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Ozone Inhalation in Rats: Effects on Alkaline Phosphatase and Lactic Dehydrogenase Isoenzymes in Lavage and Plasma

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Ozone is found in urban and rural atmospheres and is produced from a variety of natural and man-made sources. Animal studies conducted at typical ambient levels result in reproducible morphological, biochemical and functional effects.

Ozone damages type I epithelial cells, induces proliferation of type II cells (Evans et al.1976) and produces inflammation of the terminal bronchiolar-alveolar duct region (Moore and Schwartz 1981). Ozone increases lung oxygen utilization (Mustafa and Lee 1976) and increases glutathione metabolism (Chow et al. 1974). Ozone increases airway resistance (Gross and White 1983).

We measured lactic dehydrogenase (LD) isoenzymes to ascertain the tissue giving rise to the increased LD activity in lavage. We also assayed acid phosphatase, alkaline phosphatase, creatine kinase activities, and protein levels since these parameters were increased in rat lung lavage after particulate exposure (Hart et al. 1984). We determined white cell differential and red cell morphology parameters because previous investigators reported that ozone increased neutrophil/lymphocyte ratio (Bobb and Fairchild 1967).

MATERIALS AND METHODS

Experimental Groups A and B: Fischer 344 type male rats were quarantined and examined for infectious diseases by a staff veterinarian before the study. Group A consisted of 6 control, or air-exposed, rats and 7 rats which were exposed to 0.7 ppm ozone continuously for 7 days. Group B consisted of 8 control rats and 16 rats which were exposed to 0.8 ppm ozone for 18 hours. Two hours after exposure, Group A rats, four Group B control rats, and eight Group B ozone-exposed rats were sacrificed. Twenty-four hours after exposure the remaining control and ozone-exposed rats of Group B were sacrificed.

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Exposure Conditions: Fischer 344 rats were placed in a stainless steel and glass modified Rochester chamber and exposed to ozone as previously described (Nachtman, et al. 1986). Ozone was monitored by a Dasibi Ozone Monitor (Model 1003-PC, Dasibi Environmental Corp., Glendale, CA). The chamber concentrations were within 10% of target values. All ozone exposures and animal handling were performed at the Biomedical Science Department of General Motors Research Laboratories. Animals were allowed food and water during the exposure period.

Sample Preparation: After removal from the chamber, rats were injected with Nembutal (35 mg/kg i.p.). The abdomen was cut laterally above the level of the kidneys. Blood was removed by heart puncture with needle and heparinized syringe. A few drops of blood were used to prepare slides for cell counting. Plasma was separated from the cells. The trachea was cannulated with a 14 gauge stainless steel catheter. The lungs, heart and trachea were removed and the lungs were carefully lavaged with 0.9% saline. The volume used was carefully adjusted to prevent overinflation of the lung. Each of three lavage volumes was instilled and withdrawn three times in sequence and all recovered lavage fluid was pooled. The pooled extract was spun in a centrifuge at 140 x g for 10 min to remove cells.

Biochemical Analyses: Lavage samples were assayed for protein content using the method of Lowry et al. 1951. A standard curve was generated for each analysis using bovine serum albumin (Sigma Chemical Company). Plasma total protein was measured by biuret.

Phosphatase assays were carried out on a Dupont Automatic Clinical Analyzer (Dupont Co., Clinical Instrumentation Systems Division, Wilmington, DE). Acid phosphatase was assayed using thymolphthalein monophosphate substrate. Alkaline phosphatase was assayed using p-nitrophenylphosphate substrate (Roy 1970).

Creatine kinase (CK) was assayed using creatine phosphate substrate coupled to NADPH reduction (Oliver 1955). LD was measured by the lactate to pyruvate method. CK and LD were measured on a Demand system (Worthington Diagnostics, Inc.). LD isoenzymes were separated by electrophoresis on agarose gels. After separation a reaction mixture containing lactate, NAD and nitroblue tetrazolium(NBT) was layered over the gel. LD isoenzymes were detected by the blue color produced when NADH reduces NBT to a colored formazan. Beckman Paragon Electrophoresis Kit for LD isoenzymes was used (Beckman Instruments, Inc., Brea, CA).

Hematology: Red cell morphology and white cell differentials were performed on blood smears taken by cardiac puncture in heparinized syringes. Counts were based upon 100 cells.

Statistical Analysis: Parameters from Group A were analyzed by Student's t-test, Group B by Duncan's Multiple Range Test. Data were entered on the IBM mainframe computer at the computer center, Wayne State University. The significance level was set at 0.05 using statistical programs from SPSSX (Statistical Programs for Social Sciences SPSS, Inc., Chicago, IL).

RESULTS and DISCUSSION

Group A rats exposed to ozone for 0.7 ppm/7 days gained significantly more weight than controls (10.1 % vs. 2.1 %). Group B rats exposed to ozone 0.8 ppm/18 hours lost weight (approximately 4 % of their body weight) regardless of 2 or 24 hours recovery. Group B controls gained 2.2 % body weight.

Table 1. Analysis of lavage fluid from air and ozone exposed rats Mean, (S.D.)

Experimental Group	Protein (mg/lavage)	Total LD IU/L	ALKPHOS. IU/L
A-air	3.81 1.30	16.2 1.7	36.5 3.9
A-ozone	5.65 1.97	26.3 9.3	31.3 3.2
B-air	3.69 1.93	12.6 3.4	36.3 5.2
B-ozone,2hrs.	9.73 0.67[a]	16.8 6.9	15.3 1.3[a]
B-ozone,24hrs.	9.49 0.59[a]	17.7 9.5	37.0 3.3

A = 0.7 ppm ozone, 7 days; B = 0.8 ppm, ozone for 18 hours; 8 rats were sacrificed 2 hours after exposure; 8 remaining were sacrificed 24 hours after exposure.
a, ozone-treated group is significantly different from air exposed control.

In both Group A and Group B, we found lavage protein concentration the most sensitive to ozone exposure. Although this increase was not statistically significant for Group A, the pulmonary lavage protein content was elevated approximately 50% in ozone-exposed rats vs. control rats. For Group B, the increase in lavage protein content of ozone-exposed rats was significantly greater than control (Table 1). Total lactate dehydrogenase (LDT) in pulmonary lavage was also significantly increased in Group A (62%). Group B ozone-exposed rats tended to have increased LDT; however, this was not statistically significant. The distribution of LD isoenzymes in lavage was not significantly altered by ozone exposure at 0.7 ppm or 0.8 ppm. The ratios of LD4/LD5 were: Group A, control= 0.043; ozone exposed= 0.053; Group B, control=.083, ozone (2hr)= 0.091, ozone (24hr)= 0.092.

Lavage alkaline phosphatase (ALKPHOS) activity decreased after 0.7 ppm ozone exposure ($p<0.05$, Student's test) as well as after 0.8 ppm ozone exposure at a 2 hour post-exposure period. Lavage ALKPHOS activity returned to the control level 24-hours after the 0.8 ppm exposure ended. Creatine kinase (CK) and acid phosphatase (ACPHOS) activities in pulmonary lavage were not affected (Data not shown).

Plasma ALKPHOS levels 2-hours after 0.8 ppm (Group B) were significantly decreased to 145.3 U/L (air controls = 285.9) but after 24hours in clean air, returned to 244.3. Plasma LDT, CK and ACPHOS levels were unaffected by either ozone exposure.

Group B plasma LD isoenzyme results are tabulated in Table 2. The

relative percentage of plasma isoenzymes was different between control rats, ozone-exposed/ 2 hour post-exposure, and 2-hour post-exposure rats of Group B. Results show that the % M was significantly lower 2 hours after ozone exposure and further decreased 24 hours post-exposure. Group A plasma LD isoenzyme results were not significantly different between control and ozone-exposed rats.

Regression analysis revealed that Group B plasma alkaline phosphatase values were significantly correlated with plasma protein (Multiple R = 0.58;F[1,34] = 15.54, F = 0.0004) but not with % M (Multiple R =0.18; F[1,34] = 1.40, F = 0.29).

For Group B, the neutrophil to lymphocyte ratio (N/L) was dramatically elevated 2 hours after exposure (1.12 compared to control 0.38). However, 24 hours post exposure, it had returned to control (24 hour value = 0.36). Group A white blood cell counts and N/L ratio were not statistical different: the N/L ratio,however, was slightly higher in Group A exposed rats than in control rats (0.43 and 0.38, respectively). For either group, red blood cell morphology was unaffected by (Data not shown).

Table 2. Plasma Lactic Dehydrogenase (LD) from Air-and Ozone-Exposed Rats 0.8 PPM - 18 Hours (Group B)Mean (S.D.)

Parameter	Air	Ozone	Ozone
		2 Hours	24 Hours
Total LD*	119 (87.3)	86.3 (16.7)	83.7 (31.1)
LD1 (%)	6.5 (3.4)	14.7 (5.6)[a,b]	16.3 (6.3)[a]
LD2 (%)	6.3 (3.2)	10.1 (4.2)	14.9 (0.3)[b]
LD3 (%)	8.5 (9.0)	3.0 (1.5)	11.5 (11.5)
LD4 (%)	5.9 (1.9)	4.3 (0.79)	7.4 (3.9)
LD5 (%)	72.7 (10.7)	60.1 (8.4)[a,b]	49.8 (8.6)[a]
PERCENT M	82.9 (5.9)	75.3 (7.3)[a,b]	65.1 (8.8)[a]

Group B - ozone exposure, 0.8 ppm; time, 18 hours; 8 animals were sacrificed 2 hours after exposure; the remaining 8 were sacrificed 24 hours later. * LD activity is expressed as International Units per liter (U/L).

a, ozone-treated group is different from air exposed control; b, 2-hour post exposure is different from 24 hour post-exposure group.

Lavage analysis has provided biochemical indicators of pulmonary damage. Ozone increases lavage protein levels (Alpert et al 1971). Inhaled particulates will increase lavage lactate dehydrogenase (LD) (Henderson et al. 1979).

Consistent with work of Bobb and Fairchild 1967, we observed an acute increase of N/L ratios exposures at much lower levels than the 3 - 8 ppm levels they employed. The N/L ratio was much greater in Group B

rats 2 hours post-exposure than either 24 hours post-exposure or in Group A. This agrees with Bobb and Fairchild's contention that the N/L ratio reflects an acute effect of ozone.

At both levels tested, ozone exposure decreased the alkaline phosphatase (ALKPHOS) activity in lavage with a trend to lower plasma ALKPHOS values. Furthermore, we found a correlation between ALKPHOS and plasma protein. In humans, generalized malnutrition and vitamin C deficiency were associated with decreases in plasma ALKPHOS (Posen 1967; Kaplan 1972). Furthermore, guinea pigs deficient in vitamin C are more sensitive to oxidant gases such as NO_2 (Hatch et al.1986). Ozone could deplete the antioxidant Vitamin C which would account for the decrease in ALKPHOS. Another explanation is that ozone or a metabolite may have direct effect on ALKPHOS activity. For example, singlet oxygen inhibits ALKPHOS activity in vitro (Stevens et al. 1970).

LD 1 is related to aerobic oxidation (H_4) while LD 5 (M_4) is associated with anaerobic glycolysis. Percent M is a parameter indicating the amount of subunit M present on the average. Cells in tissue culture respond to hypoxia by increasing %M (Hellung-Larsen and Anderson 1968) while hyperoxia produces a decrease in % M (Guttler and Clawsen 1969). We found that ozone exposure decreased % M in ozone exposure 2 hr post exposure and further decreased 24 hours post-exposure, indicating a progressive shift to production of subunit H. In our experiments, the effect of ozone on % M is similar to hyperoxia.

Lavage LDT was increased in Group A rats (0.7 ppm/7 days). There are other reports of LDT increase. Similar to our results, Lindy et al. 1970 found that the (LD4/LD5) ratio did not change as fibrosis persisted. Because LD4 and LD5 were the only isoenzymes found in lung lavage, then we suggest that increased LD in lavage fluid is characteristic of pulmonary tissue or inflammatory cells rather than plasma exudate. We plan to investigate the contribution of these components in future studies.

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