

Effect of ozone on response to ovalbumin & its modulation by vitamins C & E in sensitized guinea pigs

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Background & objectives: Exposure to ozone and asthma are both associated with increased oxidative stress. Exposure to ozone therefore, may potentiate the airway response to allergens. We undertook this study to investigate the effect of ozone exposure on airway response to ovalbumin in sensitized guinea pigs and its modulation by dietary supplementation with antioxidant vitamins C and E.

Methods: After *in vivo* measurements of specific airways conductance (SGaw) and airway hyperresponsiveness (AHR) to inhaled histamine, guinea pigs were sensitized to ovalbumin and divided into three groups: (i) sensitized; (ii) sensitized and exposed daily to ozone; and (iii) sensitized, exposed daily to ozone and given dietary supplementation with vitamin C, 2 mg/kg body wt and E, 7 IU/kg body wt. A control group of nonsensitized animals was included. After 4 wk, AHR was measured again and animals were challenged with inhaled ovalbumin. Changes in SGaw were followed for early and late airway bronchoconstrictive responses. The following measurements were obtained: (i) parameters of oxidative stress - plasma malonaldehyde (MDA) as marker of lipid peroxidation and superoxide anion generation by leukocytes and bronchoalveolar lavage (BAL) cells; (ii) antioxidant status: red cell superoxide dismutase (SOD); and (iii) glutathione peroxidase (GPx). BAL cytology was studied.

Results: Ozone exposure resulted in an increase in AHR and early and late bronchoconstrictive responses after ovalbumin challenge, greater superoxide anion generation in BAL cells, higher plasma MDA levels and decrease in red cell SOD activity. Dietary supplementation with vitamin C and E prevented or ameliorated these responses.

Interpretation & conclusions: Exposure to ozone at concentrations of 0.12 ppm for 2 h daily for 4 wk enhances the airway response to allergens in sensitized guinea pigs. Dietary supplementation with antioxidant vitamins E and C, affords variable degree of protection against this enhancement.

Key words Airway hyperresponsiveness - antioxidants - asthma - ovalbumin - oxidative stress - ozone - vitamin C - vitamin E

Outdoor air pollution levels have been associated with a broad spectrum of adverse health effects in individuals with asthma¹. Ozone is a potent inflammatory outdoor air pollutant that mediates many of its toxic effects through free radical reactions². Increased oxidative stress has been well documented to play a

significant role in the initiation and perpetuation of the airway inflammation in asthma^{3,4}. Thus, combined ozone exposure and allergen exposure may have a synergistic effect. Ozone exposure has been shown to adversely affect asthmatics and enhance the response to allergen exposure⁵⁻⁹ in asthmatics and in animal models

though other workers have failed to corroborate these findings¹⁰.

Vitamin C or L-ascorbate is an important water-soluble antioxidant, capable of scavenging a variety of free radicals and oxidants, including ozone¹¹. Ozone-induced lung damage is potentiated in ascorbate-depleted guinea pigs¹². Vitamin E is another antioxidant scavenges peroxy radicals derived from ozone-polyunsaturated fatty acids (PUFA) interactions¹³. Ozone exposure studies on vitamin E deficient animals have provided evidence of protective role of vitamin E¹³. These vitamins, together, may therefore offer protection against ozone-allergen interaction. We therefore carried out a study to investigate whether exposure to ozone in concentrations that are often found in the ambient air enhances the response to allergen inhalation in sensitized guinea pigs and whether dietary supplementation with vitamins C and E offers any protection.

Material & Methods

Forty healthy male guinea pigs (Hartley strain) weighing from 250 - 400 g and housed in climate-controlled animal house of VP chest Institute, Delhi, were used. Water and food were given *ad libitum*. The study was approved by the Institutional Animals Ethics Committee.

Study design: *In vivo* specific airways conductance (SGaw) and airway hyper-responsiveness (AHR) to histamine were measured^{14,15}. Ten animals were kept as controls (Group A). The remaining animals were sensitized with ovalbumin¹⁶ and divided into three groups (n=10 in each) as follows: Group B – sensitized; Group C – sensitized and exposed daily to ozone, 0.12 ppm given for 2 h; Group D – sensitized and exposed daily to ozone and given dietary supplementation with vitamin C (2 mg/kg body wt) and E (7 IU/kg body wt). The above interventions were carried out for 4 wk. All animals received commercial feed throughout the study.

After 4 wk, SGaw and AHR to histamine were measured followed by study of airway response to inhaled ovalbumin the next day (about 24 h after histamine). Subsequently, the following measurements were obtained: (i) parameters of oxidative stress - plasma malonaldehyde (MDA) as marker of lipid peroxidation and superoxide anion generation by leukocytes and bronchoalveolar lavage (BAL) cells, (ii) antioxidant status: red cell superoxide dismutase (SOD)

and glutathione peroxidase (GPx). BAL cytology was studied.

SGaw and AHR to histamine: These parameters were measured in spontaneously breathing nonanaesthetized animals using a non-invasive body plethysmograph technique¹⁴. Subsequently, AHR to histamine was determined by a standard protocol involving inhalation of histamine given in doubling concentrations starting from 0.02 mg/ml till the SGaw fell by >35 per cent or till a concentration of 2.5 mg/ml was reached. Dose of histamine producing a 35 per cent fall in SGaw was calculated (ED₃₅ histamine) as described by Agrawal¹⁵.

Sensitization and measurement of airway response to inhaled ovalbumin challenge: Sensitization was carried out by injecting 100 mg aluminium hydroxide and 100 µg ovalbumin per ml of normal saline intraperitoneally and at sites of peripheral lymph nodes as described by Santing *et al*¹⁶. This method leads to sensitization that can be demonstrated after 4 wk. The animals were challenged with inhaled ovalbumin (0.2%) and changes in SGaw were followed for early and late airway bronchoconstrictive responses. We have earlier shown that challenge with inhaled ovalbumin elicits these responses in sensitized guinea pigs¹⁷. These were labelled as early asthmatic response (EAR) and late asthmatic response (LAR). The EAR, defined as ≥ 35 per cent fall in SGaw from the baseline value is usually evident at 20 min. Monitoring was continued at 1 hourly intervals for 8 h for any LAR, defined as a second episode of bronchospasm after recovery from the EAR. Finally, the SGaw was again measured at 24 h to detect any persistence of LAR.

Exposure to ozone: This was given by placing the animals in an acrylic chamber with an inlet for ozone-enriched air and an outlet attached to a suction pump. Ozone (0.12 ppm) in air was generated using an ozone generator (Sonimix 3001 a, LN Industries SA, Geneva). A soda lime absorber was placed in the chamber to avoid CO₂ accumulation. The ozone concentration in the chamber was continuously monitored by an ozone analyzer (OZ 2000G, Seres, France).

Bronchoalveolar lavage cytology: The animals were anaesthetized 24 h after challenge with ovalbumin with an intraperitoneal injection of pentothal sodium (50 mg/kg). Blood was drawn into heparinized syringes by cardiac puncture for biochemical estimations. The trachea was exposed, cannulated and the lungs were lavaged thrice by aliquots of 1 ml saline. The lavage fluid was processed and stained with Leishman's stain.

The cells were counted in a haemocytometer at 400X magnification. Smears were made in a cytocentrifuge and stained with May-Geimsa and examined at 400X for the differential cell count (DLC).

Measurement of oxidative stress and antioxidant status:

(i) Lipid peroxidation (MDA estimation): This was carried out according to the method of Jain *et al*¹⁸ in plasma as thiobarbituric acid (TBA) reactivity of malonaldehyde (MDA), an end product of fatty acid peroxidation. The results were expressed in nmoles TBARS/ml plasma.

(ii) Superoxide anion ($O_2^{\cdot-}$) generation: Leukocytes were separated from blood according to the method of Baron and Ahmed¹⁹. The viability of leukocytes harvested with this technique was greater than 95 per cent as determined by means of trypan blue exclusion.

Superoxide anion generation by leukocytes of blood and BAL cells was measured as the superoxide dismutase-inhibitable reduction of cytochrome C (Sigma) by the method of Lehmeyer *et al*²⁰. Results were expressed in nM $O_2^{\cdot-}$ produced/30 min/ 10^6 cells.

(iii) Red cell superoxide dismutase (SOD) and glutathione peroxidase (GPx): SOD assay was based on the method developed by McCord and Fridovich²¹. Results were expressed as U/g Hb. GPx assay was carried out by the method of Little *et al*²². Results were expressed as μ M NADPH oxidized/min/g Hb.

Statistical analysis: Statistical analysis was carried out using SPSS 11.0 and GraphPad Prism 4.01 (GraphPad Software Inc., USA) softwares. Group data were expressed as mean $\pm$ SD. The homogeneity of variance and distribution of data was examined. For baseline SGaw and AHR (ED_{35} Histamine), analysis of variance (ANOVA) was used to compare multiple groups followed by Bonferroni test for between-group differences if ANOVA indicated significant differences. For EAR and LAR, and biochemical and cytology parameters, nonparametric tests were used for analysis.

These included Kruskal Wallis test for comparison of multiple groups and Mann Whitney U test for between-group comparisons. Paired t test/Wilcoxon signed rank test were used to compare SGaw and ED_{35} Histamine at baseline and after sensitization, respectively. $P < 0.05$ was considered as significant.

Results

The baseline SGaw (in per sec per cm H_2O) was 0.17 ± 0.02 , 0.15 ± 0.03 , 0.19 ± 0.06 and 0.16 ± 0.05 in groups A, B, C and D, respectively (ANOVA, $P > 0.05$). Baseline AHR (ED_{35} Histamine) (mg/ml), in the four groups was, respectively: 1.08 ± 0.61 , 1.40 ± 1.03 , 1.14 ± 0.59 and 1.38 ± 0.65 (ANOVA, $P > 0.05$). The ANOVA results are shown in Table I. Post-sensitization, the SGaw was 0.14 ± 0.02 in group B, 0.17 ± 0.06 in group C and 0.15 ± 0.02 in group D. The post-sensitization values were not significantly different from the baseline values in any of the three groups. In group C, the ED_{35} histamine decreased from 1.14 ± 0.59 to 0.64 ± 0.38 mg/ml ($P < 0.01$) after sensitization. In group B, the post-sensitization ED_{35} histamine was 0.91 ± 0.74 mg/ml compared to the baseline value of 1.40 ± 1.03 and in group D, it was 0.98 ± 0.46 mg/ml compared to a baseline value of 1.38 ± 0.65 .

After challenge with ovalbumin, the EAR (% decrease from pre-challenge value of SGaw, 20 min after challenge) was 50.72 ± 7.38 , 65.18 ± 13.77 , and 57.77 ± 21.52 , respectively in the three groups B, C and D. The ozone exposed animals (group C) had a significantly greater fall as compared to the group B animals ($P < 0.01$). The LAR (% decrease from pre-challenge value of SGaw) at 6 h was 15.9 ± 17.5 , 31.0 ± 36.8 and 16.5 ± 9.60 in groups B, C and D, respectively while at 24 h, it was 5.8 ± 15.1 , 34.9 ± 36.5 and -4.4 ± 15.2 , respectively. Group C animals had significantly greater decrease in SGaw at 24 h compared to groups B and D ($P < 0.01$ for each).

Significant differences were found in lipid peroxidation (TBARS), superoxide anion ($O_2^{\cdot-}$) generation in the BAL cells, and red cell superoxide

Table I. Results of analysis of variance for baseline SGaw and ED_{35} Histamine in the four groups

		Sum of Squares	df	Mean Square	F	Significance
Baseline SGaw	Between groups	0.007	3	0.002	1.355	0.273
	Within groups	0.064	35	0.002		
	Total	0.071	38			
Baseline ED_{35} Histamine	between groups	0.816	3	0.272	0.489	0.692
	Within groups	19.480	35	0.557		
	Total	20.296	38			

dismutase and glutathione peroxidase (activity). The maximum lipid peroxidation was observed in group C as was the decrease in SOD activity. Group D animals did not differ from control group for these parameters (Table II). The differences in total counts in BAL fluid were not significant. However, both eosinophils and

neutrophils were significantly increased in groups B, C and D as compared to group A ($P < 0.001$, $P < 0.01$ and $P < 0.01$, respectively). While groups B and C did not differ, the eosinophils were significantly less in group D as compared to group B and C ($P < 0.05$ for each comparison) (Table III).

Table II. Measures of oxidative stress and antioxidants in the four groups

	Group A	Group B	Group C	Group D	Between-group differences (Mann Whitney test)
Plasma TBARS (nmol/ml)**	1.22 ± 0.41	2.40 ± 0.73	3.34 ± 1.05	1.63 ± 0.46	B vs A*, C vs A**, B vs C+, C vs D**
Neutrophil O ₂ ⁻ generation (nM O ₂ ⁻ produced/30 min/10 ⁶ cells)	2.32 ± 0.84	3.67 ± 1.66	4.04 ± 0.52	3.25 ± 0.30	Between-group differences not significant
BAL cells O ₂ ⁻ generation (nM O ₂ ⁻ produced/30 min/10 ⁶ cells)+	36.83 ± 9.86	35.87 ± 4.12	48.00 ± 8.84	46.09 ± 11.46	Between-group differences not significant
Red cell SOD levels (U/g Hb) **	4130 ± 884.1	3335.5 ± 676.3	1930.6 ± 1047.7	4115.0 ± 1175.9	B vs A*, C vs A*, B vs C*, C vs D*
Red cell GPx (μM NADPH oxidized/min/g Hb) **	198.85 ± 61.4	244.99 ± 49.5	203.47 ± 158.5	131.46 ± 29.9	B vs C*, B vs D*, A vs D*

Group A, controls; Group B, sensitized with ovalbumin; Group C, sensitized and exposed daily to ozone; Group D, sensitized and exposed daily to ozone and given dietary supplementation with vitamin C and E; Superscripts in col 1 indicate significance of Kruskal Wallis chi square statistic; $P^* < 0.01$, $P^{**} < 0.001$, $P^+ < 0.05$. Col 6 shows significant between-group differences on Mann Whitney test. Values are mean ± SD (n=10)

Table III. Bronchoalveolar lavage cytology in the four groups

	Group A	Group B	Group C	Group D	Between-group differences (Mann Whitney test)
Total Count (X 10 ⁶ /l)	4.92±1.3	7.16±2.8	6.13±3.8	6.00±3.2	Between-group differences not significant
Eosinophils (%)***	2.10±3.3	15.90±9.0	17.71±10.9	8.11±5.5	B vs A**, C vs A*, D vs A*, B vs D+, C vs D+
Lymphocytes (%) ⁺	11.90±5.3	11.60±3.9	8.86±3.9	6.89±3.2	D vs A+, D vs C+
Neutrophils (%)***	3.30±1.6	9.30±4.3	10.86±3.9	8.11±3.3	B vs A*, C vs A*, D vs A*
Macrophages (%) [*]	82.60±8.2	63.20±11.5	62.57±13.8	77.00±8.7	B vs A*, C vs A*, D vs C*

Group A, controls; Group B, sensitized with ovalbumin; Group C, sensitized and exposed daily to ozone; Group D, sensitized and exposed daily to ozone and given dietary supplementation with vitamin C and E; Superscripts in col 1 indicate significance of Kruskal Wallis chi square statistic $P^+ < 0.05$; $P^* < 0.01$, $P^{**} < 0.001$; $P^{***} < 0.0001$. Col 6 shows significant between-group differences on Mann Whitney test. Value are mean ± SD (n=10)

Discussion

The results of the present study showed that ozone exposure at concentrations of 0.12 ppm for 2 h daily for 4 wk enhanced the airway and the inflammatory response to ovalbumin in sensitized guinea pigs. These animals showed an increase in the AHR to histamine and a greater early and late bronchoconstrictive responses after ovalbumin challenge. Dietary supplementation with antioxidant vitamins, C and E, afforded variable degree of protection against this enhancement.

An increase in superoxide generation in peripheral neutrophils has been reported in asthmatics by several workers²³, including a study from our laboratory⁴. In the present study, we found it to be increased in BAL cells although not in the peripheral blood leukocytes in ozone-exposed animals. Lipid peroxidation, a marker for oxidative stress was significantly more in the ozone-exposed animals than in the vitamin-supplemented ozone-exposed animals. The higher levels in the ozone-exposed animals likely resulted from there being two causes of oxidative stress, the inflammatory response induced by the allergen challenge and the inhaled ozone itself.

The activity of SOD was decreased in both ozone-exposed and non ozone-exposed sensitized animals but to a greater degree in the former. Vitamin supplementation prevented this decrease. For GPx, the changes did not reveal any definite pattern. A review of literature shows that changes in activities of antioxidant enzyme in asthma are complex and variable in direction²³. Levels of antioxidants would at any time reflect the balance between how quickly these are consumed and resynthesized. Therefore, changes may be more marked in one or more of the antioxidants and may be in either direction, decreased or increased³. Nevertheless, any change measured in the status of antioxidants reflects a perturbation of the homeostasis of the oxidant-antioxidant balance.

Enhanced response to allergen exposure at concentrations of 0.12 ppm for 1 h⁶, 0.4 ppm ozone for 2 h²⁴, and 0.16 ppm ozone for 7.6 h⁸ has been reported in asthmatics but others have failed to corroborate these findings^{10,11}. In studies of longer exposures in sensitized animal models, early airway response increased without increased inflammation after exposure to ozone at 0.1 and 0.3 ppm for 24 wk in guinea pigs^{25,26} and a transient and small increase in airway responsiveness without change in airway inflammation in mice exposed for 5 h to 0.3 ppm

ozone²⁷ has been reported. Wagner *et al*²⁸ observed that exposure to ozone (0.5 ppm, 8 h/day) for 1 day or 3 consecutive days caused a significant inflammatory response in nasal tissues of sensitized rats after nasal challenge. Depuydt *et al*²⁹ reported enhanced inflammatory response in mice on exposure to ozone (100 ppb for 2 h). Yanai *et al*³⁰ reported increased airway responsiveness to acetylcholine and antigen with ozone (3 ppm).

Most of these studies have used ozone concentrations that are greater than those usually observed in the ambient air and thus may not be relevant for humans. We used concentrations that are often reached in urban air. In addition, we have looked at parameters of oxidative stress as a possible mechanism for the potentiation of response to ozone. Our results suggest that the current ambient levels of ozone increase the response to allergens.

Both vitamins C and E are constituents of the epithelial lining fluid and are likely to form the first line of defense against ozone. Ascorbate is consumed in an ozone-concentration and time-dependant manner³¹. It can scavenge ozone¹¹ and protects against ozone-induced lung damage in a dose-dependant manner¹². Vitamin E scavenges peroxy radicals derived from ozone-polyunsaturated fatty acids (PUFA) interactions¹³. Thus, given the different mechanisms by which vitamin C and E exert their anti-oxidant actions, a dietary supplementation with both the vitamins may be able to protect against ozone-mediated damage and thus the ozone-allergen interaction. Our results support this hypothesis. In a recent study on the effects of vitamin E on airway responses and biochemical parameters in sensitized guinea pigs, we have shown that it suppresses the increase in airway reactivity following sensitization and has membrane stabilizing actions¹⁷. In another study on isolated tracheal preparations from ovalbumin-sensitized guinea pigs, vitamin E was found to reduce the contractile effects of ovalbumin and carbachol³². Thus, antioxidant vitamins may have a therapeutic potential in conditions involving increased oxidative stress in the airways including exposure to oxidant air pollutants. This needs to be explored.

The limitation of the present study is the small number of animals in the study groups. We opted for an animal model of asthma that had been successfully developed earlier in our laboratory¹⁷. Long-term

studies of ozone exposure cannot be carried out in humans for ethical reasons. Whether results obtained in an animal model can be replicated in humans needs to be investigated. Although we have used a single concentration of ozone that is often reached in ambient air and is thus relevant for everyday exposures, use of more than one different doses would provide a greater insight into the biological effects of ozone.

In conclusion, exposure to ozone at concentrations of 0.12 ppm for 2 h daily for 4 wk enhanced the response to allergens in sensitized guinea pigs. Dietary supplementation with antioxidant vitamins E and C, afforded variable degree of protection against this enhancement.

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