

Original article

Ozone inhalation induces exacerbation of eosinophilic airway inflammation and Th2-skew immune response in a rat model of AR

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ABSTRACT

Background: Ozone (O₃) exposure elicits allergic rhinitis (AR) exacerbations by mechanisms that remain poorly understood. We used a rat model to investigate the effects of O₃ on eosinophilic airway inflammation and Th2-related response.**Methods:** Sprague-Dawley (SD) rats were sensitized and challenged with ovalbumin (OVA) to make AR models. Three groups of AR rats were exposed respectively to 0.5, 1.0, 2.0 ppm of O₃ for 2 h daily over 6 weeks consecutively and studied 24 h later. Normal rats exposed to O₃ alone were used as controls. Nasal symptoms and OVA-specific immunoglobulin E (OVA-sIgE) in the serum were evaluated. Inflammatory cells in nasal lavage fluid (NLF) were classified and counted. Cytokines protein levels in NLF were assessed by ELISA. The pathological changes in the nasal mucosa were examined by histology.**Results:** The combination of allergen and repeated O₃ exposure in rats induced a significant increase of the number of sneezes, nasal rubs, amount of nasal secretion and OVA-sIgE in the serum, accompanied by enhancement of eosinophils in NLF and nasal mucosa. The increase of interleukin-5 (IL-5), IL-13, Eotaxin and decrease of INF-γ protein levels in NLF were detected in AR rats after O₃ inhalation. Hematoxylin and eosin staining showed disordered arrangement of the nasal mucosa epithelium and eosinophilic infiltration in a concentration-dependent manner.**Conclusions:** O₃ inhalation deteriorated symptoms in AR rats, and the possible mechanism is that ozone co-exposure could enhance the expression of Th2 cytokines, eosinophilic airway inflammation dose-dependently. The observation is helpful for us to understand the synergistic effect of O₃ in the air pollution and allergen on aggravating allergic rhinitis.

1. Introduction

Allergic rhinitis (AR) is a common chronic inflammatory airway disease of nose and or sinus characterized by runny nose, repeated sneezing, nasal congestion and itching nose [1]. It is a global health problem with substantial social burden and economic costs, which negatively affects life quality and school and work performance in patients [2]. AR is a consequence of interactions between gene and environment. Furthermore, the potential effect of increase in air pollutant levels should be pay more attention to with regard to the growing

prevalence of AR. Epidemiologic studies have indicated that exposure to air pollutants particularly ozone(O₃) could play an important role in the development of AR [3].

Ambient O₃ is a gaseous component of air pollution that is generated by interaction of air pollution such as nitrogen oxide and organic compounds, by the action of sunlight. As major air pollutants, especially in warm or hot seasons, O₃ air pollution is currently considered one of the most common problems in view of the consensus of the National Ambient Air Quality Standards (NAAQS). Consequences of O₃ inhalation include increased susceptibility and hypersensitivity to respiratory

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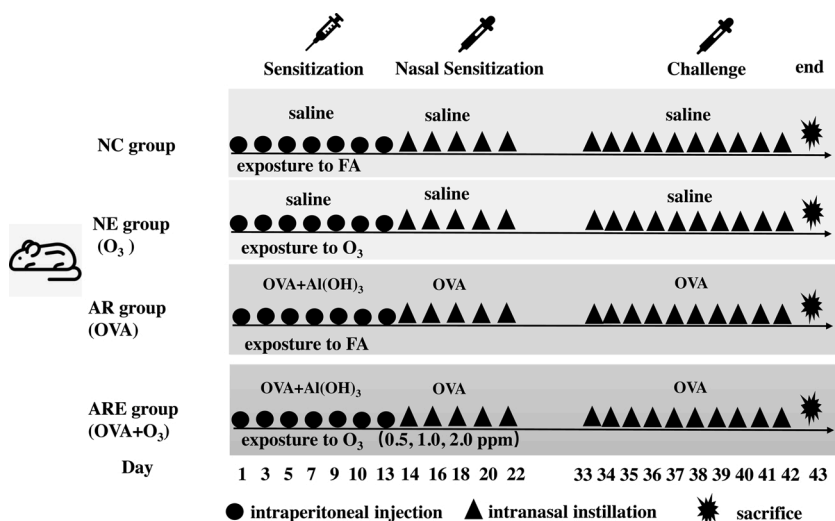


Fig. 1. Experimental protocols for inducing AR in rats and O₃ exposure.

Sixty rats were randomly assigned to six groups (n = 10 per group) (1) a negative control group (NC group) which was exposed to filtered room air and sensitized as well as challenged with sterile saline solution; (2) a group of healthy rats exposed to 1.0 ppm of O₃ and sensitized and challenged with sterile saline (NE group). (3) a group which was exposed to filtered room air and sensitized with OVA + Al(OH)₃ via intraperitoneal injection as well as challenged with OVA intranasally (AR group). (4) a group which was sensitized and challenged by OVA and exposed to O₃ of a low concentration of 0.5 ppm in 2 h per day for 6 weeks via inhalation (AREL group). (5) a group which was sensitized and challenged by OVA and exposed to O₃ of a middle concentration of 0.5 ppm in 2 h per day for 6 weeks via inhalation (AREM group). (6) a group which was sensitized and challenged by OVA and exposed to O₃ of a high concentration of 2 ppm in 2 h per day for 6 weeks via inhalation (AREH group). All specimens of nasal mucosa and NLF and blood were obtained on day 43. NC: negative control; O₃: ozone; AR: allergic rhinitis; OVA: ovalbumin; AREL: AR rats exposed to O₃ (0.5 ppm). AREM: AR rats exposed to O₃ (1.0 ppm). AREH: AR rats exposed to O₃ (2.0 ppm).

infections or allergen and exacerbations of preexisting respiratory diseases such as asthma and chronic obstructive airway disease [4].

Studies both in human and animal show that ozone inhalation exacerbates hyperresponsiveness and immune and inflammatory responses in airway of asthmatic subjects [5]. AR is frequently associated with asthma, with similar mechanisms refer to the inflammatory and pathology responses to some condition. Likewise, AR might be exacerbated by mechanisms similar to those that occur in asthmatics after ozone exposure. People with AR might be a susceptible target group of ozone exposure. In fact, epidemiological studies in the United Kingdom showed a 37 % rise in AR patients on days following high air O₃ concentrations [6].

However, the mechanisms that contribute to the deleterious effects of ozone inhalation on AR patients remain unclear. Similar studies in animal models have not been performed. Accordingly, this study was designed to explore the effect of repeated O₃ exposure in ovalbumin (OVA) sensitized/challenged AR rat model. Meanwhile, cells and cytokines in NLF as well as pathology change of the nasal mucosa were assessed to verify the hypothesis that O₃ inhalation could deteriorate Th1/Th2 imbalance in AR.

2. Materials and methods

2.1. Animals

Sprague-Dawley (SD) and specifically pathogen-free (SPF), female rats (age 6–7 weeks and about 180–200 g body weight) were purchased from Sippr-BK Laboratory Animal (Shanghai, China), and housed in the Experimental Animal Center of Fudan University (Shanghai, China). The animal study was performed after the approval of Fudan University's Institutional Animal Care and Use Committee. All animal surgery was conducted under general anesthesia to minimize suffering. Animals were housed in cages with free access to tap water and food (free from OVA) under standard conditions which temperature and relative humidity were maintained between 21–24 °C and 40–60 %, respectively. Room lights were put on a 12-h light/dark cycle beginning at 7:00 A.M. All animals were acclimatized to the environment conditions for 1 week before start of the experiments.

2.2. AR model

AR models were established according to previous method of our

team [7–9] (Fig. 1) with minor modified. Sensitization and antigen challenges were carried out systemically in three steps for 42 days. In the first stage, rats were sensitized with 3 mg OVA (Sigma-Aldrich, St. Louis, Missouri) and 30 mg calmogastin (Sigma-Aldrich) dissolved in 1 mL saline and injected intraperitoneally (i.p.) once a day for 7 days (day 1, 3, 5, 7, 9, 11 and 13). Meanwhile, rats in the control group (NC) were injected i.p. with the same volume of sterile saline. In the second stage from day 14 to 22, nasal reinforcement was conducted dropping an intranasal instillation of 25 µL OVA solution by a micropipette at a concentration of 1% OVA (1% OVA in physiologic saline solution) in each nostril once every other day. In the last stage from day 33 to 42, nasal challenge was performed by an intranasal instillation of 25 µL OVA solution in each nostril once a day. Instead, sterile saline was implemented on the NC rats under the same conditions.

2.3. O₃ exposure

Rats were exposed to filtered air or O₃ at 0.5, 1.0 or 2.0 ppm for 2 h daily over 6 weeks consecutively in a stainless steel chamber (55 × 55 × 55 cm³) which connected to an air pump (ZJ-XZ-2; ZJ Environmental Protection, Shanghai, China). The inhalation equipment sustained a dynamic air flow of 12 air changes per hour so HEPA-filtered room air was exchanged through the chamber. O₃ was generated from an electrical discharge O₃ generator (ZJC-T01; ZJ Environmental Protection, Shanghai, China). The concentration of O₃ were continuously monitored at breathing zone of the rats in the chamber with a O₃ analyzer (ZJ-1010-E; ZJ Environmental Protection, Shanghai, China) and feedback-controlled the work of O₃ generator. Calibration of the O₃ analyzer was performed by the Shanghai Environmental Monitoring Center (Shanghai, China). The chamber was maintained at temperature of 20–25 °C and humidity of 50–70 %. The concentration of O₃ in chambers for exposure to 0.5, 1.0 or 2.0 ppm and was maintained within the mean chamber concentration by less than ±10 %.

Rats in NC group and AR group inhaled filtered room air in the chamber; and for rats in NE group and three ARE groups, filtered room air was mixed with filtered room air in the chamber three different doses of O₃ and then passed through the chamber. All rats were exposed to O₃ or filtered room air in the chamber for 2 h during which time they were deprived of water and food. The protocol is shown as Fig. 1. The parameters were measured 24 h after the last O₃ exposure. Nasal symptoms and OVA-specific Ig E in the serum were evaluated. NLF inflammatory cells were classified and counted. Cytokines protein levels in NLF were

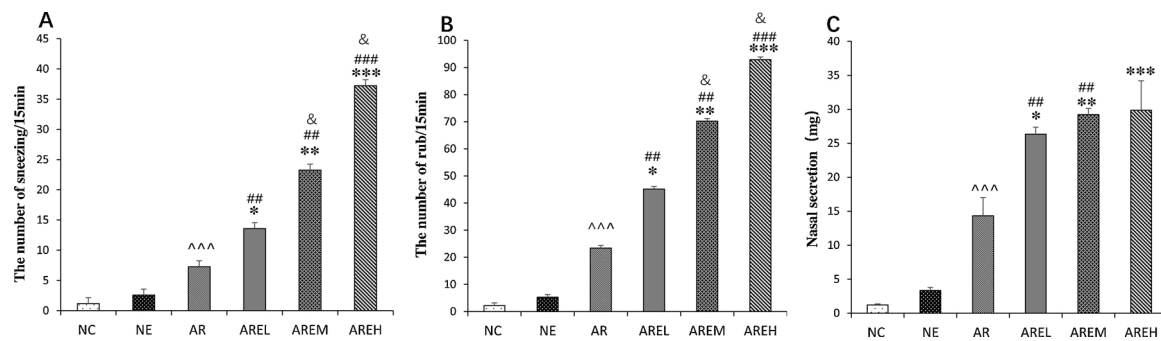


Fig. 2. A, B, C : O_3 exposure aggravates allergic nasal symptoms in AR rats. (N = 10 per group). The numbers of sneezes (Panel A), nasal scratching (Panel B) and nasal secretion (Panel C) in each rat were counted for 15 min immediately after the last nasal challenge. Data are presented as the means $\pm$ standard deviation (SD). * P < 0.05, ** P < 0.01, *** P < 0.001 (AREL, AREM, AREH groups vs. AR group), # P < 0.05, ## P < 0.01, ### P < 0.001 (AREL, AREM, AREH groups vs. NE group), ^^^ P < 0.01 (AR group vs. NC group), & P < 0.05 (AREM, AREH groups vs. AREL group). NC: negative control; O_3 : ozone; AR: allergic rhinitis; OVA: ovalbumin; AREL: AR rats exposed to O_3 (0.5 ppm). AREM: AR rats exposed to O_3 (1.0 ppm). AREH: AR rats exposed to O_3 (2.0 ppm).

assessed by ELISA. The pathological changes in the nasal mucosa were examined by histology.

2.4. Evaluation of nasal symptoms

Immediately after the last intranasal challenge of OVA or saline, the number of sneezes and nasal scratching events and amount of nasal secretion for the first 15 min were counted by two observers independently as described in our previous research [7]. The nasal secretion was collected with a piece of pre-weighed filter-paper strip which was inserted into one nostril for 5 min. The difference in weight of the absorption paper was recorded as quantity of nasal secretion [10].

2.5. Sample collection

24 h after the last O_3 exposure (day 43), rats were anaesthetized with 10 % chloral hydrate sodium 0.3 g/kg via i.p. injection. NLF was collected as previously described [9]. Briefly, the rats were fix to the anatomical plate in a supine position and received tracheotomy. Subsequently, a plastic cannula was inserted from the dissected trachea to the nasopharynx and the nasal cavity of rat were irrigated with saline solution from nasopharynx through the tracheal cannula by a micro pump (300 L/min for 6 min). NLF retrieved from anterior nares were centrifuged at $1000\times g$ for 10 min at 4 °C. The supernatant was stored at -80 °C before assay. The obtained remaining cell pellet was re-suspended in 0.5 mL PBS solution for cell count. Blood samples were collected from the abdominal aortas, and was centrifuged at $1000\times g$ for 10 min at 4 °C. The obtained serum was stored at -80 °C for further analysis. The heads of the rats were removed and sectioned down the middle beside the nasal septum in a sagittal plane. The septum mucosa was dissected and fixed in 4% paraformaldehyde and embedded in paraffin for histological and morphometric analysis.

2.6. Measurement of cell numbers in NLF

The NLF cell pellet was re-suspended in 0.2 mL of PBS for cell count. The numbers of total leukocyte were calculated using a standard hemocytometer with an optical microscope (BX-43; Olympus Optical, Tokyo, Japan). 10 μ L NLF cells PBS were stained with Giemsa stain. The numbers of leukocyte classification (eosinophils, macrophages, lymphocytes and neutrophils) were determined under $\times 400$ magnification by a single observer in a blinded manner with morphological criteria.

2.7. Measurement of OVA-Specific IgE in serum and IL-5, IL-13, Eotaxin, IFN- γ in NLF

The levels of interleukin (IL)- 5, IL-13, Eotaxin, Interferon gamma

(IFN- γ) in NLF, and OVA specific Immunoglobulin E (OVA-s Ig E) in Serum were measured by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions of commercial kits (ELISA Kit for Rat IL-5, IL-13, Eotaxin, IFN- γ , and OVA-sIgE, Xitang Biotech, Shanghai, China).

2.8. Histopathology of nasal mucosa

After decalcification, the tissue block was embedded in paraffin, and 4-mm thick sections were made. Hematoxylin-eosin (HE) stain was performed to assess morphological changes of Nasal Mucosa and identify inflammatory cell infiltration in the sections under a light microscope (BX-43, Olympus, Tokyo, Japan). The number of total leukocytes, macrophages, neutrophils and eosinophils in all stained samples were calculated. Five non-overlapping fields in the lamina propria of the nasal mucosa were randomly chosen per section. They were counted by two independent investigators under $\times 400$ magnification from two separately nasal mucosa sections per rat [8]. Cell counts in each stained sample were recorded as the mean of the counts for 5 non-overlapping in the same area. The proportion of each kind of leukocytes in the total leukocytes in each group were compared between groups.

2.9. Statistical analysis

Data were expressed as the mean $\pm$ standard deviation (SD). Statistical analysis and graphics were performed with SPSS 20.0. One-way analysis of variance (ANOVA) was performed for comparisons between multiple groups. P < 0.05 was considered to be statistically significant.

3. Result

3.1. O_3 exposure aggravated AR symptoms in rats

During 15 min after OVA challenge, significantly more nasal symptoms, including sneezes, nasal rubs, and nasal secretion were observed in the rats of the AR group than NC group (p < 0.001) (Fig. 2A–C). These results showed that the AR model was made correctly. O_3 exposure in concentration of 0.5, 1.0, 2.0 ppm significantly augmented the sneezing response, nasal rubs, and nasal secretion in ARE group in comparison with NC group, NE group, and AR group without O_3 -exposure. (p < 0.05). The concentration-dependent effect of O_3 exposure on the sneezing response and nasal rubbing was shown in Fig. 2AB. However, there was no statistically significant difference in the nasal secretion between different dose of O_3 exposure groups (p > 0.05; Fig. 2C). Briefly, inhalation of O_3 aggravated the sneezing response and nasal rubbing in a concentration-dependent pattern. Additionally, the numbers of sneezes

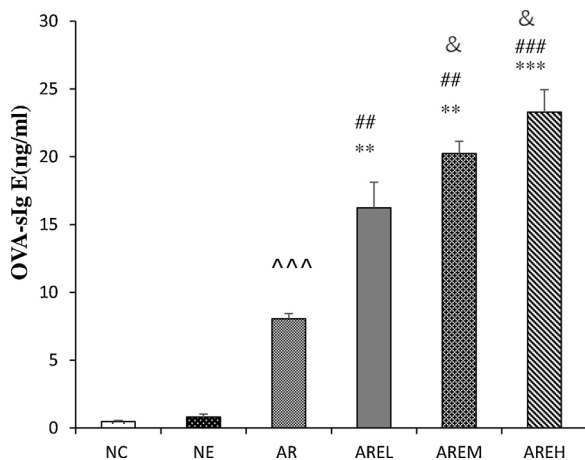


Fig. 3. O₃ exposure enhanced OVA-specific IgE levels in serum of AR model (N = 10 per group). Data are presented as the means ± standard deviation (SD). ** $P < 0.01$, *** $P < 0.001$ (AREL, AREM, AREH groups vs. AR group), ## $P < 0.01$, ### $P < 0.001$ (AREL, AREM, AREH groups vs. NE group), ^ ^ ^ $P < 0.01$ (AR group vs. NC group). NC: negative control; O₃: ozone; AR: allergic rhinitis; OVA: ovalbumin; AREL: AR rats exposed to O₃ (0.5 ppm). AREM: AR rats exposed to O₃ (1.0 ppm). AREH: AR rats exposed to O₃ (2.0 ppm).

and scratching and nasal secretion in the rats of NE group were only mildly higher than those in the NC group but did not reach statistical significance ($p > 0.05$).

3.2. O₃ exposure enhanced OVA-specific IgE levels in serum of AR model

ELISA detection result displayed the levels of serum OVA-specific IgE were higher significantly in AR group than NC group ($p < 0.001$) (Fig. 3) which indicated that the AR model was successfully established. In AREL, AREM and AREH group, O₃ exposure enhanced higher OVA-

specific IgE levels in serum of AR model compared with the AR and NC groups. There was no statistically significant difference between different dose of O₃ exposure groups ($p > 0.05$). The difference in OVA-specific IgE between the NE and NC groups was not statistically significant ($p > 0.05$).

3.3. O₃ exposure induced exacerbation of eosinophilic airway inflammation in AR rats

3.3.1. Effects on Cell Numbers in NLF after O₃ exposure

The number of inflammatory cell count and classification in NLF were shown in Fig. 4. OVA challenge and ozone exposures led to an increase in total leukocytes in AREL, AREM, AREH group which were much more higher than AR group and NE group ($p < 0.05$) (Fig. 4A). The combined effect of O₃ exposure and OVA treatment enhanced eosinophils, neutrophils and lymphocytes more obviously ($p < 0.05$) compared with negative control group (Fig. 4BDE). The number of eosinophils in three different ARE groups increased in a concentration-dependent pattern compared with the AR, NC, NE groups, whereas the numbers of neutrophils and lymphocytes between the difference dose ARE group was statistically insignificant, which reflecting an increase in eosinophilic airway inflammation. Compared with the control group, eosinophilic inflammation was markedly enhanced in AR rats ($p < 0.05$; Fig. 4B). Otherwise, O₃ exposures caused slightly but not significantly elevated eosinophilic inflammation, compared with negative control group. In addition, numbers of neutrophils in NE and ARE group were significantly increased, compared with negative control group and AR group, indicating the presence of neutrophilic inflammation following ozone exposure ($p < 0.05$; Fig. 4D). Changes in macrophages in NLF were significantly elevated in NE group (Fig. 4C). No eosinophilia could be found in NLF of normal rats.

3.3.2. Effects on histopathology of nasal mucosa after O₃ exposure

HE staining of the pathological sections of the nasal mucosa in the NC group, the cilia of nasal mucosa were arranged neatly, the tissue level

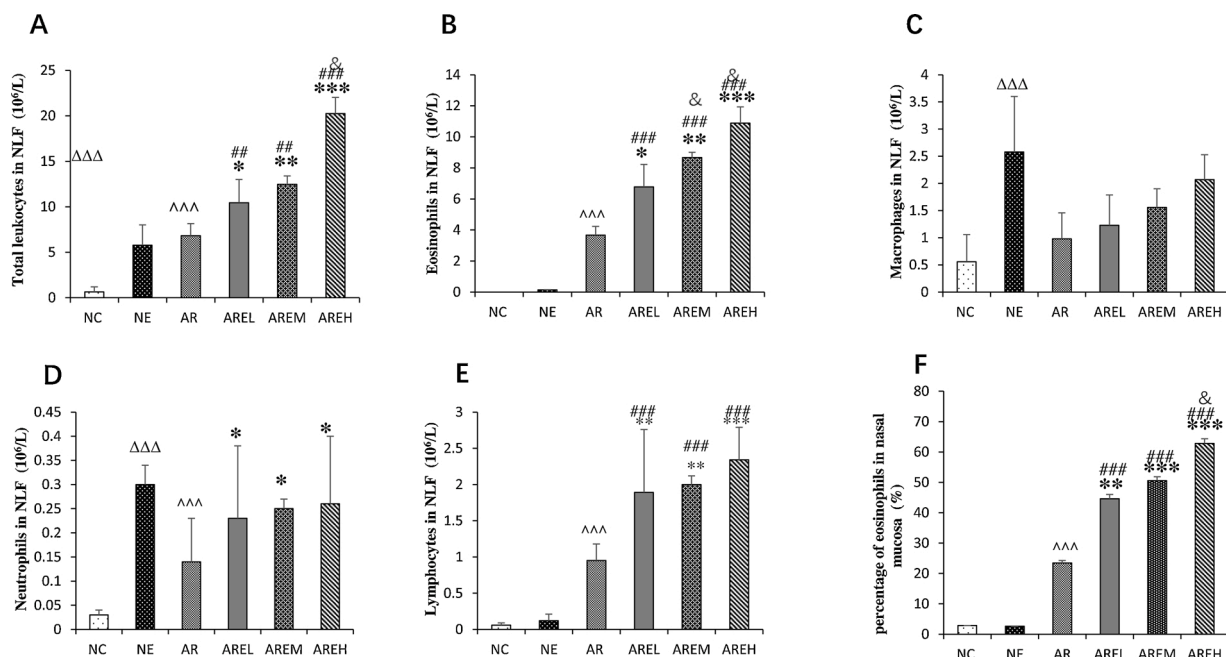


Fig. 4. O₃ exposure induced exacerbation of eosinophilic airway inflammation in AR rats (N = 10 per group). Effects on Cell Numbers in NLF after O₃ exposure (A–E) and the percentage of eosinophils in nasal mucosa after O₃ exposure (F). Data are presented as the means ± standard deviation (SD). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (AREL, AREM, AREH groups vs. AR group), # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ (AREL, AREM, AREH groups vs. NE group), ^ ^ ^ $P < 0.05$, ^ ^ ^ $P < 0.01$, ^ ^ ^ $P < 0.001$ (AR group vs. NC group), ^ ^ ^ $P < 0.05$, ^ ^ ^ $P < 0.01$, ^ ^ ^ $P < 0.001$ (AREM, AREH groups vs. AREL group). NC: negative control; O₃: ozone; AR: allergic rhinitis; OVA: ovalbumin; AREL: AR rats exposed to O₃ (0.5 ppm). AREM: AR rats exposed to O₃ (1.0 ppm). AREH: AR rats exposed to O₃ (2.0 ppm).

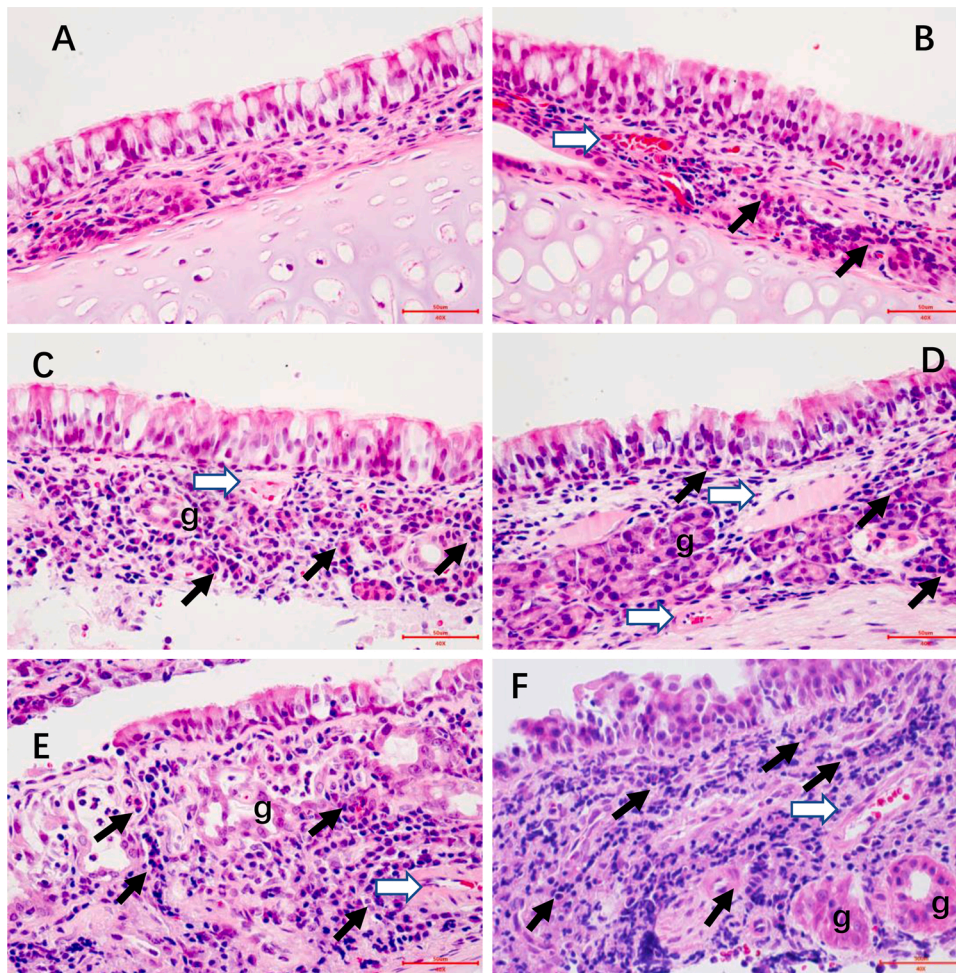


Fig. 5. Effects on histopathology of Nasal Mucosa after O_3 exposure ($N = 10$ per group). Representative photomicrographs of nasal mucosa (Panel AF, 400X) with eosinophilic infiltration (black arrows) and hyperaemia (open arrows) in hematoxylin and eosin-stained sections. g: hyperplasia of gland in lamina propria. Scale bar: 50 μ m.

(A) NC group. (B) NE group (C) AR group (D) AREL group (E) AREM group (F) AREH.

was clear, and the cell structure was intact. In the AR group, the epithelial cells and cilia of the nasal mucosa were disordered, some epithelial cells were shed, the mucosa lamina propria was infiltrated by a small number of eosinophils, and the glands were slightly swollen. In the ARE group, with OVA and Ozone combination treatment, the nasal mucosal epithelial cells were disordered significantly, the mucosal basement membrane was thickened, the submucosal lamina propria was swollen, and the number and percentage of eosinophils was significantly increased, accompanied by vascular congestion and dilatation and glandular hyperplasia compared with NC, NE, AR group (Fig. 5).

Histological examination of nasal mucosa tissue was concordant with the findings in NLF: tissue infiltration of eosinophils in each group was in the same trend as eosinophilia in NLF. OVA sensitization and challenge enhanced eosinophils infiltration in ARE group, compared with ozone-exposed alone in NE group ($p < 0.05$) and with OVA sensitization and challenge alone in AR group ($p < 0.01$). Compared with the control group, eosinophils percentage was markedly enhanced in AR rats ($p < 0.01$). The percentage of eosinophils increased in three doses of O_3 exposure groups in a concentration-dependent manner compared with the AR group ($p < 0.05$) (Fig. 4F). The percentages of neutrophils, macrophages, and lymphocytes in each section were also counted, and the trends of change between groups were similar to those observed in NLF, so the histograms were not shown in the paper.

3.4. O_3 inhalation elevated the expression of Th2 cytokines and Eotaxin in AR rats

The level of cytokines in the NLF was detected by ELISA. The

expressions of IL-5, IL-13 and Eotaxin in NLF were significantly increased in three ARE groups in dose-dependent manner, whereas IFN- γ was reduced when compared with those in the AR and NE group ($p < 0.05$) (Fig. 6 A to D). And there was not significant differences between the three ARE groups under different concentrations ($p > 0.05$).

4. Discussion and conclusion

Ambient ozone is the primary photochemical smog in air pollution, and its level often exceeds the health standard in many countries, especially in summer. With the high-speed development of economy, the acceleration of urban industrialization and the increasing motor vehicles, the haze characterized by the high concentration of particulate matter 2.5 (PM_{2.5}) has changed to the compound pollution. In recent years, the photochemical smog pollution characterized by O_3 is becoming increasingly prominent. Especially in summer, ozone has replaced PM as the primary pollutant in major cities of the world [11]. Meanwhile, O_3 pollution causes ever-increasing public health concerns in recent decades. Except for PM_{2.5}, O_3 was the unique air pollutant considered as a risk factor in the Global Burden of Disease Study in view of its independent effects on airway diseases mortality in the American Cancer Society Cohort Study [12].

Here we confirmed that O_3 exposure exacerbated AR symptoms in a concentration-dependent manner. We hypothesized that such exacerbation of AR symptoms may be due to increased Th2 cytokines expression and eosinophilic airway inflammation.

In this study, the O_3 -inhaled groups were exposed to a low dose (0.5 ppm), medium dose (1 ppm), and high dose (2 ppm) of O_3 respectively in

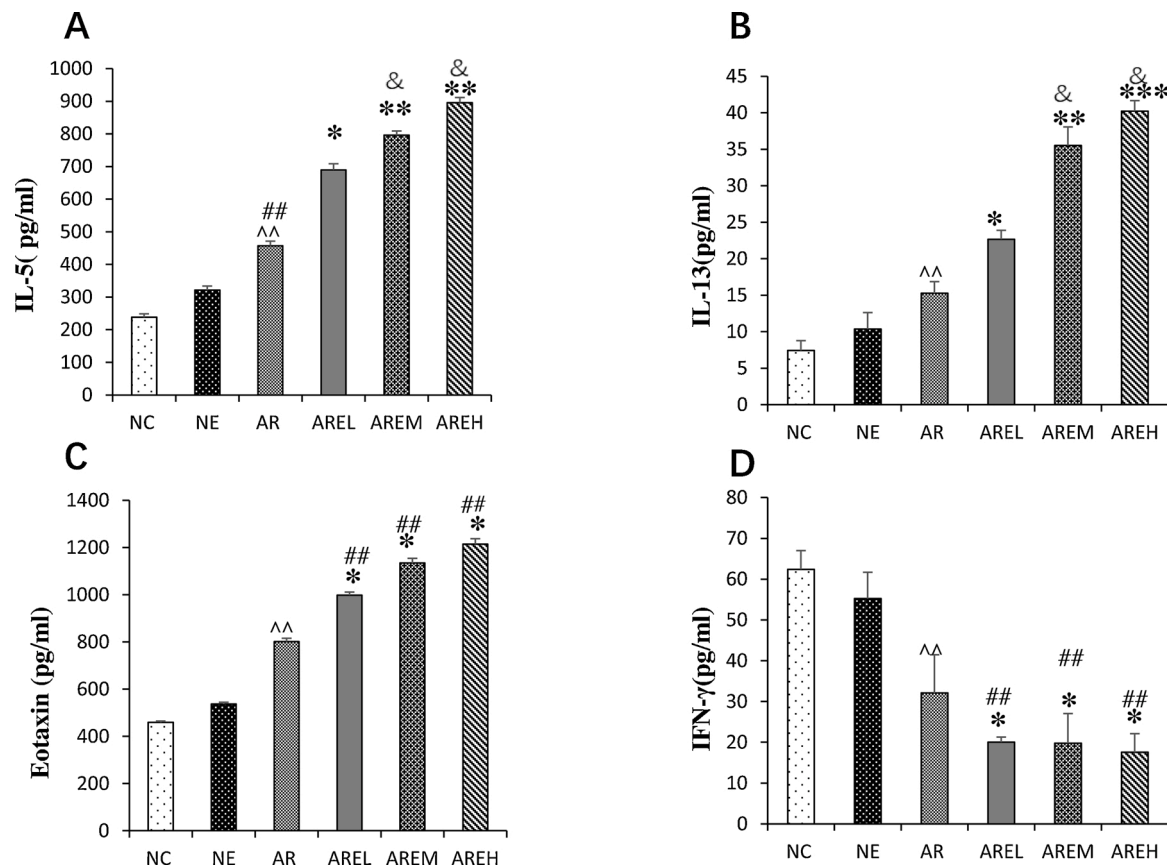


Fig. 6. O₃ inhalation elevated the expression of Th2 cytokines in AR rats (N = 10 per group). Cytokine profile in NLF after O₃ exposure (Panel AD—), **P < 0.01, ***P < 0.001 (AREL, AREM, AREH groups vs. AR group), ##P < 0.01 (ARL, ARM, ARH groups vs. NE group), ^^P < 0.01 (AR group vs. NC group), &P < 0.05 (AREM, AREH groups vs. AREL group). NC: negative control; O₃: ozone; AR: allergic rhinitis; OVA: ovalbumin; AREL: AR rats exposed to O₃ (0.5 ppm). AREM: AR rats exposed to O₃ (1.0 ppm). AREH: AR rats exposed to O₃ (2.0 ppm).

a whole-body inhalation chamber for 2 h per day over 6 weeks. It is worth to note that the O₃ exposure was applied during the OVA sensitization and challenge process synchronously. Thus, this exposure protocol would mimic the scenarios in which AR patients are triggered by continuous exposure to mild, moderate-severe, or serious levels of O₃ in the atmosphere [13].

According to the U.S. Environmental Protection Agency (EPA), 0.25–0.4 ppm (8 h averaging time) is detrimental concentration to human health. However, the previous research did not find airway inflammation when mice inhaled 0.4 ppm or lower levels of O₃. Because the deposition fraction of O₃ which in humans and rodents is 100 % and 25 % respectively [14]. Previous studies in mouse have confirmed that the concentrations of O₃ arousing airway inflammation are about 4–5 times higher than the concentration required to evoke comparable inflammation in exercising human individual [15,16]. Accordingly, the levels of O₃ exposure in our study were about 7- (AREL), 15- (AREM), and 30-fold (AREH) higher than those recommended by NAAQS of the United States (8 h averaging time 0.07 ppm). The three doses were respectively related to mild, moderate-severe, and serious O₃ levels in real life situation. Additionally, we limited the exposure time per day to 2 h (instead of 8 h) because rats did not have access to food and water during inhalation to avoid indirect effects by consumption of O₃-exposed food and water. As shown by several studies in many species, including mice and humans, acute exposure to O₃ not more than one day induced the release of inflammatory mediators and promote airway inflammation in a rapid, but transient manner, with accumulation of neutrophils, increased epithelial cells in bronchoalveolar lavage (BAL) fluid [17,18]. These changes frequently peaked at 6–12 hours, with recovery in the following 24–48 hours [14]. Hence the exposure

course last 6 weeks here, was chronic exposure and accord closely with pathogenesis of AR.

Current evidence demonstrates that levels of inflammatory cells and Th2 cytokines in NLF are improved when allergic subjects are exposed to O₃. In both healthy and asthmatic patients, O₃ exposure provoked nasal inflammation represented by the enhancement of eosinophils and inflammatory cytokines in NLF. However, these inflammation markers challenged by O₃ were greater in asthmatics than in non-asthmatics subjects [19]. These results were consistent with our findings in AR rats. Briefly, repeated exposure of O₃ manifested the inflammation and enhanced the sensibility of the nasal mucosa to specific allergen and nonspecific stimulus of O₃. The present research showed that O₃ exacerbated nasal allergic response in a concentration-dependent manner accompanied by increasing eosinophilic airway inflammation. This result is consistent with the findings in OVA-challenged asthmatic mouse model under O₃ exposures (3 ppm for 3 h on 4 days) [20]. The possible mechanism maybe prolonged survival of eosinophils in the nasal airway. As reported previously in mice, enhanced eosinophilia was associated with reduced expression of Fas and FasL and decreased of apoptotic eosinophils which may be due to amplified release of IL-5, GM-CSF and G-CSF in the BAL fluid of mice that received O₃ and allergen co-exposure [14].

In addition, numbers of neutrophils in NE group (Fig. 3D) were significantly increased compared with negative control group and AR group, indicating neutrophilic influx following ozone exposures in normal rats which was different with OVA and O₃ combination intervention in AER groups.

We also made morphology and histopathology examination to evaluate effect of O₃ exposure on AR individual. HE staining of the nasal

mucosa in the ARE group, with OVA and O₃ combination treatment, the nasal mucosal epithelial cells were disordered significantly, the mucosal basement membrane was thickened, the submucosal lamina propria was swollen, and the infiltration of eosinophils was significantly increased, accompanied by vascular congestion and dilatation and glandular hyperplasia.

In addition, O₃ exposure amplified OVA-specific IgE levels in serum in a concentration-dependent manner in AR rats. Many factors could contribute to IgE production, such as antigen-presenting activity, differentiation and proliferation of Th2 cells and B cells, cytokines, and chemokines. It was confirmed that O₃ enhanced the activity of antigen-presenting activity of BAL cells in rats by expressing increased cell-surface molecules associated with antigen presentation after O₃ exposure, [21] which contributed to increased antibody and eventually aggravated the AR symptoms and histopathology change.

Our results demonstrated that there was a significant synergistic effect of O₃ and OVA on the elevation in cytokines protein of IL-5, IL-13 and Eotaxin in NLF which displayed the Th2-dominant immune response. In a research of *Aspergillus fumigatus*(Af)-sensitized murine models, O₃ exposure of 3.0 ppm for 2 h exacerbated airway responsiveness of by increasing viability of eosinophils and inhibited eosinophil apoptosis accompanied increasing IL-5, granulocyte-macrophage-colony stimulating factor (GM-CSF) and G-CSF protein levels [14]. As we know, a number of Eotaxin have been identified as forceful mediators for eosinophil transferring into the airways in AR and asthmatic diseases. A previous research put 3-month old monkeys under ozone and house dust mite (HDM) aerosol inhalation and found expression of Eotaxin in the lung and lavage samples were enhanced by ozone and allergen co-exposure [22]. O₃ is a strong oxidant agent. Numerous studies have placed their focus on the influence of oxidative stress after O₃ exposure. It is known that chronic O₃ exposure in mice causes activation of oxidative pathways resulting in cell death and a chronic bronchial inflammation with emphysema [23]. Another research in asthmatic mouse model of O₃ exposure (1.0 ppm for 3 h) during OVA challenge period found exacerbation of pulmonary inflammation along with increase of cytokines such as IL-6, IL-13 and IFN- γ , and that oxidative stress mediates the O₃-enhanced allergic immune response [24]. This pathway may be involved in the pathological process of O₃-aggravated AR, which will be verified in our future studies.

Although there have been several studies assessing the O₃ effects on the asthmatic inflammation in mice [13,14,17], our research is unique in the animal protocol to mimic the real-life scenarios of O₃-induced exacerbation in AR patients. Moreover, our experimental results provide the first evidence in AR rat model that consecutive chronic ozone exposure for 6 weeks could enhance the allergic symptoms and Th2 inflammatory. The results demonstrate that O₃ and allergen could enhance the effects of the other. Thus, AR patients may be more sensitive to ambient O₃ than non-AR individuals. In summary, the study incurs the concern that ambient O₃ is able to exacerbate allergic airway inflammation and Th2-skew immune response in AR individuals which may be the underlying mechanism of susceptible individuals to ambient O₃. Effective strategies need to be adopt to improve air quality and avoid influence of air pollutions such as O₃ for prevention of AR exacerbations.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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