

Effects of repeated ozone exposure on pulmonary function and bronchial responsiveness in mice sensitized with ovalbumin

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Received 2 January 2004; accepted 13 June 2004

Available online 31 July 2004

Abstract

We examined the effect of repeated ozone exposure on the respiratory system in mice, sensitized with ovalbumin (OA). OA-sensitized mice and saline-treated control mice were exposed to 1.0 ppm ozone or clean air for 6 h daily and five days weekly for five weeks. Subsequently, these mice were exposed to 1.0 ppm ozone for 1 h, and pulmonary function was evaluated by pneumotachography during ozone exposure and arterial blood gas analysis and histopathological examination were performed. OA sensitization or repeated ozone exposure did not affect baseline pulmonary function. In OA-sensitized mice receiving repeated ozone exposure, acute ozone exposure increased respiratory resistance and decreased dynamic compliance, with significant differences in both parameters compared with, after repeated ozone exposure without OA sensitization. In mice without repeated exposure, there was no difference in the effect of acute ozone exposure according to the use of OA sensitization. After 1-h exposure, arterial blood gas analysis showed a significant decrease in PaO₂ in mice undergoing OA sensitization alone, with no significant reduction of this parameter in mice receiving repeated exposure. Histopathologically, alveolar epithelial hyperplasia occurred in repeatedly exposed mice. These results indicated that respiratory allergy might be a risk factor, which aggravates the effects of repeated ozone exposure.

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Keywords: Ozone; Ovalbumin; Asthma; Pulmonary function; Blood gas analysis

1. Introduction

Ozone is a typical air pollutant along with nitrogen dioxide and suspended particulates that are responsible for air pollution in urban areas. Ozone

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is a potent oxidative gaseous substance that is associated with photochemical air pollution. Ozone is known to have adverse effects on human health, such as mucosal irritation and adverse respiratory effects, and to induce symptoms including irritable cough, substernal pain, reduction of forced vital capacity (FVC) and forced expiratory volume in one second (FEV_{1.0}), and neutrophilic inflammation in airway submucosa (A Committee of the Environmental and Health Assembly of the American Thoracic Society, 1996). In a recent large-scale epidemiological study, there was a strong association between atmospheric ozone level and asthma-related hospital admission (Petroeschevsky et al., 2001). In a cohort study, it was shown that, in communities with high concentrations of ozone, children who play for longer periods of time outdoors are more likely to develop asthma (McConnell et al., 2002).

In asthmatics, ozone exposure induces decreased FEV_{1.0} (Kreit et al., 1989; Scannell et al., 1996) and increased airway resistance (Scannell et al., 1996), and is associated with higher occurrence of inflammatory reactions (Basha et al., 1994). It is thus considered that asthmatic subjects are more apt to suffer from the effects of ozone exposure compared with healthy individuals (Basha et al., 1994; Scannell et al., 1996). In particular, ozone exposure, even at low levels, is more likely to induce respiratory symptoms in children on maintenance medication for asthma (Gent et al., 2003).

Animal studies also showed that ozone exposure decreased dynamic compliance in the lung, increased respiratory rate and induced neutrophilic inflammation (Coleridge et al., 1993; Arito et al., 1997). It was also reported that, during the sensitization process, ozone exposure increased specific antigens and bronchial hypersensitivity (Matthew, 1995; Sun et al., 1997). Yamauchi et al. (2002) showed that sensitization with ovalbumin (OA) was a risk factor that increased the likelihood of the adverse respiratory effect of acute ozone exposure. These animal experiments examined the effects of ozone exposure for relatively short periods of time, and it is not well-known how repeated, long-term ozone exposure affects the respiratory system.

The present study simulated conditions that resembled actual ozone exposure in humans in urban areas. OA-sensitized mice were repeatedly exposed to ozone for five weeks to examine the effects of ozone exposure and OA sensitization on the respiratory system.

2. Methods

2.1. Animals

A total of 30 male C57BL/6 mice (six weeks old; body weight: 20–22 g) were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and used in the present study.

2.2. Study protocol

These mice were divided into four groups, and experiments were performed as shown in Fig. 1. Groups 1 and 2 received 1 mg of Al(OH)₃ (Wako Pure Chemical Industries; Osaka, Japan) alone on days 0 and 7 and inhalation of 0.9% saline for 30 min three times on days 14, 21, 28 and 35 with an ultrasonic nebulizer (NE-U12; Omron, Tokyo, Japan). Groups 3 and 4 received intraperitoneal doses of OA (SIGMA, Tokyo, Japan) at 50 µg and 1 mg of Al(OH)₃ and exposure to an aerosol of OA (10 mg/mL) in 0.9% saline for 30 min three times on days 14, 21, 28 and 35 with an ultrasonic nebulizer (Foster et al., 1996). Groups 2 and 4 were exposed to 1.0 ppm ozone for 6 h daily, five days a week for five weeks, and groups 1 and 3 were exposed to clean air during the same period. A Hinner-type stainless steel chamber was used for ozone or clean air exposure. Ozone was produced with an ultraviolet lamp that was attached inside the air duct at the upper part of the chamber. Ozone level in the chamber was continuously determined with an ozone monitor (EG-2001; Ebara Jitsugyo, Tokyo, Japan) and the voltage of the ultraviolet lamp was fed back and controlled, so that ozone level was maintained at approximately 1.00 ppm (0.90–1.02 ppm) throughout the exposure period.

2.3. Pulmonary function analysis

Similar to a study by Yamauchi et al. (2002), physiological parameters of pulmonary function in mice were determined under spontaneous respiration. A previous study showed that bronchial hypersensitivity to acetylcholine occurred from 3 to 24 h after the final OA inhalation and was more potent at 3 h compared with at 24 h (Yamaguchi et al., 1994). Based on this report, pulmonary function was examined 3 h after the final sensitization with OA inhalation in the present study.

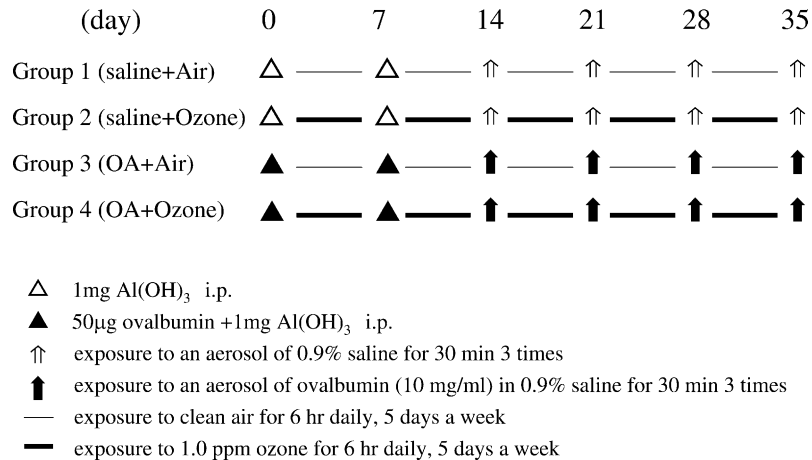


Fig. 1. Experimental design for ovalbumin sensitization and repeated exposure to ozone.

Mice were anesthetized with urethane (1.5 g/kg, i.p.), and a short polyethylene tube was inserted into the trachea and another polyethylene tube was inserted into 1/3 of the lower esophagus. Air flow signal was measured with a Lilley-type pneumotachograph that was connected to the intratracheal tube. Lung capacity was obtained from the electrical integral of the air flow signal. Intraesophageal pressure was used to replace intrathoracic pressure. Body temperature was maintained at 36 °C during the experiment. These data were transferred to a computer via an A/D converter (MacLab/400; AD Instruments, Castle Hill, Australia). After stabilization of respiration for at least 5 min, respiratory resistance, dynamic compliance, respiratory rate, tidal volume and minute ventilation were determined and served as baseline pulmonary function values (Onodera et al., 1997).

2.4. Acute effect of ozone exposure on pulmonary function

After the determination of baseline pulmonary function values, mice were exposed to 1.0 ppm ozone for 1 h and pulmonary function was examined with time during this exposure period. Ozone was aspirated with an air compressor from the chamber where ozone level was maintained at 1.0 ppm and sent into the polyethylene tube that was inserted into the trachea at a constant flow rate.

Pulmonary function parameters during 1 min were averaged at 10-min intervals for 60 min from the start

of exposure for comparison. For the evaluation of the time profile of exposure-induced changes, results are expressed as ratios to baseline values.

2.5. Arterial blood gas analysis

After ozone exposure for 1 h, arterial blood was sampled from the abdominal aorta and immediately analyzed with a blood gas system analyzer (Model-ABL500; Radiometer, Copenhagen, Denmark).

2.6. Histopathology

After ozone exposure and blood sampling from the abdominal aorta, the lung and other thoracic organs were removed en bloc. The trachea was cannulated and 10% formalin was gradually injected into the tube so that the pleura became smooth. After tissue fixation, paraffin sections were prepared and stained with hematoxylin and eosin, and the preparations were microscopically examined.

2.7. Statistical analysis

Data are shown as mean ± S.E.M. The analysis of variance was used to compare baseline pulmonary function values and blood gas parameters. When the results were significant, between-group comparison was performed according to the Tukey's method. A split-plot design was used for comparison of the

time-profile of changes in pulmonary function values and the *P*-value was adjusted according to the Huynh–Feldt method. When there were significant differences among the four groups, the contrast's test was used for between-pairs of groups. Results were significant at $P < 0.05$ in all tests.

3. Results

3.1. Pulmonary function at baseline

Table 1 shows pulmonary function values after respiratory stabilization for at least 5 min after the start of pulmonary function test. Respiratory resistance was slightly higher in group 3, in which mice were sensitized with OA, and slightly lower in groups 2 and 4 where mice were repeatedly exposed to ozone, with no significant difference among the four groups. There was no significant difference in dynamic compliance, respiratory rate, tidal volume, or minute ventilation per gram of body weight among the four groups.

3.2. Time profile of changes in pulmonary function during ozone exposure

After the determination of baseline pulmonary function values, the time profile of changes in pulmonary function values was examined during 1-h exposure to 1.0 ppm ozone. Results are shown in Fig. 2(a–e).

There was almost no change in respiratory resistance during ozone exposure for 60 min as compared with at baseline in groups 1 and 2. In group 3, respiratory resistance slightly increased to 40 min and sub-

sequently after the start of ozone exposure, with no significant difference compared with other groups. In group 4, respiratory resistance increased from 10 min after the start of ozone exposure and reached 144% of the baseline value, showing a significant increase compared with group 2 (Fig. 2(a)).

Dynamic compliance showed almost no change as compared with at baseline throughout ozone exposure in groups 1 and 2. In groups 3 and 4, dynamic compliance decreased after the start of ozone exposure and reached 78% and 77% of the respective baseline values 60 min after exposure initiation. Compared with group 2, dynamic compliance was significantly reduced in group 4, while there was no significant difference in group 3 compared with other groups (Fig. 2(b)).

Respiratory rate slightly decreased to 92% of the baseline value after 60 min of ozone exposure in group 3. Compared with at baseline, there was almost no change in the other three groups, with no significant difference among the four groups (Fig. 2(c)).

Tidal volume decreased from 30 min after the start of ozone exposure and reached to 94% of the baseline value in group 3. In group 4, on the other hand, tidal volume slightly increased to 105% of the baseline value 50 min after exposure initiation. There was no significant difference among the four groups throughout the 60-min ozone exposure period.

Minute ventilation per gram of body weight increased to 115% of the baseline value 40 min after the start of ozone exposure and decreased thereafter in group 1. In group 4, minute ventilation slightly increased to 111% of the baseline value after 60 min of ozone exposure. There was no significant difference among the four groups (Fig. 2(e)).

Table 1
Pulmonary function at baseline

Group	Group 1 (saline + air) (n = 6)	Group 2 (saline + ozone) (n = 8)	Group 3 (OA + air) (n = 8)	Group 4 (OA + ozone) (n = 8)	<i>P</i> -value
Respiratory resistance (cmH ₂ O/ml s ⁻¹)	0.930 (0.150)	0.833 (0.199)	1.285 (0.265)	0.716 (0.190)	0.260
Dynamic compliance (ml/cmH ₂ O)	0.026 (0.008)	0.035 (0.008)	0.026 (0.008)	0.047 (0.012)	0.366
Respiratory rate (per min)	295.3 (13.3)	289.1 (20.0)	275.1 (14.7)	295.5 (16.0)	0.799
Tidal volume (ml)	0.194 (0.065)	0.323 (0.071)	0.189 (0.051)	0.309 (0.063)	0.291
Minute ventilation/body weight (ml/min g ⁻¹)	2.660 (0.985)	4.515 (1.014)	2.295 (0.710)	4.272 (0.810)	0.203

All values are means with S.E.M. shown in parentheses.

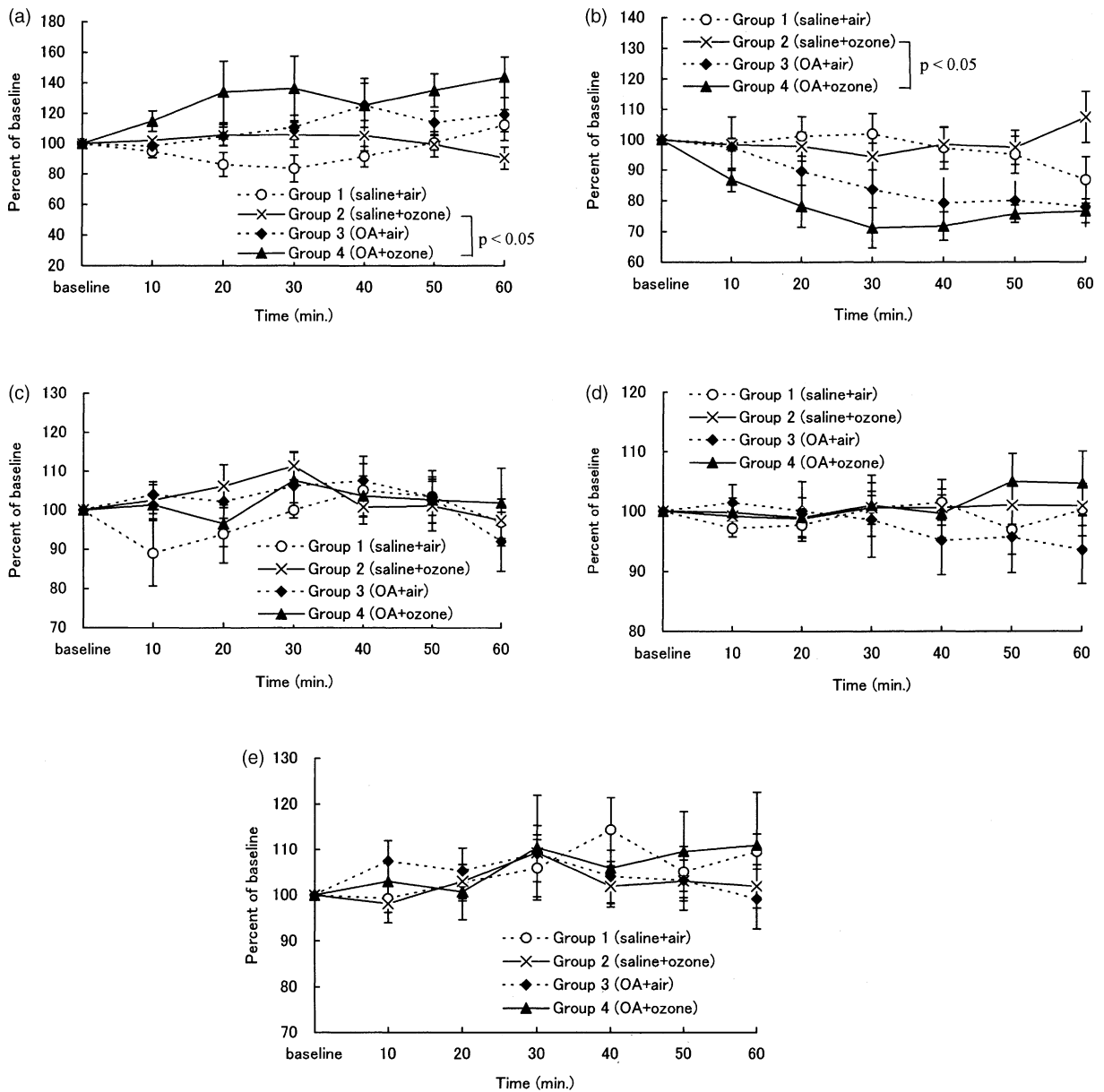


Fig. 2. Time course of pulmonary responses to 1.0 ppm ozone for 1 h. (a) Respiratory resistance; (b) dynamic compliance; (c) respiratory rate; (d) tidal volume; and (e) minute ventilation per gram body weight. All values are means with S.E.M. Each parameter expressed as percent of baseline. Number of animals in the four groups is as follows: group 1 ($n = 6$), group 2 ($n = 8$), group 3 ($n = 8$), and group 4 ($n = 8$). To compare values in repeated measurements, the split-plot method was used. The significance of differences between experimental groups was evaluated using contrast's test.

Table 2
Effect on arterial blood gases

Group	Group 1 (saline + air) (n = 6)	Group 2 (saline + ozone) (n = 8)	Group 3 (OA + air) (n = 8)	Group 4 (OA + ozone) (n = 8)	P-value
pH	7.25 (0.02)	7.25 (0.02)	7.28 (0.03)	7.27 (0.02)	0.670
PaO ₂ (mmHg)	142.4* (4.9)	132.6* (8.6)	100.5 (9.7)	113.2 (7.8)	0.008
PaCO ₂ (mmHg)	40.4 (2.0)	39.6 (1.7)	39.3 (3.8)	40.9 (3.3)	0.977
HCO ₃ ⁻ (mEq/l)	17.2 (0.5)	17.2 (0.4)	17.7 (1.1)	18.3 (0.9)	0.742

All values are means with S.E.M. shown in parentheses.

* $P < 0.05$ vs. group 3.

3.3. Effect on arterial blood gas

In group 3, PaO₂ was 100.5 mmHg, showing a significant reduction compared with groups 1 and 2. In group 4, PaO₂ was 113.2 mmHg, showing a slightly lower value compared with groups 1 and 2, but the differences were not significant. There was no difference in pH, PaCO₂ or HCO₃⁻ among the four groups (Table 2).

3.4. Histopathology

Compared with group 1, alveolar epithelial hyperplasia was observed in group 2. In group 3, eosinophils and histiocytes infiltrated around blood vessels compared with group 1. In group 4, perivascular infiltration of eosinophils and histiocytes as well as alveolar epithelial hyperplasia were observed (See Fig. 3).

4. Discussion

Recent epidemiological and clinical studies showed that ozone has various effects on the pathophysiology of asthma (Molfini et al., 1991; Mortimer et al., 2002; Bosson et al., 2003; Gent et al., 2003). Yamauchi et al. (2002) reported that OA-sensitized mice, after 1-h exposure to 1.0 ppm ozone, presented with reduction of dynamic compliance and minute ventilation compared with non-sensitized mice, showing that OA-sensitized mice are more apt to suffer from the effects of ozone exposure. The study by Yamauchi et al. (2002) evaluated only the acute effects of ozone exposure for 1 h, not examining the effects of repeated ozone exposure that are experienced every day by residents in urban areas. Our present study reproduced conditions that are similar to actual living conditions in urban areas, and

OA-sensitized mice were repeatedly exposed to ozone 6 h daily, five days a week for five weeks, in order to examine the effects of ozone exposure and OA sensitization on the respiratory system.

Our sensitization protocol was similar to a study by Yamauchi et al. (2002), which has been confirmed to be sufficiently effective because OA-sensitized mice had demonstrated accelerated bronchial hyper-reactivity to methacholine compared with non-sensitized mice. At baseline, respiratory resistance was slightly larger in OA-sensitized groups and slightly lower in the two groups undergoing repeated ozone exposure, but there was no significant difference. No significant difference was also found in any other parameter among the four groups. These values almost agreed with the previous reports (Henderson et al., 1996; Onodera et al., 1997; Yamauchi et al., 2002). It was considered from these results that repeated ozone exposure alone had no adverse effects on pulmonary function at rest.

After further exposure to 1.0 ppm ozone, respiratory resistance increased and dynamic compliance was reduced in the repeated ozone exposure group after OA sensitization (group 4), with significant differences compared with the ozone exposure group with no OA sensitization (group 2). In previous animal studies, there was no change in respiratory resistance after ozone exposure at approximately 1 ppm (Tepper et al., 1990; Yamauchi et al., 2002). In the present study, respiratory resistance slightly increased and dynamic compliance decreased after 1-h ozone exposure in the OA sensitization alone group (group 3). There was no significant difference in any of the two parameters and the results were similar to those in the study by Yamauchi et al. (2002). In OA-sensitized mice that were repeatedly exposed to ozone, acute ozone exposure was associated with significant changes of increased respiratory resistance and reduced dynamic

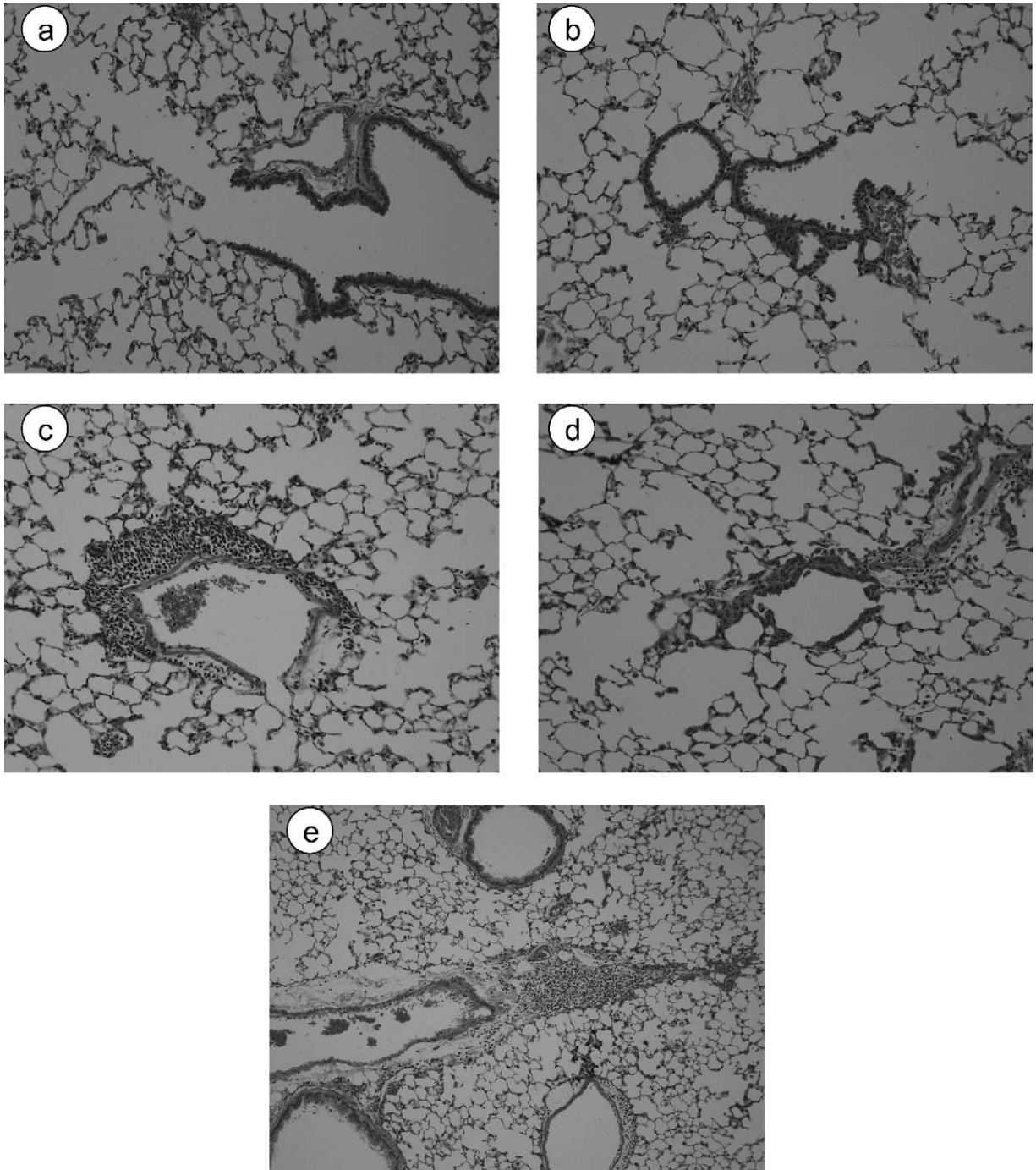


Fig. 3. Photomicrographs of mouse lung of the four groups. (a) No representative finding is shown in group 1 (saline + air); (b) alveolar epithelial hyperplasia is observed in group 2 (saline + ozone); (c) eosinophils and histiocytes infiltrate around blood vessels in group 3 (OA + air); (d) alveolar epithelial hyperplasia, and (e) perivascular infiltration of eosinophils and histiocytes are represented in group 4 (OA + ozone).

compliance, showing that this animal group was apt to receive the acute effect of ozone exposure.

It was reported that ozone exposure increased respiratory rate (McDonnell et al., 1983; Tepper et al., 1990), but another report described that there was no change in respiratory rate after 24-h ozone exposure in mice (Currie et al., 1998). It was also reported in humans and animals that ozone exposure was not associated with changes in ventilation (Tepper et al., 1990). Yamauchi et al. (2002), in contrast, described reduction of minute ventilation after acute ozone exposure in OA-sensitized mice. In the present study, there was no significant effect of OA sensitization or repeated ozone exposure on respiratory rate, tidal volume or minute ventilation. It is considered that changes in these parameters differ according to animal species and conditions of sensitization and ozone exposure, and further research is needed in view of these factors.

No consensus has been developed for the effect of ozone exposure on arterial blood gas values. In sheep, 3-h exposure to 1–4 ppm ozone had no effect on arterial blood gas values (Schelegle et al., 1990). Paterson et al. (1992) found significant changes of PaO₂ reduction and increased PaCO₂ in rats that were exposed to 3 ppm ozone for 4 h. Yamauchi et al. (2002) reported that, in OA-sensitized mice, PaO₂ significantly decreased after 1-h exposure to 1.0 ppm ozone with no change in PaCO₂, suggesting the occurrence of ventilation–perfusion ratio mismatch (Coleridge and Coleridge, 1994). Also in the present study, PaO₂ was significantly reduced after 1-h exposure to 1.0 ppm ozone with no change in PaCO₂ in the group of OA sensitization alone (group 3), showing similar results as those in the study by Yamauchi et al. (2002). In the group of OA sensitization and repeated ozone exposure (group 4), PaO₂ slightly decreased with no significant difference compared with other groups. While no definite cause was known, this phenomenon might result from adaptive reaction to ozone (Kirschvink et al., 2002) due to repeated exposure.

Histopathologically, Hiroshima et al. (1987) reported peribronchiolar fibrillation and fibrous strand deposition to alveolar ducts in rats that were exposed to 0.5 ppm ozone for 3–12 months. In the present study, alveolar epithelia hyperplasia occurred after repeated exposure to 1.0 ppm ozone for five weeks. In OA-sensitized mice, pulmonary tissues presented with eosinophilic and histiocytic infiltration around blood

vessels. In the group of OA sensitization and repeated ozone exposure (group 4), both of the above findings were found. It was reported in asthmatic subjects undergoing allergen loading that eosinophilic bronchial inflammatory reaction increased after 24-h ozone exposure (Vagaggini et al., 2002). Garssen et al. (1997) suggested that ozone exposure leads to an increased severity and incidence of pulmonary infectious diseases. In the present study, OA-sensitized mice that were repeatedly exposed to ozone were more likely to suffer from the effects of acute ozone exposure, showing that allergens and ozone exposure may synergistically affect the lung.

In conclusion, there was no change in any pulmonary function value in OA-sensitized mice after repeated exposure to 1.0 ppm ozone for five weeks. After 1-h acute ozone exposure, however, these mice presented with increased respiratory resistance and decreased dynamic compliance, with significant differences in both parameters compared with non-OA-sensitized mice after repeated ozone exposure. In mice without repeated ozone exposure, there was no effect of OA sensitization. These results suggest that OA sensitization is a risk factor that increases the effect of repeated ozone exposure and may explain the synergistic effect of allergens and ozone exposure.

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