in other diseases characterized by increased oxidative stress. Considering the very important role an active lifestyle plays in maintaining health, it is important to understand the optimal approach to exercise while limiting the potential adverse effects of physical activity in high oxidant environments.

Acknowledgments

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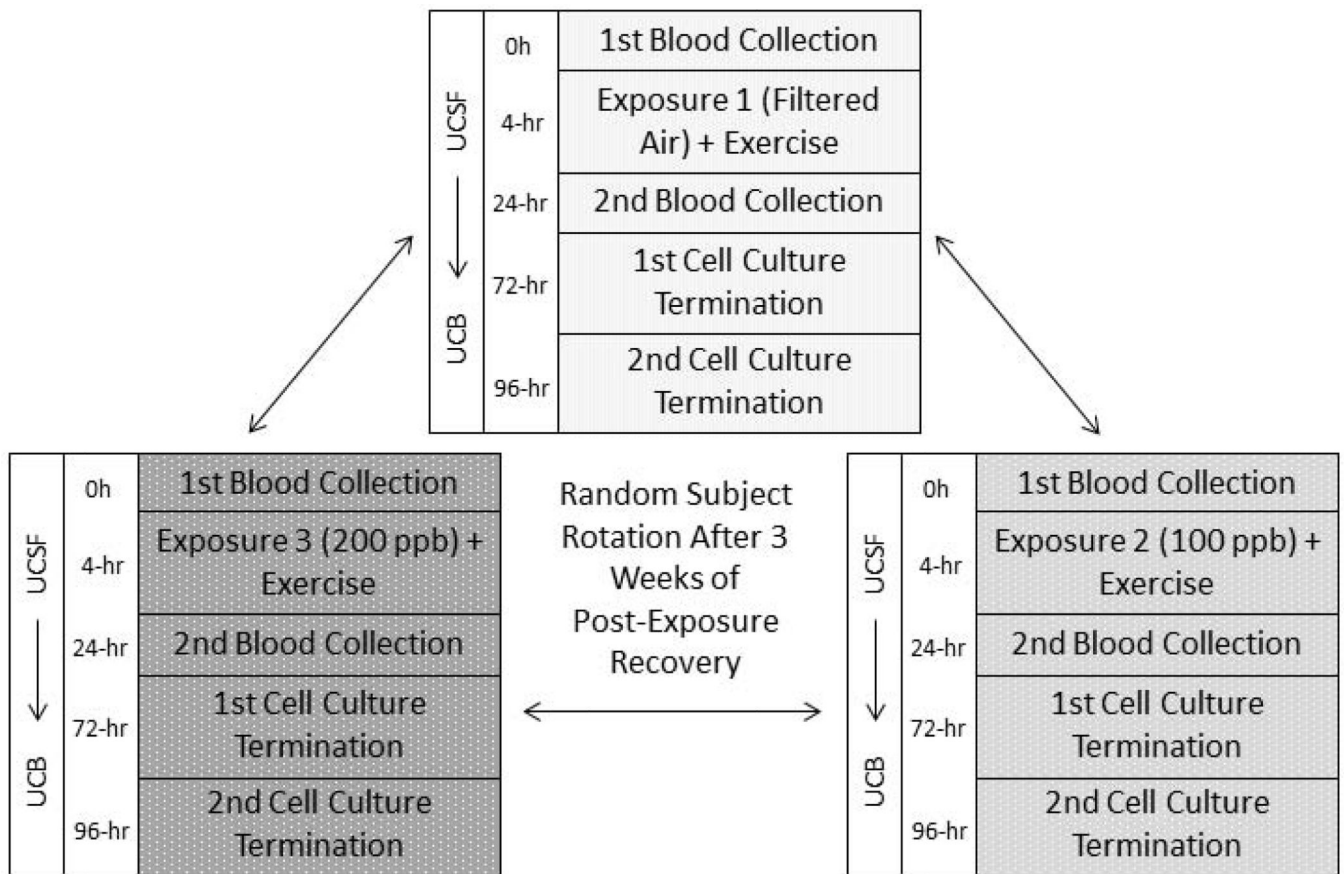
Figure 1. Repeated measures study design
Subjects (n=22) were rotated through three exposure protocols (filtered air, 100 ppb O_3, and 200 ppb O_3) in a random order, with three weeks of recovery between each trial. Blood was collected before and 24-hr post exposure at the University of California, San Francisco (UCSF). Lymphocytes from pre- and post-exposure blood were cultured and scored for cytogenetic markers at the University of California, Berkeley (UCB).
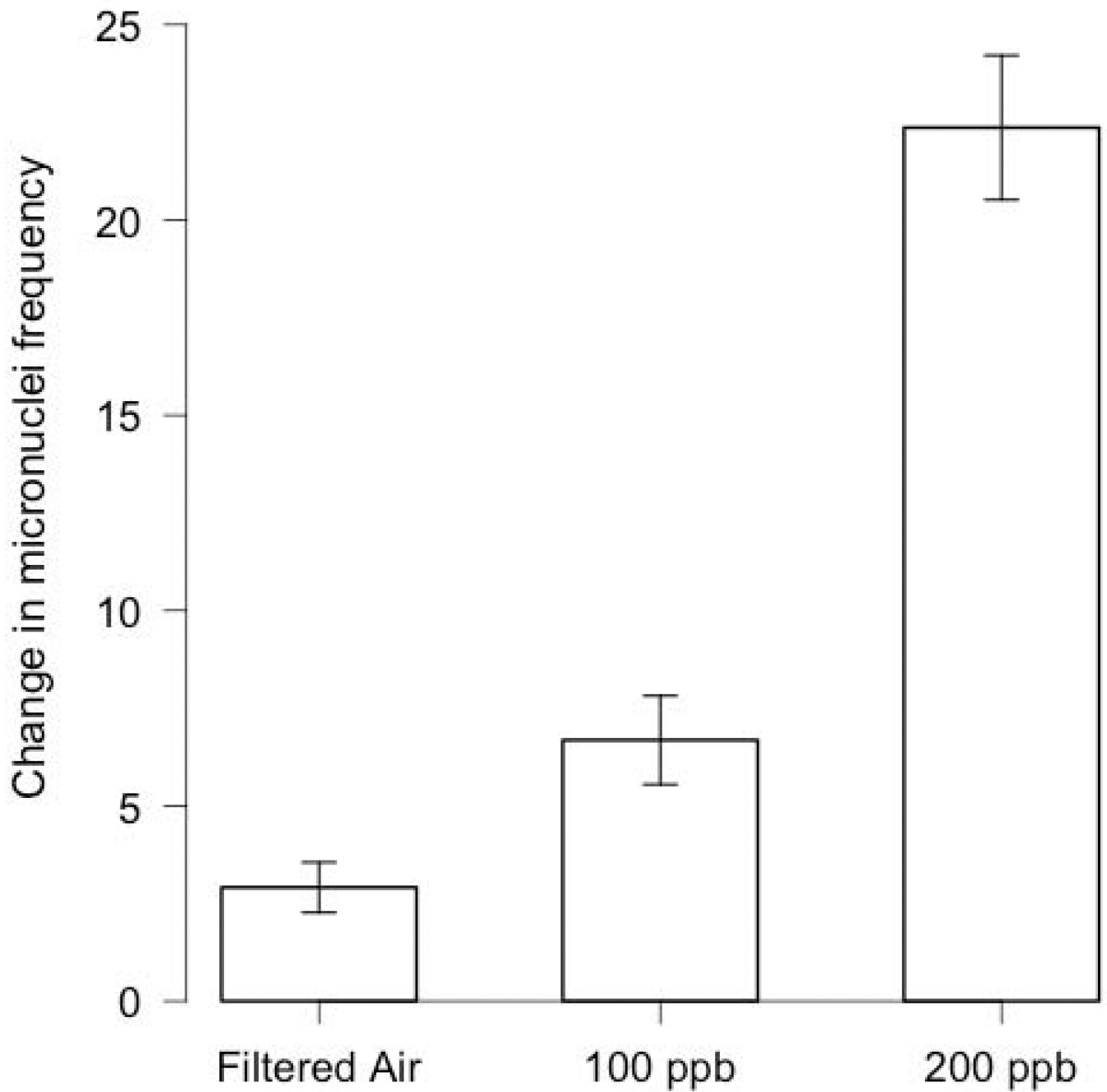
Figure 2. Micronuclei by ozone exposure
Graph represents the differences between post- and pre-exposure numbers of observed micronuclei per 1000 cells with bars showing 95% confidence intervals and strong evidence of a positive trend ($P < 0.001$).
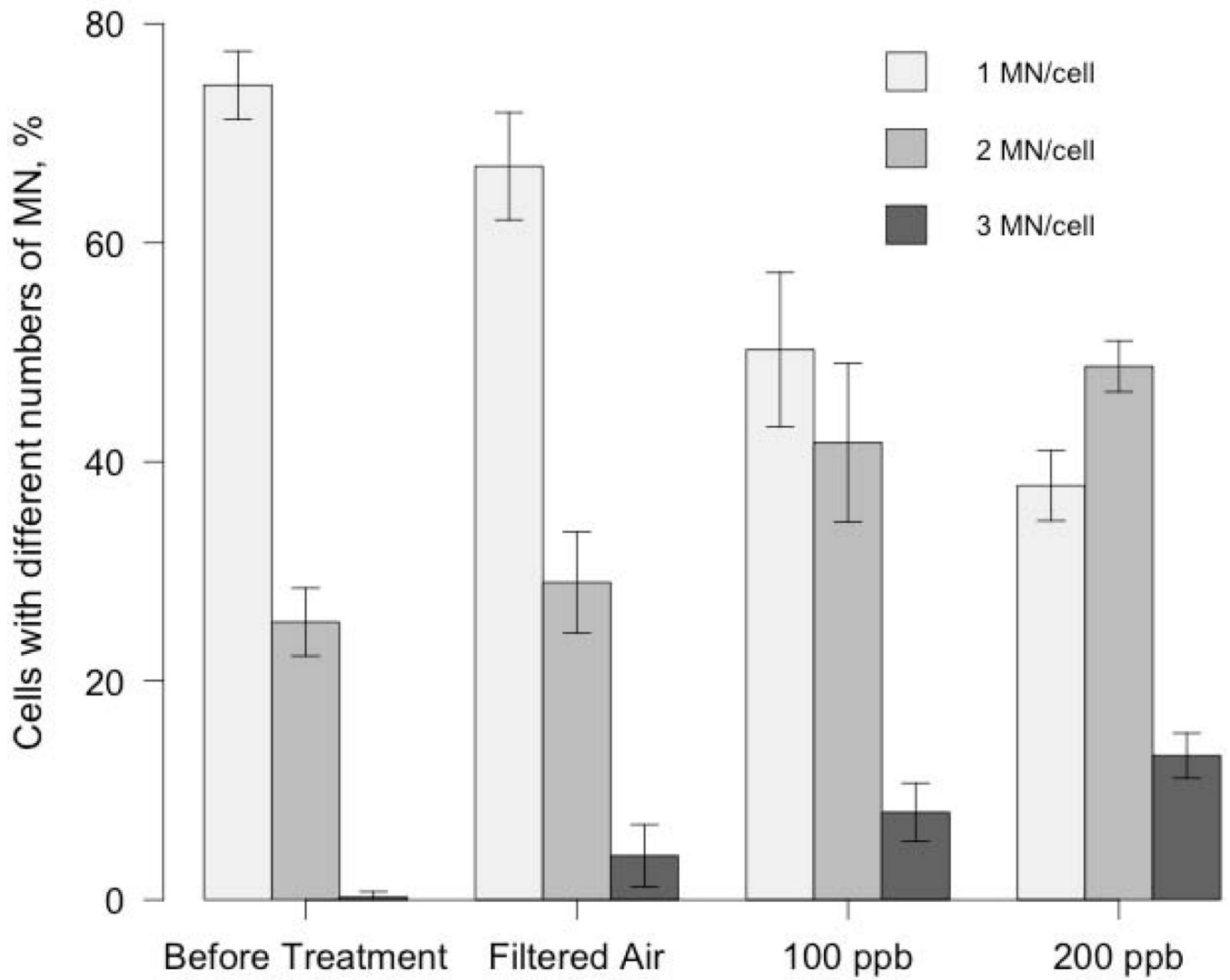
Figure 3. Micronuclei in binucleated cells by ozone exposure
Graph represents the mean percentage of binucleated cells with 1, 2, or 3 micronuclei (MN) before and after exposure with bars showing 95% confidence intervals. With increasing exposure, we see evidence of a downward trend in the percent of binucleated cells with 1 MN ($P < 0.001$) and upward trends in the percent with 2 or 3 MN ($P < 0.001$, for each).
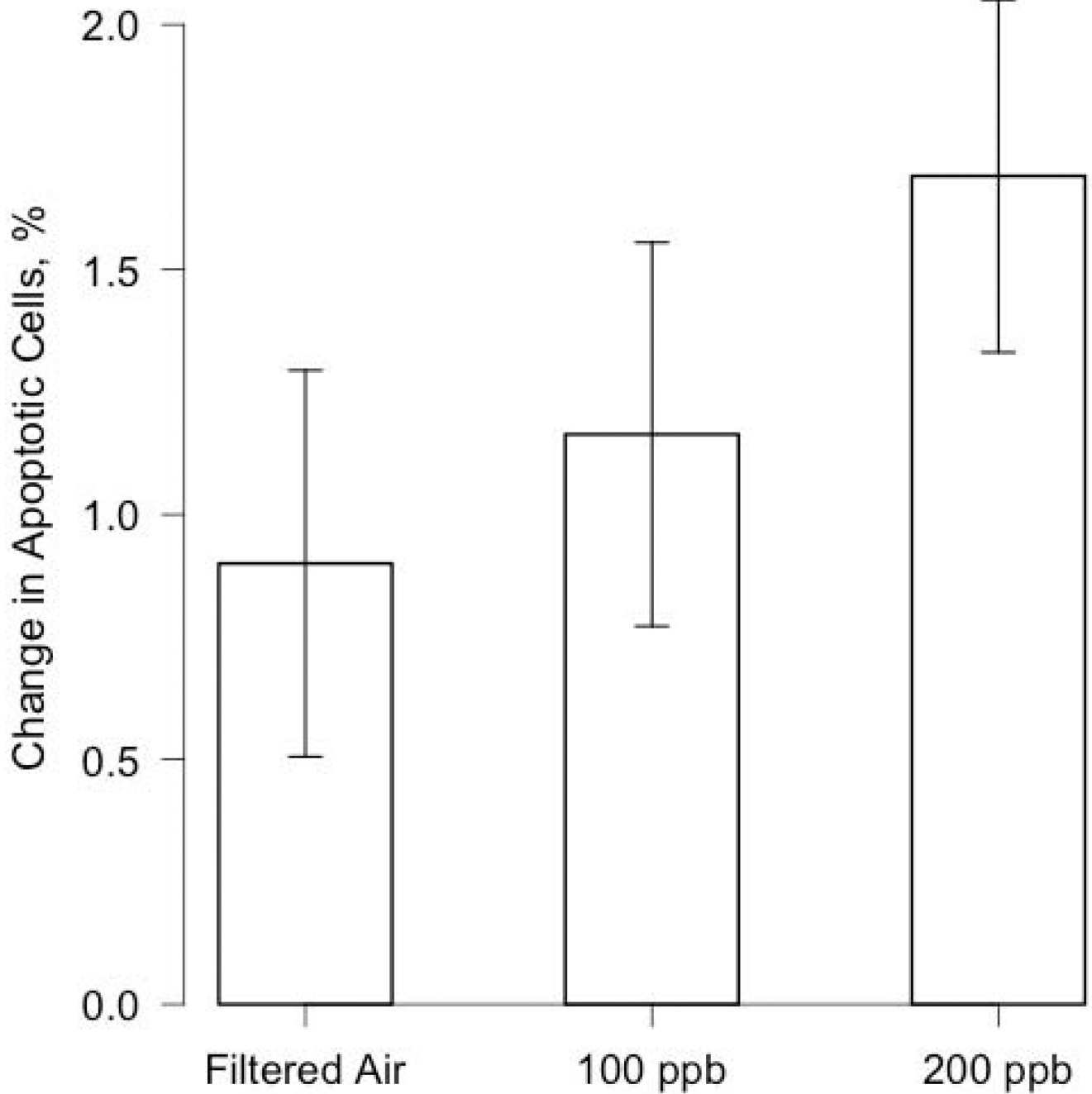
Figure 4. Cellular apoptosis by ozone exposure
Graph represents the differences between post- and pre-exposure percentages of apoptotic cells, with bars showing 95% confidence intervals and evidence of a positive trend ($P < 0.005$).
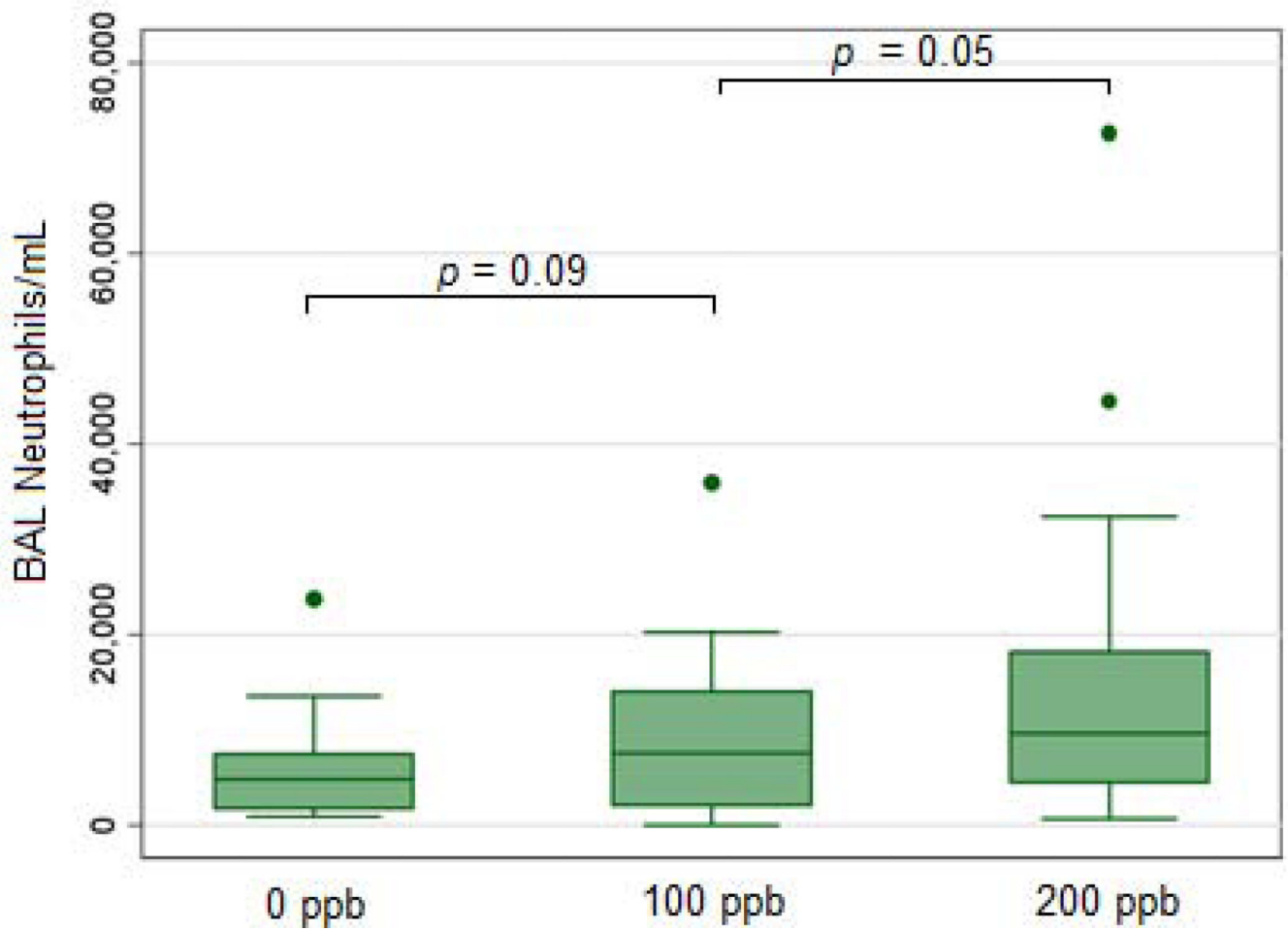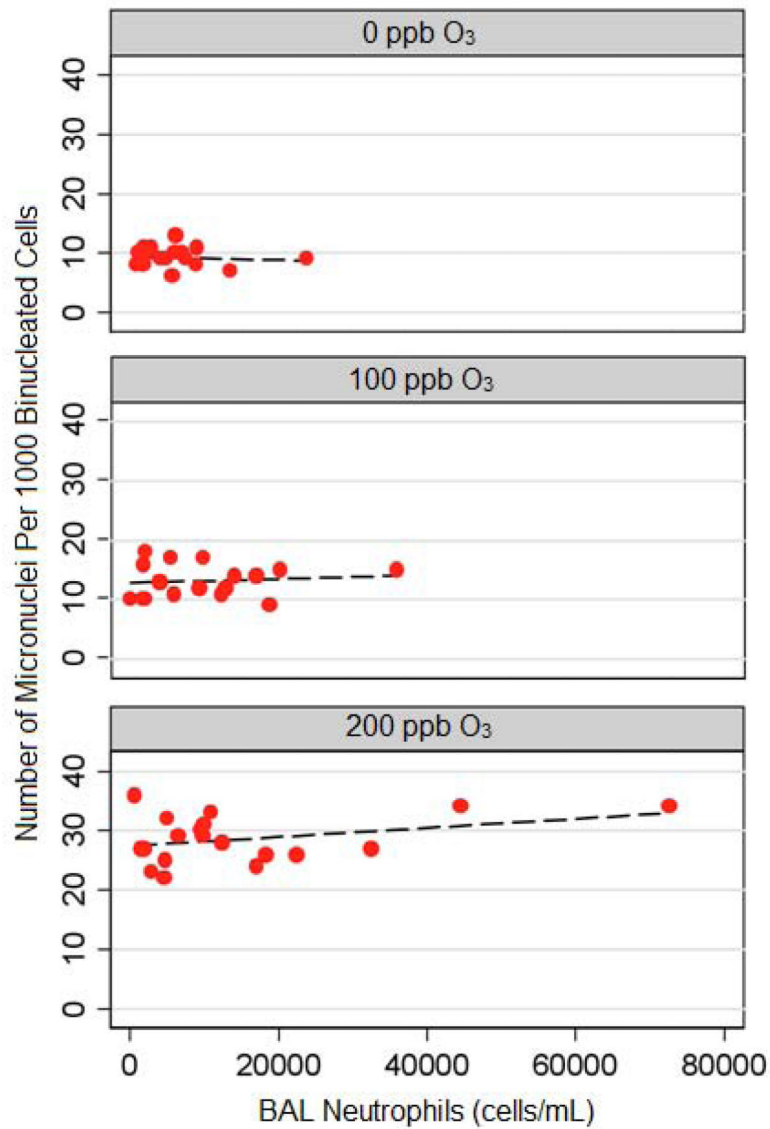
Figure 5. Ozone-induced BAL inflammation measured by the presence of neutrophils
The concentration of BAL neutrophils was higher after exposure to 100 ppb O_3 relative to filtered air ($P = 0.09$), and even higher after exposure to 200 ppb O_3 ($P = 0.05$).

Predictor	Estimate (SE)	*p*-value
O$_3$ (ppb)	0.09 (0.007)	<0.0001
Neutrophils (10^4 cells/mL)	0.85 (0.396)	0.033

Figure 6. Association of MN with BAL neutrophils is independent of effects of O$_3$ exposure
Both O$_3$ exposure and BAL neutrophil concentration were independently statistically significantly associated with MN frequency, but no evidence of synergy was observed.

Table I

Subject Characteristics

Subject Characteristic	All Subjects (n=22)	Female (n=12)	Male (n=10)
Age (years)	33.0 ± 7.4^{1}	32.6 ± 8.3	33.3 ± 6.6
Height (cm)	169.4 ± 9.45	163.6 ± 6.1	176 ± 7.6
BMI $(kg/m^2)^2$	26.1 ± 6.5	26.6 ± 8.0	25.6 ± 4.1
BSA $(m^2)^2$	1.87 ± 0.28	1.69 ± 0.31	1.93 ± 0.17
V_E (L/min/m^2 BSA)^2	19.98 ± 0.46	19.9 ± 0.27	20.1 ± 0.64
Mild Asthmatics [N (%)]	10 (40%)	5 (36%)	5 (45%)

BMI: body mass index; BSA: body surface area; V_E expired minute ventilation

Table II

MN Assay Results in Lymphocytes from Subjects Exposed to Ozone (n=22)

Cell Type	Filtered Air		100 ppb		200 ppb		Beta Exposure		P-value[1]	
	0-hr (Range)	24-hr (Range)	0-hr (Range)	24-hr (Range)	0-hr (Range)	24-hr (Range)	0-hr	24-hr	0-hr	24-hr
Binucleated cells										
MN/1000 cells	6.36 (5 – 9)	9.27 (6 – 13)	6.91 (6 – 9)	13.04 (9 – 18)	8.0 (6 – 10)	28.73 (22 – 36)	0.008	0.097	<0.0001	<0.0001
MN cells/1000 cells	5.09 (4 – 7)	6.77 (5 – 8)	5.68 (5 – 8)	8.27 (6 – 13)	6.18 (5 – 8)	16.59 (12 – 21)	0.005	0.049	<0.0001	<0.0001
Bridges/1000 cells	7.72 (6 – 9)	14.68 (6 – 22)	8.04 (6 – 10)	14.82 (7 – 20)	8.50 (6 – 16)	14.54 (0 – 19)	0.004	-0.0007	0.169	0.917
Nuclear Buds/1000 cells	1.95 (1 – 3)	4.59 (1 – 8)	2.04 (1 – 3)	4.91 (2 – 7)	2.59 (0 – 7)	5.5 (3 – 8)	0.003	0.004	0.064	0.07
All Cells										
% Apoptotic	2.3 (1.4 – 3.2)	3.2 (1.4 – 4.2)	2.22 (1.4 – 3)	3.46 (1.4 – 4.2)	2.40 (1.6 – 3.8)	3.99 (2.6 – 4.8)	0.005	0.004	0.526	0.001
% Necrotic	6.63 (5.6 – 8)	7.91 (5.9 – 9.2)	6.22 (5 – 7.2)	7.42 (5 – 9.2)	6.75 (5 – 9.2)	7.79 (7.2 – 9.2)	0.006	-0.0006	0.623	0.674
RI	1.62 (1.6 – 1.7)	1.52 (1.5 – 1.7)	1.63 (1.6 – 1.7)	1.49 (1.5 – 1.7)	1.61 (1.5 – 1.7)	1.49 (1.4 – 1.5)	0.001	-0.0002	0.367	0.783

[1] All 0-hr to 24-hr means are significantly different.

MN: micronuclei; ppb: parts per billion; RI: replicative index