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Genotoxicity in B6C3F1 Mice Following 0.5 ppm Ozone Inhalation

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ABSTRACT *: To determine whether ozone is genotoxic at environmentally relevant exposure level, B6C3F1 mice were exposed to 0.5 ppm ozone for 12 weeks, 6 hr/day. Chromosomal aberration, supravital micronucleus and hprt mutation assays were performed. The percentage of abnormal cells was significantly increased at 0.5 ppm ozone when compared to unexposed control in chromosome aberration assay. Significant increase in the frequencies of micronucleated reticulocytes and 6-thioguanine-resistant (TG^r) lymphocytes was also observed in supravital micronucleus assay using peripheral blood and lymphocyte hprt mutation assay, respectively. The results indicate, that under our experimental conditions, 0.5 ppm ozone are genotoxic in exposed B6C3F1 mice.*

Key Words *: 0.5 ppm ozone, Chromosomal aberration assay, Supravital micronucleus assay, Lymphocyte hprt mutation assay, Genotoxicity*

I. INTRODUCTION

Ozone is a common urban air pollutant to which human are routinely exposed. This environmental air pollutant, which is generally associated with large cities, can cause damage to the lung when inhaled. The national ambient air quality standard for ozone, 0.12 ppm for a daily 1-h average, is exceeded in more than several cities in the worldwide (U.S. Environmental Protection Agency, 1986). Laboratory animal and human clinical studies have demonstrated that ozone causes reversible decrements in pulmonary function, increased permeability of the epithelium, influx of inflammatory cells, impaired pulmonary defense capacity and tissue damage (Lippmann, 1989). Ozone is mutagenic and has carcinogenic potential in rodents, since it causes DNA damage by oxidative stress from hydroxyl radicals, superoxide, singlet oxygen, and hydrogen peroxide (Lippmann, 1989).

Ozone is also genotoxic to microorganisms, plants and *in vitro* cell (Lippmann, 1989). The results from cytogenetic studies with laboratory animals after ozone exposure are somewhat contradictory. Chromosome aberrations in lymphocytes, but not sister-chromatid exchanges (SCEs), have been demonstrated in Chinese hamsters but not in mice (Zelac *et al.*, 1971; Gooch *et al.*, 1976). Chromatid deletions were induced in pulmonary macrophages in rats (Rithidech *et al.*, 1990). No cytogenetic effects have been reported for bone marrow cells or spermatocytes. Limited experimental and epidemiological studies with human subjects do not allow a conclusion on the cytogenetic effects of ozone in human lymphocytes (Merz *et al.*, 1975; Guerrero *et al.*, 1979; Sarto and Viola, 1980). No life-long cancer studies have been performed with ozone. However, after 4 and 6 months of inhalation exposure, lung adenomas were induced in strain A/J mice, but not in Swiss-Webster mice (Victorin, 1992).

The present study describes the genotoxic potential of 0.5 ppm ozone in chromosomal aberration assay, supravital micronucleus assay in peripheral bloods and *hprt* mutation assays.

II. MATERIALS AND METHODS

1. Animals

Male and female B6C3F1 mice were obtained from Dae-Han Laboratory Animal Co., Ltd. (Seoul, Korea) at 4-5 weeks of age and were acclimated for about 7

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days before initiation of chemical exposure. Before and after ozone exposures, the mice were housed 5 per cage in polycarbonate cages with bottom wire net. Food and water were provided *ad libitum* except the period of ozone exposures. Rooms were maintained at 20~22°C, with a relative humidity of 20~50% and a 12-h light/dark cycle. All of the methods used in this study were approved by the Animal Care and Use Committee at SNU and conform to NIH guidelines (NIH publication No. 86-23, revised 1985).

2. Exposures

Mice were exposed in 1.5 m^3 whole-body inhalation exposure chambers (Dusturbo, Seoul, Korea). Animals were exposed for 6 hr per day (between 9:00 AM and 3:00 PM), 5 days per week for 12 weeks, 0.50±0.02 ppm ozone. Ozone (CAS NO. 10028-15-6) was generated from pure oxygen using a silent arc (corona) discharge ozonator (Model KDA-8, Sam-Il Environment Technology Inc., Korea) and was monitored by gas detection system with O_3 gas sensor (Analytical Technology Inc., USA). Measurements were taken at 12 locations in each chamber to ensure uniformity of ozone distribution and uniformity was enhanced by use of a recirculation device. Airflow in the chambers was maintained at 15 changes per hour. Ambient ozone was removed from the air entering all chambers using a potassium permanganate filter and charcoal and HEPA filters were used to further filter the air. The mice of test group were exposed to the same concentration of ozone used in the National Toxicology Program (NTP) carcinogenesis study (National Toxicology Program, 1994).

3. Chromosomal aberration assay

Ozone-exposed and control mice were euthanized after 12 weeks exposure by sodium pentobarbital overdose. Spleens were dissected out from each animal and splenocytes were collected by goring the spleen through a sterilized nylon filter and washing the filter to collect the cells in phosphate buffered solution (PBS). The cell suspension was then gently layered on a same volume of Histopaque 1077 (Sigma) and centrifuged at 800 × g for 15 min. The interface was carefully collected, resuspended in PBS and centrifuged at 200 × g for 15 min. The cell pellet was once more resuspended in PBS and centrifuged at 200 × g for 10 min. Finally, the cell pellet was resuspended in 1 m*l* of PBS. The cell concentration was calculated by using a haemocytometer.

Splenocyte cultures were initiated at a concentration of 1 × 10^6 cells/ml in complete medium. Growth medium consisted of RPMI 1640 (Gibco, UK) supplemented with 15% fetal bovine serum (Life Technology, Sweden), 2 mM L-glutamine and antibiotics. Concanavalin A (Con A, Sigma) at a final concentration 5 μg/m*l* was used as a mitogen. At least, two different cultures were prepared from each animal for each experimental point. Cultures were allowed to grow at 37°C in a 5% CO_2 atmosphere with 95% humidity. 44~72 hr later, mitotic cells were blocked with colcemid and cells were harvested by centrifugation at 400 × g for 10 min. The supernatant was removed and the cell pellet was resuspended in 10 m*l* prewarmed hypotonic solution KCl (0.075 M). After 20 min at 37°C, the tubes were centrifuged for 8 min at 400 × g. The supernatant was removed and cell pellet was resuspended in 10 m*l* fixative (acetic acid:methanol, 1:3, V:V). The fixative was changed 4 times and the cells were resuspended in 0.3~0.5 m*l* fixative prior to slide preparation. Samples of cell suspension were added to precleaned slides, air dried and chromosomes stained with diluted Giemsa (1:20) and evaluated by a single observer.

A total 100 well-spread metaphase cells (50 cells per tube) with 40±2 chromosomes per animal were scored for gaps, breaks, exchanges, chromatid breaks and chromatid exchanges. Chromosome and chromatid aberrations were scored separately and the total percentage of abnormal cells were expressed for statistical analysis. Gaps were recorded but not included in the total chromosome aberration frequency.

Statistical significance of the difference in various types of chromosome aberrations among each group was evaluated with repeated Student *t*-test. A significance level of $p<0.05$ and $p<0.01$ was used to determine if exposed groups were different from unexposed control group.

4. Supravital micronucleus assay in peripheral blood

Ten microliters of acridine orange (AO, 1 mg/m*l*, Sigma, USA) was spread on glass slide which was

prewarmed to 70°C and stored at room temperature until use. An adaptation of the method described by Hayashi *et al.* was used. Briefly, blood samples were taken, via small cut in lateral tail vein, from five male and five female animals per each treated- and control-groups after terminal exposure. Five microliters blood was dropped on the central area of AO coated slides and clean coverslip was placed carefully over the drops. Then, slides were incubated at 4°C for 2 hours. The slides were examined under oil immersion optics using fluorescence microscope fitted with blue excitation filter and yellow barrier filter. Only type I, II and III erythrocytes were observed. At least 1,000 erythrocytes per animals were examined for micronuclei. The frequencies of micronucleated reticulocyte provide an index of induced genetic damage.

Statistical significance of the difference in micronucleus occurrences among various treated groups was checked by the method of Kim *et al.*, 2000. We used software program for the Toxicologists easy use of a statistical procedure. A significance level of $p<0.05$ was used to determine if the exposed groups were different from unexposed control group.

5. Lymphocyte *hprt* mutation assay

In this study, the T-cell cloning assay was used for measuring mutation frequencies (MFs) at the hypoxanthine-guanine phosphoribosyltransferase (*hprt*) locus of lymphocytes isolated from spleen of mice following exposure to ozone for 12 weeks.

The procedures for isolating lymphocytes from spleen and culturing *hprt* mutant T-cell colonies were modified in detail previously (Skopek *et al.*, 1992; Skopek and Walker, 1992). Briefly, T-cells were isolated by macerating spleens individually in 12-well plates, layering the cells on a histopaque 1077 and washing the recovered cells with RPMI 1640 medium. The cells were then resuspended in primary culture medium for mitogenic stimulation for 36~40 hours. For the stimulaton and growth of mouse T-cells in the present study, both primary culture medium and mutant plating medium were modified by the addition of a conditioned medium from Con A- stimulated mouse splenocyte and blood cultures (Skopek and Walker, 1992). After primary culture, cells were then enumerated using a haemocytometer and cultured in 96-well U-bottom microtiter plates with supplemented medium to determine clonal efficiency (CE) and to identify *hprt* mutants. To measure cloning efficiencies for T-cells from mice, aliquots of primed cultures were diluted in cloning medium so that there were 5 cells/well cultured in the presence of 1×10^5 lethally irradiated mouse splenic lymphocytes (feeder cells)/well. Excess lymphocytes isolated from untreated mice were used as a source of feeder cells. To isolate *hprt* mutants, primary cultures were diluted to 1×10^5 cells/m*l* using mutant plating medium supplemented with 1 mg 6-thioguanine (TG)/m*l*, and then seed in 96-well plates at 100 m*l* per well for incubation. Plates were scored for colony growth at 40× magnification (and confirmed at higher magnification as necessary) on day 10~15 days. *Hprt* mutant frequencies (MFs) were calculated as described previously (Albertini *et al.*, 1985) and following; (a) P(0) = P0 = number of negative wells/total number of wells; (b) mutant fraction (Mf) = ($-ln\ P_0$ in TG-plates)/(1×10^5); (c) clonal efficiency (CE) = ($-ln\ P_0$ in CE-plates)/(5 cells/well); (d) mutant frequency (MF) = mutant fraction (Mf)/clonal efficiency (CE).

III. RESULTS

1. Chromosome aberration assay

The number and type of chromosome aberration, and the frequencies of cells with chromatid break and with chromatid exchange in male and female mice exposured to 0.5 ppm ozone are shown in Table 1 and 2. Ozone caused a significant increase of chromosome aberration when compared to control ($p<0.01$).

2. Supravital micronucleus assay in peripheral blood

The effects of ozone exposure on male and female mices peripheral blood micronucleus frequency are presented in Table 3 and 4. Ozone induced micronucleated reticulocytes and the frequencies of micronucleated reticulocyte were significantly higher than those of control group ($p<0.05$).

3. Lymphocyte *hprt* mutation assay

Male mice, treated with ozone and mixture were

Table 1. Chromosome aberrations in male B6C3F1 mouse lymphocytes after *in vivo* exposure to test materials for 12 weeks[#]

Treatment	Dose	Aberration/cell				Gaps/100 cell (%)[a]	Aberrant cells (%)[b, c]
		Chromatid type		Chromosome type			
		Break	Exchange	Break	Exchange		
Control	0	0.002	0.004	0.006	0.006	0.20±0.45	1.60±0.89
Ozone	0.5 ppm	0.026	0.03	0.026	0.012	1.00±0.71	8.40±1.52**

[a]Total chromatid and chromosome gaps/100 cells at each concentrations were recorded but not included as aberrations.
[b]Cells with at least 1 aberration.
[c]Statistical significance of difference from control group (**p<0.01; Student *t*-test).
[#]Results are for 5 animals at each group (100 cells/animal).

Table 2. Chromosome aberrations in female mouse lymphocytes after *in vivo* exposure to test materials for 12 weeks[#]

Treatment	Dose	Aberration/cell				Gaps/100 cell (%)[a]	Aberrant cells (%)[b, c]
		Chromatid type		Chromosome type			
		Break	Exchange	Break	Exchange		
Control	0	0.004	0.008	0.002	0.006	0.60±0.55	1.40±0.55
Ozone	0.5 ppm	0.018	0.018	0.024	0.018	1.20±0.45	7.00±1.58**

[a]Total chromatid and chromosome gaps/100 cells at each concentrations were recorded but not included as aberrations.
[b]Cells with at least 1 aberration.
[c]Statistical significance of difference from control group (**p<0.01; Student *t*-test).
[#]Results are for 5 animals at each group (100 cells/animal).

Table 3. Peripherial blood supravital micronucleus test in male B6C3F1 mice treated with test materials for 12 weeks

Treatment	Dose	No. of mice	No. of micronucleated reticulocyte[a]
Control	0	5	6.11±1.27
Ozone	0.5 ppm	5	8.79±3.73*

[a]Mean SD/1,000 reticulocyte of 3 slides per mouse.
Statistical significance of difference from control group (*p<0.05).

Table 4. Periperal blood supravital micronucleus test in female B6C3F1 mice treated with test materials for 12 weeks

Treatment	Dose	No. of mice	No. of micronucleated reticulocyte[a]
Control	0	5	6.78±2.11
Ozone	0.5 ppm	5	10.89±3.41*

[a]Mean SD/1,000 reticulocyte of 3 slides per mouse.
Statistical significance of difference from control group (*p< 0.05).

assayed for the mutation frequency of 6-thioguanine-resistant (TG^r) spleen lymphocytes. The frequency of TG^r lymphocytes in ozone-treated groups (2.858×10^{-6}) was higher than the concurrent control frequency (1.387×10^{-6}), with increasing range from about 2.1-fold above the control (Table 5).

IV. DISCUSSION

In this study, *in vivo* genotoxic effect of ozone was evaluated in B6C3F1 mice exposed to 0.5 ppm ozone by inhalation for 12 week exposures. Our results of chromosome aberration test are in accordance with

Table 5. Mutant frequency of *hprt* gene in splenic cells from test materials treated B6C3F1 mice during 12 week[a]

Treatment	Dose	Positive wells/total wells in 6-thioguanine plates	Po[b]	CE	Mutation fraction[c]	Mutation frequency[d]
Control	0	16/504	0.97	0.23	0.323×10^{-6}	1.387×10^{-6}
Ozone	0.5 ppm	26/504	0.95	0.19	0.530×10^{-6}	2.858×10^{-6}

[a]1×10^5 cells/well used for selective medium containing 1 μg/ml 6-thioguanine.
[b]Po = P (o) = Number of negative wells/total number of wells.
[c]Mutant Fraction = (-*ln* Po in TG-plates)/(1×10^5 cells/well). CE (Clonal Efficiency) = (-*ln* Po in CE-plates)/(5 cells/well).
[d]Mutation frequency (MF) = Mutation Fraction/CE.

those of other *in vivo* studies on ozone genotoxicity (Tables 1, 2). In fact, results from the cytogenetic studies with laboratory animals up to now are contradictory. Zelac *et al.* noted chromosome aberrations, but Tice *et al.* only saw chromatid-type aberrations at similar doses in Chinese hamsters. Sister chromatid exchanges (SCEs) were not induced by the same treatment. In mice, no aberrations of either type were observed (Gooch *et al.*, 1976). So our finding of the increase in the level of both chromatid- and chromosome-type aberration in lymphocytes of mice exposed to ozone and mixtures is quite interesting. The reasons underlying the discrepancy between other studies and the present study may be related to several factors, including (i) different tissues and cell types were used, (ii) aberrant cells were determined at different times exposure. However, with regard to the positive findings of our results, *in vivo* genotoxic effects cannot be ruled out.

The significantly increased micronucleated reticulocyte frequency was affected by ozone in the current study (Tables 3, 4). It may be explained that micronuclei arise from acentric fragments that fail to incorporate into the daughter nuclei during cell division or are formed by entire chromosomes that lag behind during mitosis due to a failure of mitotic spindle, or by complex chromosomal configurations that pose problems during anaphase after treatment of each test material (Stephnou *et al.*, 1998).

The specific response to ozone was investigated by determining the *hprt* gene T-cell mutants from control, ozone-exposed mice for 12 weeks. The present study showed that the frequency of TG^r lymphocytes in treated groups was higher than that of control group (Table 5). An important component in the application of lymphocyte *hprt* assays for the study of *in vivo* mutation is the characterization of DNA sequence changes responsible for the mutant phenotypes. The generation of mutation spectrum, *i.e.* the relative frequency of the different types of DNA sequence alterations and their distribution over the sequence of the target gene, is generally considered to be mutagen specific. This specificity is related to the types of DNA lesions induced, the sites where lesions are formed, the mutagenic potency of the lesion and the rate at which the lesions are repaired (Anane *et al.*, 1997). The present study showing increased mutation frequency of ozone in B6C3F1 mice suggests that chemical specificity of mutations should be elucidated by further study.

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