



Effects of ozone repeated short exposures on the airway/lung inflammation, airway hyperresponsiveness and mucus production in a mouse model of ovalbumin-induced asthma



Wuping Bao^{a,b,1}, Yingying Zhang^{a,b,1}, Min Zhang^{b,1}, Aihua Bao^b, Xia Fei^b, Xue Zhang^b, Xin Zhou^{a,b,*}

^a Department of Respiratory Medicine, Shanghai General Hospital of Nanjing Medical University, Shanghai, China

^b Department of Respiratory Medicine, Shanghai General Hospital, Shanghai Jiao Tong University, Shanghai, China

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ABSTRACT

Objective: The purpose of this study is to explore the influence of ozone repeated short exposures on airway/lung inflammation, airway hyperresponsiveness (AHR) and airway hypersecretion in ovalbumin (OVA) sensitized/challenged asthmatic mouse model.

Methods: OVA sensitization was performing by intraperitoneal injection. Ozone exposures (3ppm for 3hours) were given one hour after aerosolized OVA challenges (once every other day, 4 times totally). Methacholine (MCH) bronchial provocation tests, Liu's staining of BALF cell smears, hematoxylin-eosin (HE) staining and Periodic Acid-Schiff (PAS) staining of lung tissue were performed. Interleukins (ILs; IL-4, IL-13, IL-1 β , and IL-18) protein (ELISA) and mRNA expression levels (RT-qPCR) in murine lung, 8-hydroxy-2'-deoxyguanosine (8-OHdG, ELISA), malondialdehyde (MDA, thiobarbituric acid assay), reduced glutathione (GSH, spectrophotometric method) in bronchoalveolar lavage fluid (BALF), and GSH1 mRNA relative expression levels (RT-qPCR) in lung tissue were analyzed.

Result: Repeated ozone exposures down-regulated the AHR to MCH in mice undergoing OVA sensitization and challenge, however not all parameters associated with asthma were decreased since obvious mucus hypersecretion was induced and airway inflammation increased slightly, especially around small airways. Following ozone co-exposure, the increase of IL-4 and IL-13 levels in murine lung caused by OVA sensitization/challenge were reversed. Instead, levels of IL-1 β in BALF remained, higher than negative control group. Ozone repeated short exposures also induced significant increase of 8-OHdG in BALF in OVA sensitized and challenged mice.

Conclusion: For asthmatic mice undergoing ozone exposures, AHR is not an accurate indicator of the severity of asthma. Repeated short ozone exposures increase mucus hypersecretion, possibly via an increase in oxidative stress and immune dysregulation.

1. Introduction

Bronchial asthma is an inflammatory airway disease characterized by reversible airflow limitation, airway hyperresponsiveness (AHR), chronic airway inflammation, mucus hypersecretion, and remodeling [1]. It is a major and increasing global health problem that affects about 300 million patients worldwide currently [2–4]. High concentrations of ozone are associated with the increases in mortality, hospitalizations,

and emergency-department visit rates of asthmatics [5–7]. Notably, compared with non-asthmatic patients, the decrements of forced expiratory volume in one second (FEV₁) and the increases of airway responsiveness are more significant in asthmatics following high concentration ozone exposure (0.4 ppm, 2 h) [8,9]. Greater ozone-induced decrements are found in asthmatics who had lower baseline FEV₁ divided by forced vital capacity (FVC) and lower baseline FEV₁ [10,11]. Subjects with preexisting asthma and rhinitis are known to be

Abbreviations: 8-OHdG, 8-hydroxy-2'-deoxyguanosine; AHR, airway hyperresponsiveness; ANOVA, analysis of variance; BALF, bronchoalveolar lavage fluid; ELISA, enzyme-linked immunosorbent assay; FEV₁, forced expiratory volume in one second; FVC, forced vital capacity; GSH, reduced glutathione; HE, hematoxylin-eosin staining; IL, interleukin; i.p., intraperitoneal; MCH, methacholine; MDA, malondialdehyde; OVA, ovalbumin; O₃, ozone; OVA, ovalbumin; PAS, periodic acid-schiff stain; PBS, phosphate buffered saline; R_L, lung resistance; RT-qPCR, real-time quantitative PCR; SD, standard deviation; Th1, helper T cell 1; Th2, helper T cell 2; Th17, helper T cell 17

* Corresponding author at: Department of Respiratory Medicine, Shanghai General Hospital of Nanjing Medical University, No. 100, Haining Road, Shanghai, 200080, China.

E-mail address: xzhou53@163.com (X. Zhou).

¹ These authors contributed equally to this work.

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particularly vulnerable to ozone and are at risk of exacerbations [12]. Collectively, asthmatics have increased susceptibility to ozone. The underlying mechanisms are only partially understood.

Eosinophilic inflammation, antigen-specific IgE, AHR and mucous hypersecretion have been improved in allergic asthma models typically induced by chicken egg ovalbumin (OVA) [13,14]. Single [15–20] or repeated [15,21–27] ozone exposures led to significant decreases in spirometry and increased AHR [15,16], BALF neutrophilia [17,18,21–23], peribronchiolar and perivascular inflammatory cell infiltration [17,18,21–23], airway mucus production, and goblet cell hyperplasia [15,16,19] in mice without allergen inhaled. IL-13 [21], IL-1 beta [17,20,25–27], IL-18 [26], caspase-3 [21], and IFN- γ [18,21] were increased, indicating Th2 inflammation and inflammasome/IL-1 pathway were involved. However, the additive effect of ozone exposure on the development of allergen-induced asthma and the role of Th2 inflammation and inflammasome/IL-1 pathway is not exactly clear.

Accordingly, we performed this study to explore the influence of ozone repeated short exposure on airway/lung inflammation, AHR, and airway hypersecretion in ovalbumin (OVA) sensitized/challenged asthmatic mouse model. Further, markers of oxidative stress, pro-inflammatory cytokines, and antioxidant were analyzed to verify the hypothesized links between deleterious effect of ozone exposure and oxidative stress-immunity imbalance network in the asthmatics.

2. Materials and methods

2.1. Animals

Pathogen-free, male C57/BL6 mice (age 8–10 weeks) were purchased from SLRC Laboratory Animal (Shanghai, China), and housed within filter-topped cages under controlled conditions of temperature (21–25 °C) and humidity (40–60%), administered food and water ad libitum (free from OVA). Mice were allowed to acclimatize for 1 week before beginning the experiments with a strict 12-h light–dark cycle.

All animal experiments were performed at Shanghai General Hospital and carried strictly in accordance with the recommendations of Shanghai Committee for Accreditation of Laboratory Animal. All surgery was performed under anesthesia, and all efforts were made to minimize suffering. The experimental protocols were approved by Shanghai General Hospital Institutional Review Board, Shanghai, China.

2.2. Induction of allergic airway inflammation and ozone exposures

C57/BL6 mice were randomly grouped (4 subgroups, 8 mice per each subgroup). Sensitization was performed with an intraperitoneal (i.p.) injection of 20 μ g OVA (Grade V, Sigma-Aldrich, St. Louis, MO) dissolved in 0.2 mL of Dulbecco phosphate buffered saline (PBS) emulsified in 2 mg aluminium hydroxide (Sigma-Aldrich) as an adjuvant on days 1 and 7. Challenge in mice was induced with exposure to 5% aerosolized OVA (8 mL) (Grade II, Sigma-Aldrich, St. Louis, MO) in a plastic chamber linked to an ultrasonic nebulizer (Clenny2 Aerosol, Medel, S. Polo di Torriale, Italy) for 20 min on days 14, 16, 18, and 20. Control mice were sensitized and challenged with the same volume (0.2 mL for sensitization and 8 mL for challenge) of PBS as vehicle.

C57/BL6 mice were exposed to ozone at a concentration of 3 ppm (ppm) or ambient air in a sealed Perspex container one hour after each OVA/PBS challenges on days 14, 16, 18, and 20. Ozone was generated from an ozoniser (model 300 Ozoniser, Aqua Medic, Germany), and was mixed with air. Every exposure lasted for 3 h. Ozone concentration was continuously monitored and controlled by OS-4 ozone switch (Ecosensors, Kwj Engineering Inc, California, USA). Control groups were exposed to air.

2.3. Airway hyperresponsiveness measurements

On day 21, twenty-four hours following the final ozone/air exposure, mice were anaesthetized with anesthetic solution (3 μ L/mg) containing ketamine (20 mg/mL, Wanhe Medical Inc., Anhui, China), xylidinothiazoline (3.5 mg/mL, MoDa animal drugs Inc., Jilin, China), dihydroetorphine (3.5 mg/mL, MoDa animal drugs Inc., Jilin, China), and haloperidol (0.25 mg/mL, MoDa animal drugs Inc., Jilin, China), via i.p. injection. Mice were tracheostomised and cannulated endotracheally using improved scalp needles. Tracheostomised mice were ventilated (MiniVent, Hugo Sach Electronic, Germany) at 250 breaths/min and tidal volume of 250 mL in a whole body plethysmograph with a pneumotachograph connected to a transducer (EMMS, Hants, UK). Transpulmonary pressure was assessed via an oesophageal catheter (EMMS, Hants, UK). Pulmonary airway resistance (R_L) was recorded for 3 min after increasing concentrations (4–256 mg/mL) of aerosolized methacholine (MCH, Sigma, UK). R_L was expressed as percentage change from baseline R_L measured following nebulized PBS. The MCH concentration required to increase R_L by 100% from baseline was calculated (PC_{100}). $-\log PC_{100}$ was taken as a measure of AHR [25,28].

2.4. Blood collection, bronchoalveolar lavage and measurements of bronchoalveolar lavage fluid (BALF) cells

After bronchial provocation tests, mice were sacrificed with an overdose of sodium pentobarbitone (200 mg/kg, i.p.). Blood was obtained from right ventricle, and was centrifuged at 2500 r/min for 10 min at 4 °C. The serum was stored at -80 °C for further analysis.

Mice were lavaged with three aliquots of 0.6 mL ice-cold PBS via an endotracheal cannula (0.72 mm inner diameter; 1.22 mm outer diameter), and bronchoalveolar lavage (BAL) fluid was retrieved [27]. Return volume was recorded and was consistently $\geq 70\%$ of the instilled volume. The BALF was then centrifuged at 2500 r/min for 10 min at 4 °C. The supernatant was stored at -80 °C for further assay. Total cell counts were calculated utilizing a standard hemocytometer under an optical microscope (Olympus BH2; Olympus Optical, Tokyo, Japan). The remaining cell pellet was re-suspended in 0.5 mL PBS solution. Cytospin slides (Cytospin 2; Shandon, UK) of BALF cells were stained with Liu's stain set (Baso diagnostics. Inc., Zhuhai, China). Total leukocyte counts and classification (macrophages, eosinophils, lymphocytes, and neutrophils) were performed according to morphological criteria under $\times 400$ magnification in a blinded manner [27].

2.5. Histological and morphometric analysis

The lungs were dissected out. The left lungs were inflated by injecting fresh 4% paraformaldehyde to provide 25 cm of water pressure, immersed overnight in paraformaldehyde, embedded in paraffin, and sectioned (5 μ m thickness) to expose the maximum surface area of lung tissue in the plane of the bronchial tree [29]. Hematoxylin-eosin (HE) stain and Periodic Acid-Schiff (PAS) stain were performed to assess morphological changes of lung and airway epithelium, inflammatory cells infiltration, and goblet cell hyperplasia as a measure of airway mucus hypersecretion in a double-blind screen performed by two independent investigators. The measurement of airway epithelium thickness and the length of basement membrane was performed by using Image-Pro Plus (version 6.0, Media Cybernetics Inc., Warrendale, PA, USA).

Peribronchiolar and perivascular inflammation observed in the HE-stained lung sections was scored from 0 to 3, as described in Chung KF's articles [25,29]: 0 = no inflammatory response; 1 = mild inflammation with foci of inflammatory cells in bronchial or vascular wall and in alveolar septa; 2 = moderate inflammation with patchy inflammation or localized inflammation in walls of bronchi or blood vessels and alveolar septa, and less than one-third of lung cross-sectional area was involved; and 3 = severe inflammation with diffuse inflammatory cells

in walls of bronchi or blood vessels and alveoli septa; between one-third and two-thirds of the lung area is involved.

Total leukocytes, neutrophils and eosinophils located along lobar bronchi and segmental bronchi were counted in 15 randomly selected, non-overlapping fields under the magnification of $400\times$ from two separately lung sections per animal. Total length of bronchi observed should be more than 10mm. Average width of observed area was 100 μ m. The airway inflammatory cells infiltration density (per mm) was calculated as the ratio of cells to the whole length of bronchial wall countered.

For PAS stain, blue/purple goblet cells were determined as PAS-positive. The numbers of PAS-negative and PAS-positive cells were counted around the internal perimeter of the airway. Percentage of PAS-positive cells in total goblet cells was calculated to reflect goblet cell metaplasia. A morphometrical analysis of volume density of PAS positive material (Image J, Maryland, USA) in lobar bronchi and segmental bronchi was performed to evaluate mucus hypersecretion. The data are expressed as percentage of PAS positive material area in total epithelial area.

2.6. Protein isolation and determination of protein concentration

The right lungs were used for ELISA, RT-PCR and oxidative stress analysis. Lung tissues for ELISA were flushed with PBS (0.01M, PH 7.4) before being frozen.

Frozen lung tissue was homogenized in lysis buffer containing protease and phosphatase inhibitors using a polytron-type homogenizer, and was centrifuged at 12,000 rpm at 4°C for 10 min. Protein concentrations in serum, supernatants of homogenates, and supernatants obtained from the BALF for each animal were determined using Pierce bicinchoninic acid Protein Assay Kit (ThermoFisher Scientific, Waltham, USA) according to the manufacturers' instruction.

2.7. Cytokines and biomarkers of oxidative stress assays

The levels of Interleukin (IL)-1 β , IL-4, IL-13 and IL-18 in the supernatants of lung tissue homogenates were quantified by enzyme-linked immunosorbent assay (ELISA) using commercially-available kits (for IL-1 β and IL-4: ANOGEN, Ontario, Canada; for IL-13 and IL-18: RayBiotech inc. Minnesota, USA). The detected samples were diluted (4-fold for IL-13 and IL-18; 2-fold for IL-1 beta and IL-4) with appropriate diluent according to the user manuals. 100ul sample was added into every well for IL-13 and IL-18; 50ul for IL-1 beta and IL-4. Biomarkers of oxidative stress analyzed in supernatants of BALF included 8-hydroxy-2'-deoxyguanosine (8-OHdG), malondialdehyde (MDA), and reduced glutathione (GSH). Levels of 8-OHdG were determined by ELISA with StressXpress® EIA kit from StressMarq Biosciences Inc. (Victoria, Canada). The concentrations of MDA were measured by thiobarbituric acid assay and GSH by spectrophotometric method. MDA and GSH assay kits were from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) and chemical colorimetric analyses were performed according manufacturer's instructions.

The results of ELISA are presented as pg/mg or ng/mg. The results of MDA, GSH and SOD activity are presented as nmol/ml, mg/gprot and U/mgprot, respectively.

2.8. RNA isolation and real-time quantitative PCR

Lung tissues were washed three times with PBS, and intracellular RNA was extracted from frozen lung tissue using TRIzol (Invitrogen, Carlsbad, CA, USA). Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Promega, Madison, USA) and oligo(dt) 18 primer were employed for cDNA synthesis in a PTC-200 Peltier Thermal Cycler (MJ Research, Watertown, Massachusetts, USA), following the manufacturer's guidance. Transcript levels of IL-4, IL-13, IL-18, IL-1 β , and GSH1 were determined by real-time Quantitative PCR (RT-qPCR)

Table 1

Forward and reverse primer sequences and PCR conditions for real-time quantitative PCR.

Gene	Forward sequences	Reverse sequences
IL-1 α	5' GCT GTC TGG GTA TTC TTA CA	5' AGG GGA AAC ACT GAT TAC AT
IL-13	5' GGT CCA CAC AGG GCA ACT GA	5' AGG GCA TAG GCA GCA AAC CA
IL-18	5' TGG GGG TTC TCT GTG GTT C	5' GCT TTC TTT GTC CTG ATG CT
IL-4	5' CAT GCT TGA AGA AGA ACT CT	5' ATG TGG ACT TGG ACT CAT T
GSH1	5' CTT TTC CTT CCC TCC TCA G	5' CAG AGT CAT TTT CCC CAA TA

PCR conditions: 5mins@95 °C, 15s@95 °C, 30s@60 °C $\times$ 40 cycles, 15s@95 °C, 30s@60 °C, 15s@95 °C.

Abbreviations: IL: interleukin; GSH: reduced glutathione.

using Absolute SYBR Green ROX Mix Reagent (ThermoFisher Scientific, Waltham, USA) in ViiA7 Real Time PCR System (Life Technologies, Carlsbad, USA). Primers are listed in Table 1. Run method of PCR was as follow: holding stage, 5 min at 95 °C; cycling stage, step one 15 s at 95 °C, step two 30 s at 60 °C, repeating step one and step two 40 times; melt curve stage, 15 s at 95 °C, 30 s at 60 °C, and 15 s at 95 °C. Mouse 18s RNA was used as endogenous control and the RT-qPCR results were quantified using relative quantification ($\Delta\Delta$ CT method).

2.9. Statistical analysis

Data are expressed as the mean $\pm$ standard deviation (SD), unless otherwise specified. The statistical analysis and graphics were performed using GraphPad PRISM, version 5.0 (GraphPad Software, San Diego, CA). Kolmogorov-Smirnov test with Dallal-Wilkinson-Lilie for P value was used to test if the values come from a Gaussian distribution. The Kruskal-Wallis One-way analysis of variant (ANOVA) with Bonferroni's post hoc test (for equal variance) or Dunnett's T3 post hoc test (for unequal variance) was used to evaluate the differences of variances between multiple groups. A post-test Mann-Whitney analysis was performed if necessary. $P < 0.05$ was defined to be statistically significant.

3. Results

3.1. Effect of ozone exposure on spontaneous activity of animals

Preset ozone concentration range was 2.75–3.25 ppm and the detected average concentration was 3.06 ± 0.14 ppm. At the beginning of ozone exposure, restlessness, irritability, increased activity, sneezing, and nasal secretions were observed in animal. About 20 min later, somnolence was obvious for all the animal. After exposure, decreased of activity, inhibition of food consumption, and lassitude persisted for 5–8 h, accompanied with significant decrease of body weight. Body weights were measured before and 8-hour after each ozone/air exposure. Significant loss of body weight was proved after ozone exposures (1.55 ± 1.09 , for ozone + saline group, $p < 0.001$, compared with air + saline group; 1.04 ± 1.02 , for ozone + OVA group, $p < 0.01$, compared with air + OVA group;). Feeding and movement activity improved in 8–12 h.

Compared with baseline, no significant changes in feeding, and movement activity were observed after air exposure in the control groups. No significant changes of body weight were found after air exposed (-0.14 ± 1.91 , for air + saline group; -0.10 ± 1.01 , for air + OVA group).

3.2. Airway resistance and bronchial hyperresponsiveness of mice

One in OVA + ozone group was died during anesthesia and tracheostomize. No airway responsiveness test and dissection was performed. So, the data about this mouse was excluded.

There were no significant differences in baseline lung resistance (R_L)

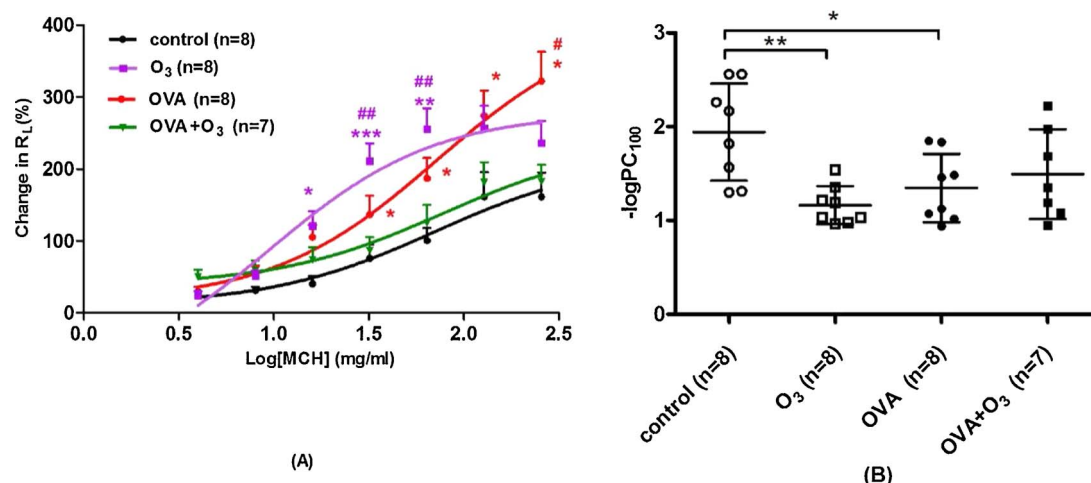


Fig. 1. Concentration-response curves to MCH. Mean percentage increase in lung resistance (R_L) to increasing concentrations of methylcholine is shown in Panel A. Individual and mean $-\log PC_{100}$ of the four groups are shown in Panel B. Data are expressed as mean $\pm$ SD. * $P < .05$, ** $P < .01$, and *** $P < .001$ compared to negative controls group, # $P < .05$ and ## $P < .01$ compared to OVA+O₃ group.

R_L : lung resistance; MCH: methylcholine; OVA: ovalbumin; O₃: ozone; PC_{100} : the concentration of methylcholine required to increase lung resistance by 100% from baseline; SD: standard deviation.

values following PBS challenge in the four groups. After OVA sensitization/challenge, without ozone exposure, animals were demonstrated a leftward shift of the concentration responsiveness to MCH, indicating an increase in bronchial responsiveness to MCH compared with negative control mice (Fig. 1, Panel A). Compared to negative control group, OVA sensitization/challenge increase R_L at the 32 mg/ml, 64 mg/ml, 128 mg/ml, and 256 mg/ml concentrations of MCH ($P < 0.05$ for all). $-\log PC_{100}$ was decreased 30.73% after OVA sensitization/challenge compared with negative control mice ($P < 0.05$, Fig. 1, Panel B).

Ozone 3ppm for 3 h also increased R_L at the 16mg/ml, 32mg/ml and 64mg/ml concentrations of MCH ($P < 0.05$, $P < 0.001$, and $P < 0.01$, respectively; Fig. 1, Panel A), and down-regulated $-\log PC_{100}$ 40.20% ($P < 0.01$, Fig. 1, Panel B), compared to negative control group.

Surprisingly, ozone exposures down-regulated the AHR of mice undergoing OVA sensitization and challenge according to R_L analysis (for 256mg/ml, $P < 0.05$, compared with OVA alone; for 32mg/ml and 64mg/ml, $P < 0.05$, compared with ozone exposed group; Fig. 1, Panel A). There was no significant difference of $-\log PC_{100}$ between OVA + ozone group and negative control group ($P > 0.05$, Fig. 1, Panel B).

3.3. Effect of OVA sensitization/challenge and ozone exposures on leukocytes numbers and classification in BALF

Results of BALF inflammatory cell count and classification were shown in Fig. 2. OVA sensitization/challenge and ozone exposures led to an increase in total leukocytes (3.61-fold, $P < 0.01$; and 3.97-fold, $P < 0.05$, respectively; Fig. 2, Panel A), reflected an increase in airway inflammation. Combination of ozone and OVA treatment up-regulated leukocytes more obviously (4.46-fold, $P < 0.01$) compared with negative control group, whereas the difference between OVA + ozone group and single treatment groups was statistically insignificant. Changes of macrophages in BALF were similar with total leukocytes (Fig. 2, Panel B), since macrophages constitute the majority of immune cells in airway.

Compared with the control group, eosinophilic inflammation was markedly enhanced in OVA sensitization/challenge asthmatic mice (31.17-fold, $P < 0.001$; Fig. 2, Panel C). Otherwise, ozone exposures caused slightly but not significantly elevated eosinophilic inflammation, compared with negative control group. In contrast, no increase following ozone exposures was observed in eosinophil numbers of BALF

in mice undergoing OVA sensitization and challenge. A similar increase of lymphocytes was noted in OVA treated groups (compared with negative control group, 5.31-fold, $P < 0.01$; and 6.81-fold, $P < 0.01$, respectively; Fig. 2, Panel E), whereas ozone repeated short exposures cannot cause significant up-regulation of lymphocytes.

In addition, numbers of neutrophils in BALF of saline + ozone group (31.77-fold, $P < 0.001$; Fig. 2, Panel D) and OVA + ozone group were significantly increased (21.48-fold, $P < 0.05$), compared with negative control group, indicating the presence of neutrophilic inflammation following ozone exposures. However, the difference between OVA + air group and OVA + ozone group did not reach statistical significance, probably due to large intragroup variability of OVS + ozone group and small group size.

3.4. Effect of OVA sensitization/challenge and ozone exposures on peribronchiolar and perivascular inflammatory cell infiltration

A histopathological analysis revealed there was lack of inflammation cells infiltration in the lung tissue of negative-control mice (Fig. 3, Panel A and E), as expected.

Peribronchiolar and perivascular infiltration of mononuclear cells was observed in ozone or OVA treated mice (Fig. 3, Panel B–D, F–H; red arrows), accompanied by bronchial wall thickening and hyperemia in pulmonary vessels (black arrows). Semi-quantitative analysis and quantitative analysis showed, compared with the negative control group, OVA sensitization and challenge resulted in a significant increase in the inflammation score (56.31-fold, $P < 0.001$; Fig. 3, Panel I) and eosinophils infiltration density ($P < 0.001$; Fig. 3, Panel K). Significant increase in neutrophils infiltration density was observed in ozone-exposed mice (277.78-fold, $P < 0.001$; Fig. 3, Panel J). With OVA and Ozone combination treatment, mice evoked significant increases in total inflammation scores, neutrophils, and eosinophils infiltration density, compared with negative control group ($P < 0.001$ for all). For ozone exposed mice, OVA sensitization and challenge enhanced the increase of eosinophils infiltration ($P < 0.001$, compared with ozone alone).

Higher density of neutrophilic aggregation was proved around small airways with diameters ≤ 2 mm (35.20 ± 15.21 , and 47.81 ± 20.40 per mm, for ozone group and ozone + OVA group, respectively; Fig. 5, Panel A) of ozone exposed groups, compared with central airways (18.59 ± 7.46 and 21.37 ± 8.16 per mm; $P < 0.01$ and $P < 0.001$, respectively). No obvious difference of eosinophils distribution was

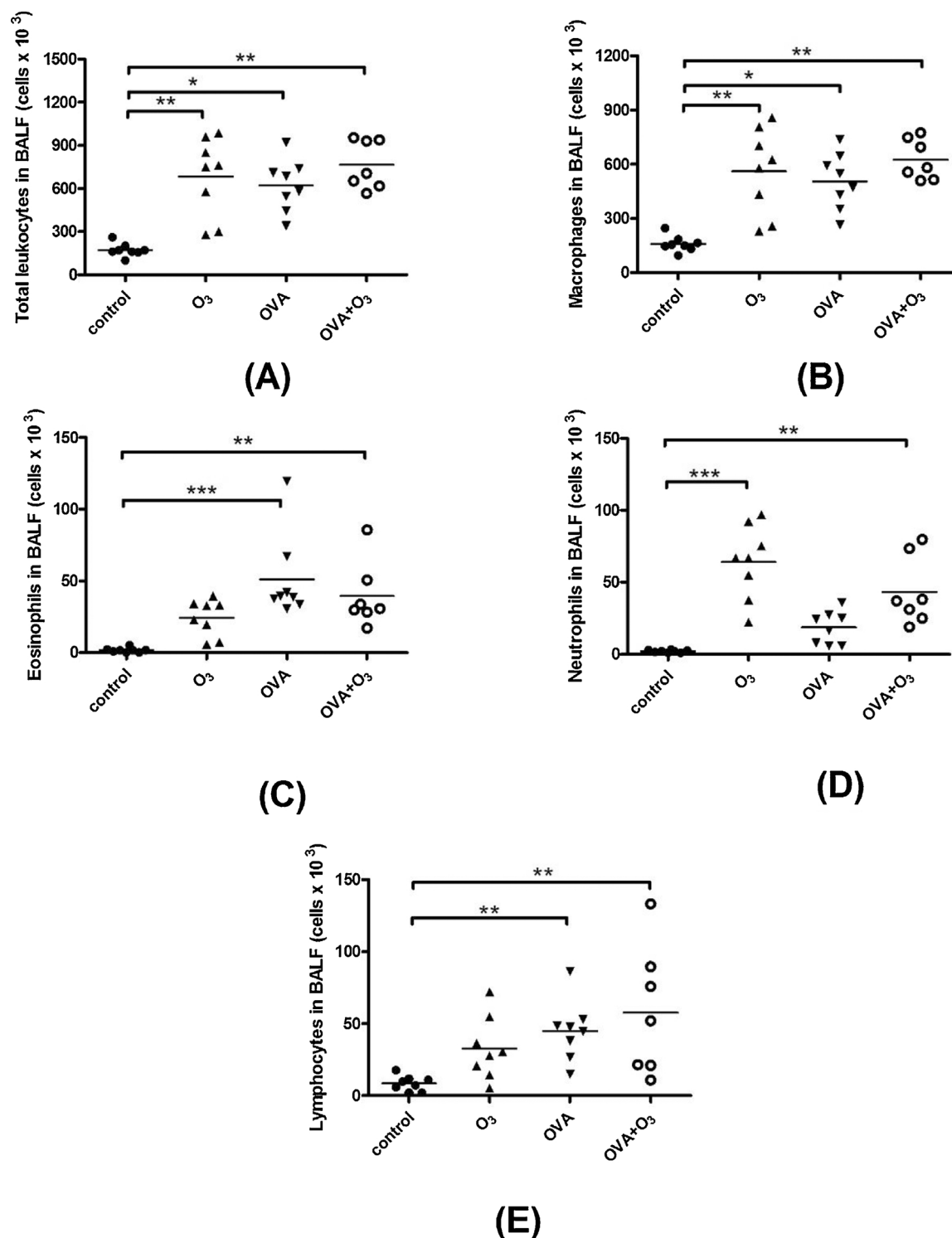


Fig. 2. Effect of ovalbumin (OVA) sensitization/challenge and ozone on airway inflammatory cells. Individual and mean numbers of total leukocytes (A), macrophages (B), eosinophils (C), neutrophils (D), and lymphocytes (E) in mouse bronchoalveolar lavage fluid (BALF) samples. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. OVA: ovalbumin; O₃: ozone.

proved between small airways and central airways in our four groups (Fig. 5, Panel B).

3.5. Effect of OVA sensitization/challenge and ozone exposures on airway mucus production and goblet cell hyperplasia

PAS staining was used to determine the mucus overproduction induced by goblet cell hyperplasia. There was lack of mucus secretion and goblet cell hyperplasia in the lung tissue of negative-control mice

(Fig. 4, Panel A and E), as expected.

Consistently, there was a strong bright violet stain (Fig. 4, Panel B–D, F–H; black arrows) in the tissue sections of mice after ozone and OVA treatment, indicating mucus overproduction in the bronchial airways compared with negative control group. Moreover, the extent of mucus staining was markedly evoked in OVA-induced mice combined with ozone exposures.

The results of positive area rate (positive staining area/epithelial cell area ratio; Fig. 4, Panel I) and PAS positive cell rate (positive cell

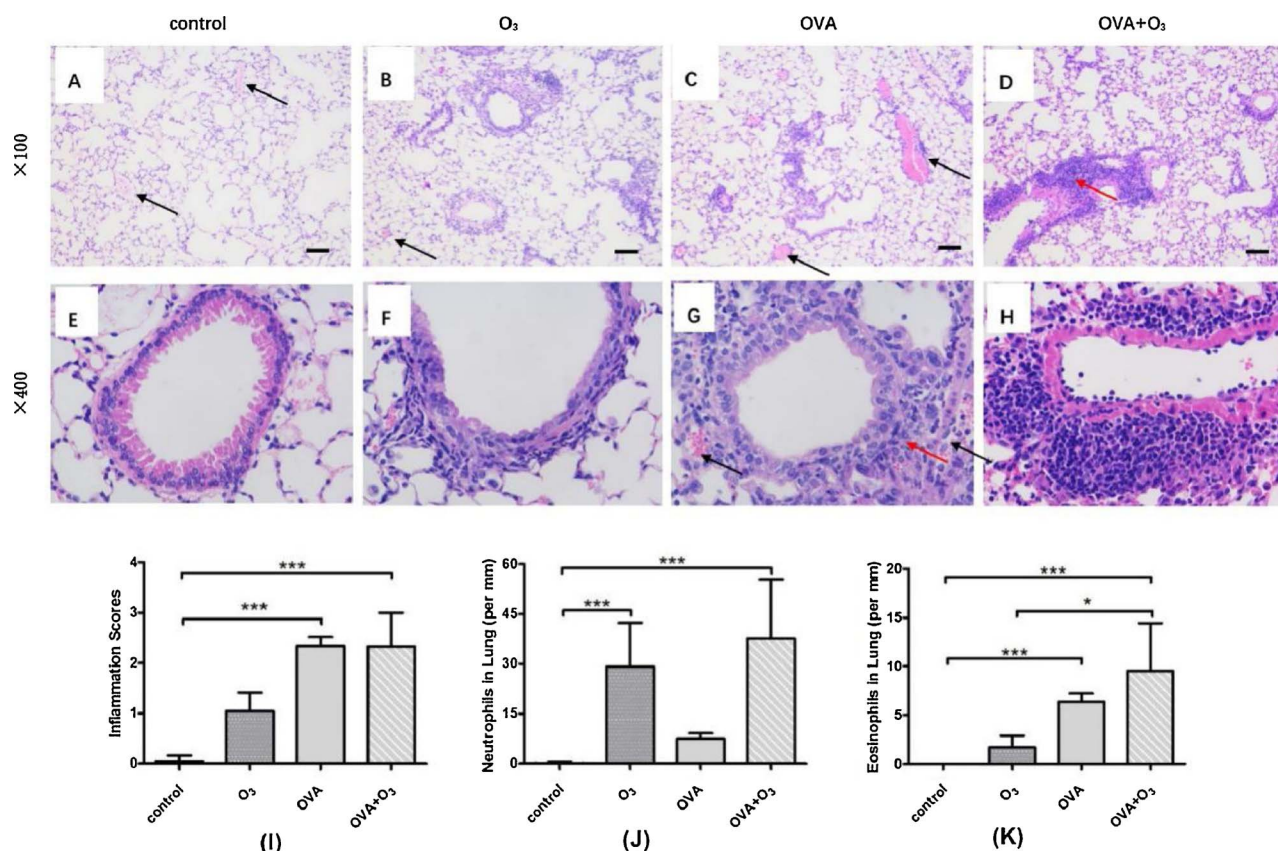


Fig. 3. Representative photomicrographs of lung (Panel A–D, 100X; Panel E–H, 400X) with inflammatory cells infiltration (red arrows) and hyperemia (black arrows) in hematoxylin and eosin-stained sections, airway inflammatory scores (Panel I) and inflammatory cells infiltration density (neutrophils and eosinophils, Panel J and Panel K, respectively). Scale bar: 30µm. Data in Panel I–K are expressed as mean $\pm$ SD. * $P < 0.05$, *** $P < 0.001$. OVA: ovalbumin; O₃: ozone; SD: standard deviation.

number/epithelial cell number ratio; Fig. 4, Panel J) were calculated. Obvious increases of PAS positive cell rate and positive area rate were confirmed after OVA sensitization and challenge (99.70-fold, $P < 0.05$, and 96.89-fold, $P < 0.05$, respectively). We further found greater elevated PAS cell rate and positive area rate after ozone exposures in OVA treated mice, compared with OVA solely treated animal (7.87-fold, and 11.45-fold, respectively, $P < 0.01$).

Higher elevation of PAS positive area rates and PAS positive cells rates were found around small airways (26.17 ± 7.74 , and 54.90 ± 17.60 , respectively; Fig. 5, Panel C, D) of combined treated group, compared with central airways (13.07 ± 2.79 and 19.30 ± 4.97 , respectively; $P < 0.001$). Similar difference of PAS positive cells rates was proved between small airways and central airways in OVA group (Fig. 5, Panel D), but the difference of PAS positive area rates was not significant in this group.

3.6. Effect of OVA sensitization/challenge and ozone exposures on cytokines in lung tissue

The levels of pro-inflammatory cytokines (IL-4, IL-13, IL-1 β , and IL-18) in the lung homogenate of mice were detected by Elisa, and the corresponding mRNA expression was detected by RT-qPCR method (Fig. 6).

In OVA sensitized/challenged mice, lung levels of IL-4 and IL-13 were increased compared with negative control mice (For ELISA, $P < 0.01$ and $P < 0.05$, respectively, Fig. 6, Panel A and Panel B; For mRNA, $P < 0.001$, for both, Fig. 6, Panel E and Panel F). For OVA-induced asthmatic mice, ozone exposures led to a markedly decrease of IL-4 and IL-13 in protein analysis ($P < 0.01$ and $P < 0.001$, respectively), whereas the changes of mRNA relative expression were statistically insignificant.

In ozone exposed mice, IL-18 protein level was increased in lung tissues compared with negative control mice ($P < 0.05$, Fig. 6, Panel C). For mRNA relative expression, this difference did not reach statistical significance due to large intragroup variability and small group size (Fig. 6, Panel G). OVA sensitization/challenge in ozone exposed mice reduced the increase of IL-18 protein level compared with ozone solely treated group ($P < 0.01$). Similarly, this difference in mRNA expression was insignificant. For OVA-induced asthmatic mice, although ozone exposures led to a slight but statistically significant decrease of IL-18 in protein analysis ($P < 0.05$), the change in mRNA expression was contrary, accordingly we cannot make a consistent conclusion about the influence of ozone in OVA-induced asthmatic mice.

IL-1 β level of OVA sensitized/challenged group was significantly higher (For ELISA, $P < 0.05$, Fig. 6, Panel D; For mRNA, $P < 0.01$, Fig. 6, Panel H), compared with negative control group. Similarly, OVA sensitization/challenge caused an up-regulation of IL-1 β mRNA expression ($P < 0.01$), but difference of protein levels from ELISA analysis between the groups was not significant. For OVA-induced asthmatic mice, ozone exposures cannot cause a significant influence to IL-1 β .

3.7. Effect of OVA sensitization/challenge and ozone exposures on chemokines of oxidative stress

MDA, one of several by-products of lipid peroxidation process, and 8-OHdG, a marker of oxidative DNA damage, were measured in supernatant of BALF to reflect the redox imbalance in asthmatic airways.

A substantial increase in 8-OHdG was observed in saline-treated mice and OVA-treated mice upon ozone exposures ($P < 0.05$ for both, Fig. 7, Panel A). Similarly, a slight but insignificant up-regulation was

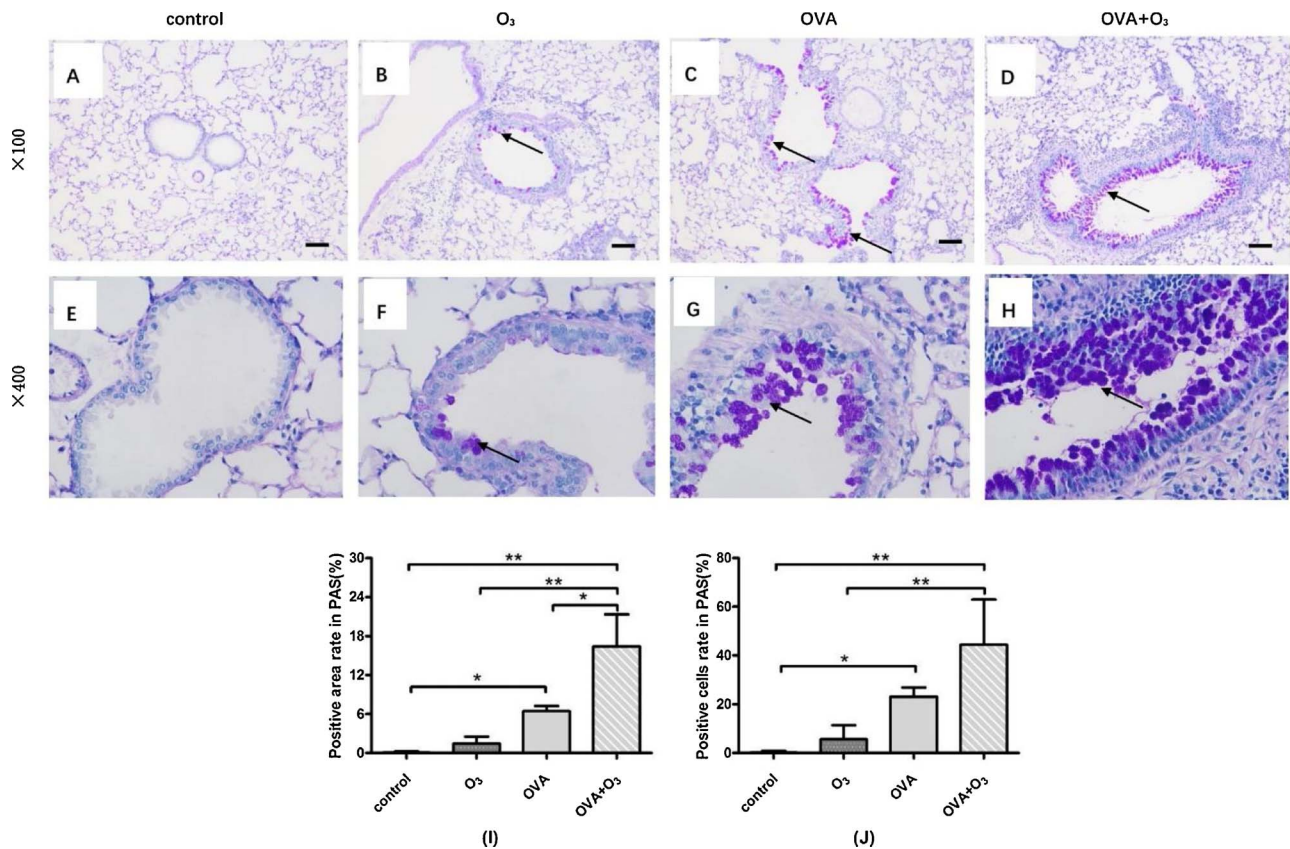


Fig. 4. Representative photomicrographs of lung (Panel A–D, 100X; Panel E–H, 400X) with airway mucus production and goblet cell hyperplasia (black arrows) in Periodic Acid-Schiff (PAS) staining sections, positive area rate (Panel I) and positive cells rate (Panel J). Scale bar: 30µm. Data in Panel I–J are expressed as mean $\pm$ SD. *P < 0.05, **P < 0.01, ***P < 0.001. OVA: ovalbumin; O₃: ozone; SD: standard deviation.

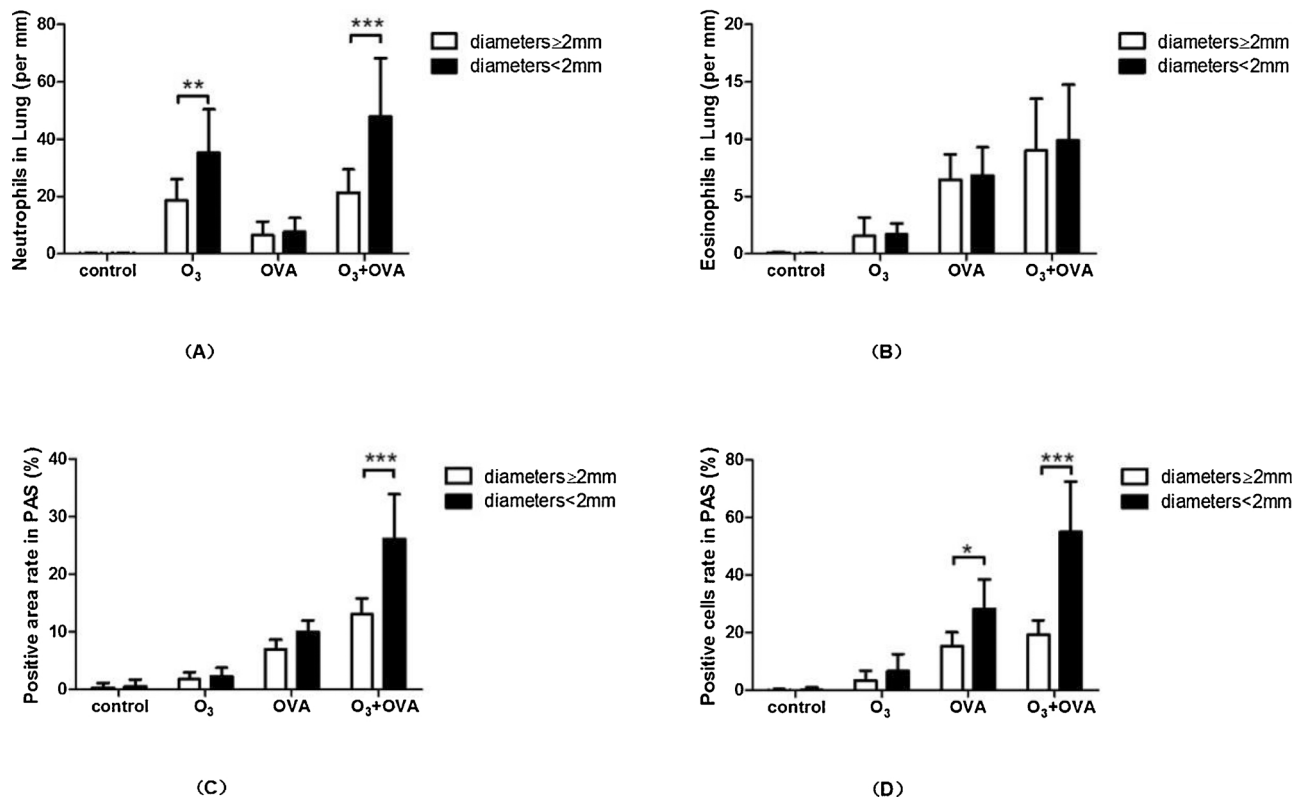


Fig. 5. Neutrophils positive area rate (Panel A), eosinophils (Panel B), Periodic Acid-Schiff (PAS) (Panel C), and PAS positive cells rate (Panel D) in airways less than 2 mm in diameter and in airways greater than 2 mm in diameter from four groups. Data are expressed as mean $\pm$ SD. * P < 0.05, **P < 0.01, ***P < 0.001. OVA: ovalbumin; O₃: ozone; SD: standard deviation.

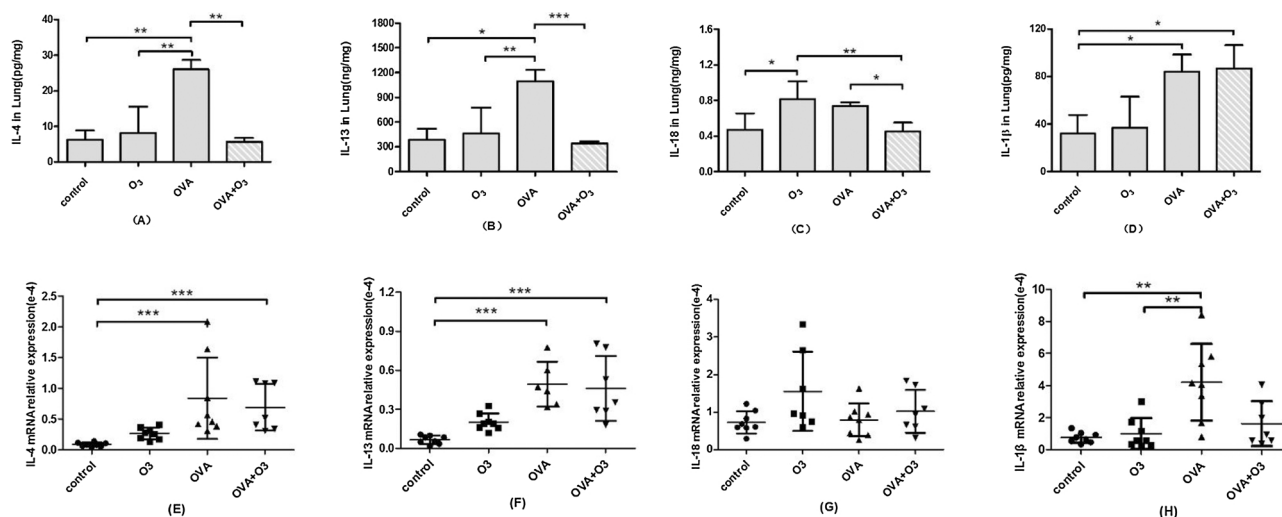


Fig. 6. Cytokine protein (Panel A–D, measured by enzyme-linked immunosorbent assay) and mRNA (Panel E–H, measured by real-time quantitative PCR) expression levels in the lung tissue. IL-4 (Panel A and E), IL-13 (Panel B and F), IL-18 (Panel C and G), and IL-1β (Panel D and H). Data in Panel A–D are expressed as mean $\pm$ SD. Individual data, mean and SD are shown in Panel E–H. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. OVA: ovalbumin; O₃: ozone; IL: interleukin; SD: standard deviation.

confirmed in MDA after ozone exposures in OVA sensitized/challenged mice (Fig. 7, Panel B).

3.8. Effect of OVA sensitization/challenge and ozone exposures on GSH concentration and GSH1 mRNA expression

Compared with negative control group, GSH concentration of ozone groups decreased significantly ($P < 0.001$; Fig. 8, Panel A). Although a similar down-regulation was induced by OVA sensitization/challenge, it is statistically insignificant between OVA treated group and negative control group. Combination treatment showed no influence in GSH concentration, compared to OVA- or ozone-treated group.

In contrast, an increase of GSH1 mRNA expression obviously induced by ozone exposures, compared with negative control group ($P < 0.001$; see Fig. 8, Panel B). No significant influence of OVA sensitization/challenge on GSH1 mRNA expression was confirmed in our study.

Taken together, ozone exposures caused an increase of oxidative stress, consumption of GSH, and up-regulation of GSH1, which is the enzyme synthesizing the anti-oxidative GSH. The up-regulation of GSH1 maybe act as a protective feedback response in the glutathione metabolism to the oxidative stress induced by ozone exposures.

4. Discussion and conclusions

In this study, we investigated the potential effect of ozone exposures on airway/lung inflammation, AHR, mucus secretion, oxidative stress, and pro-inflammatory cytokines in an OVA sensitization/challenge asthmatic model. AHR in OVA/ozone combined treated asthmatic mice was not obviously increased compared with OVA group. Although, the increase of neutrophilic inflammation induced by ozone exposure was not statistically significant in asthmatic mouse, a different of neutrophilic distribution between small airways and central airways was demonstrated in OVA/ozone combined treated animals. We further found increased mucus overproduction after ozone exposures in OVA-induced asthmatic mice, which was also mainly around small airways. Neutrophilic inflammation and mucus hypersecretion were complied with a markedly lower levels of Helper T cell (Th2)-type cytokines (IL-4 and IL-13) in lung tissue, and an obviously up-regulated of 8-OHdG, indicating ozone exposures can induce oxidative stress, DNA damage, and immune dysregulation in asthmatic mice.

It has been noted that changes in airways reactivity and inflammation/injury in response to ozone are not always codependent in rodents [30] or in human subjects [31,32]. Airway reactivity showed no further increase, but significant decrease after ozone exposures in OVA sensitized/challenged mice in our experiment. Similar results were

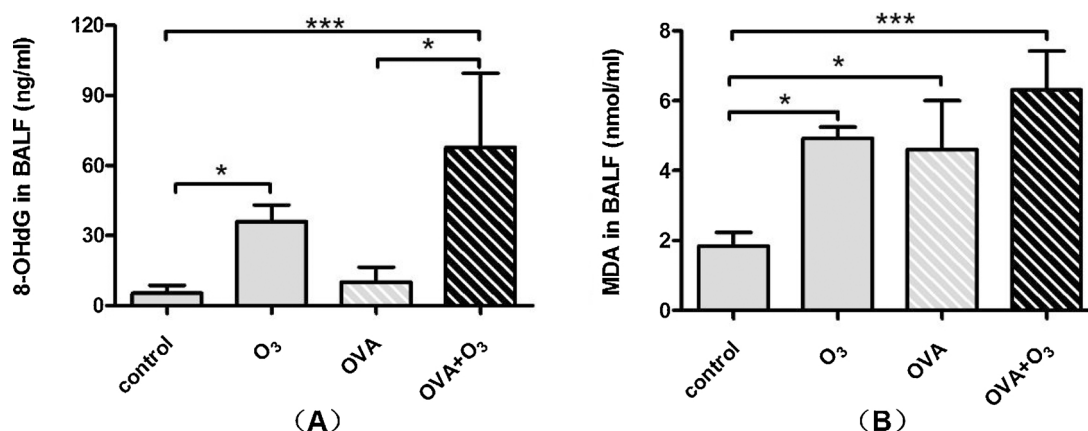


Fig. 7. 8-hydroxy-2'-deoxyguanosine (8-OHdG, measured by enzyme-linked immunosorbent assay) (Panel A) and malondialdehyde (MDA, measured by thiobarbituric acid assay) (Panel B) concentrations in bronchoalveolar lavage fluid (BALF). Data are expressed as mean $\pm$ SD. Individual data, mean and SD are shown in Panel F–J. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. OVA: ovalbumin; O₃: ozone; IL: interleukin; BALF: bronchoalveolar lavage fluid; SD: standard deviation.

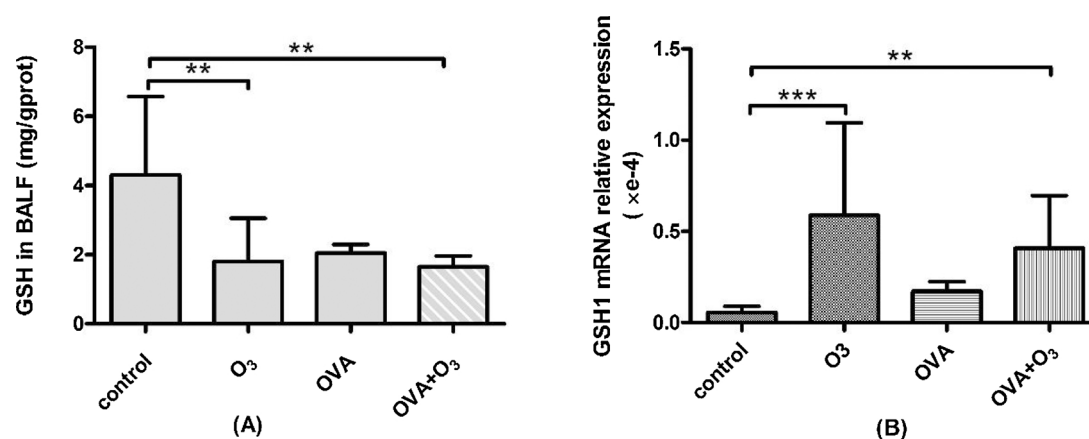


Fig. 8. Glutathione concentrations (GSH, measured by spectrophotometric method) in bronchoalveolar lavage fluid (BALF) (Panel A) and GSH 1 mRNA relative expression levels in lung tissue (Panel B, measured by real-time quantitative PCR). Data are expressed as mean $\pm$ SD. ** P < 0.01, *** P < 0.001. OVA: ovalbumin; O₃: ozone; GSH: reduced glutathione; BALF: bronchoalveolar lavage fluid; SD: standard deviation.

found in a study confirming acute effects of ozone exposure on lung function in mice sensitized and challenged to OVA [33]. A possible reason of the reduction of airway resistance and airway reactivity to inhaled MCH is mucus overproduction after ozone exposure, which maybe blocks transmission of MCH from airway to smooth muscles, but scant literature is available to support this point [33]. A wire myography used to examine bronchial responses of isolated bronchial rings in vitro is needed to eliminate the influence of mucus. Further, airways reactivity to allergen is strain-dependent [30]. C57BL/6 mice used in current two studies have greater eosinophilic inflammation, mucous cell metaplasia, and expression of genes related to type 2 immunity and airway mucus hypersecretion, but lower acetylcholine reactivity at baseline and after OVA sensitization and challenge, especially in response to inhaled MCH, as compared to similarly exposed BALB/c mice [30,34,35]. Further investigations on different strain should provide insight to the role of ozone on airway reactivity.

Mucus hypersecretion by hyperplastic goblet cells is another pathophysiological feature of asthma causing severe narrowing of the airways and contributing to the morbidity and mortality of asthma [36,37]. A growing body of evidence in experimental animal models supports that ozone exposure is also responsible for mucus overproduction and epithelial damage [38,39]. Repeated short exposures of ozone induced an increase in airway mucus secretion, but the highest degree of hypersecretion was seen when OVA sensitized/challenged mice were exposed to ozone in our study. Similar results were observed in mice and respiratory epithelium exposed to a combination of OVA and lower concentration of ozone [38,39]. The increases in goblet-cell metaplasia and mucus hypersecretion were found 24 h after exposure to OVA and 100 or 250 ppb ozone, whereas a higher concentration of 500 ppb ozone induced just mucus hypersecretion, without goblet-cell metaplasia in OVA exposed mice. These data indicated that high concentration of ozone triggered release of mucin from the goblet cells most likely due to a toxic effect of ozone on the goblet cells of OVA pre-treated mice, rather than goblet-cell metaplasia [39].

The cytokines, IL-4 and IL-13, are regarded as indicators of Th2 responses in pulmonary inflammation and play important roles in asthma by regulation of inflammatory cells' infiltration, immunoglobulin (Ig)E switching, chemokine's release, and mucus hypersecretion in the bronchiolar epithelial region [40–43]. As expected, the present study demonstrated increased eosinophilic levels in BALF and lung tissue, accompanied by significant high levels of IL-4 and IL-13 expression in the lung tissue of mice after OVA sensitization and challenge. There is strong evidence of the pathogenic role of Th2 cytokines orchestrating the eosinophilic and allergic inflammatory processes [44–48].

The elevations of IL-13 and IL-4 expression were inhibited in OVA

sensitized/challenged mice after repeated short ozone exposures. Instead, levels of IL-1 β remained high. There is a growing body of evidence to suggest a role for NLRP3 inflammasome and its products, pro-inflammatory cytokines IL-1 β and IL-18, in the inflammation of asthma patients [49–52]. The processing and subsequent release of IL-1 β and IL-18 via caspase 1 is dependent on LPS-mediated priming of NLRP3 and NLRP3 Inflammasome activation [53]. However, the regulatory mechanisms controlling IL-18 and IL-1 β expression are distinct. IL-18 is constitutively expressed in the blood monocytes, macrophages, dendritic cells, endothelial cells, intestinal epithelial cells, and keratinocytes with a cellular pool already existing before an inflammatory stimulus, which is ready to be activated and released by the inflammasome [54]. Unlike IL-18, IL-1 β is only induced in response to stimulation with TLR ligands and other cytokines (e.g., TNF) [54]. Significant increase of IL-18 protein levels after ozone exposure and significant upregulation of IL-1 β protein and mRNA levels after OVA sensitization/challenge were found in our study, indicating IL-1 cytokines involved in inflammation induced by OVA sensitization/challenge and ozone, which were possibly regulated by different inflammatory signals. Qifang Zhu et al found that IL-18 and IL-1 β can be induced by various TLR ligands and controlled by distinct regulatory pathway, even if the exact mechanisms remain unclear [54]. While we observed no detectable changes in levels of IL-18 mRNA expression and those of IL-1 β protein and mRNA expression, significant decrease of IL-18 protein was proved from lung tissue in the combination compared to OVA alone. Low levels of sputum supernatants [55] and serum [56] IL-18 protein were also found in patients with severe refractory asthma. The possible reason is cellular pool of IL-18 exhaustion, which will be investigated in our future work with assessment of caspase-1 activation, pro-caspase-1, NLRP3, and immunoblot analysis of pro-IL-18 and pro-IL-1 β .

Small airways dysfunction has been related to the occurrences of asthma, disease control, severity of symptoms, and especially the risk of exacerbation [57–61]. Its underlying pathophysiology remain to be clarified. Although inflammation was seen around the bronchus in our study, the major change in inflammation and PAS staining was around small airways. Higher density of neutrophils was proved around small airways after ozone exposure, compared with central airways. Higher elevation of mucus production was also found around small airways of combined treated group, compared with central airways. Our study provides evidence of small airway involvement in ozone induced exacerbation of asthma, which may be independent of AHR. Investigation of Neutrophilic inflammation, mucus hypersecretion, and goblet cell metaplasia in small airway of asthmatic model will be involved in our ongoing work to get more complete comprehension of ozone's influence in asthmatic patients.

There are several limitations of the current study. First, without spirometric assessments, we were unable to examine the presence of airflow obstruction, which is an important consequence of airway mucus hypersecretion. Moreover, it should be noted that the concentration of ozone tested, 3ppm, exceeds the average levels of ozone tested in urban environments and in the workplace. Further studies using lower concentrations are warranted to imitate the actual environmental conditions in which asthma patients live and fully define a role for ozone in asthma inflammation and bronchial responsiveness. In addition, ELISA analysis was run on different plates. A standard curve was generated with each run. As the standard is prepared separately every time if there are minor differences in the preparation of reagents used, the values received can be different. The only way to control for plate-to-plate variability is to run the same sample on all plates used and to show that the quantitation is the same on all plates, which were not involved in this experiment, unfortunately. The protocols of ELISA analysis will be updated accordingly. Further, the exact mechanisms of stimulated mucus hypersecretion after ozone exposures in OVA sensitized/challenged mice are still unclear. The involvement of oxidative stress and neutrophil serine proteases is just a hypothesis. Possible relationship of neutrophil serine proteases, oxidative stress, and epithelial function should be investigated in the future. Finally, we only used male mice in this study and further studies using female mice are planned.

In conclusion, OVA sensitization/challenge induced an obvious AHR and airway inflammation, which are typical characteristic changes of asthma. Our results provided the evidence that repeated short ozone exposures increased mucus hypersecretion, possibly via an increase in oxidative stress and a mixed Th2/Th17 response. Ozone could increase the risk of sensitization and the responses to inhaled allergen in airway epithelium of individuals with asthma. AHR to MCH was decreased following the combination exposure of ozone and OVA. In OVA sensitized/challenged and ozone exposed mice, AHR is not an accurate indicator to judge the severity of airway damage. Airway inflammation and epithelial mucus hypersecretion are more noticeable. Consequently, identification of potential drug targets for treatment of airway neutrophilic inflammation and hypersecretion in asthma exacerbation is warranted to avoid influence of air pollutions such as ozone. In view of this evidence supporting increased sensitivity to ozone exposure in asthmatics over that observed in non-asthmatic subjects, individuals with asthma should be urged to avoid unnecessary outdoor activity and to stay indoors on days when ozone levels are forecasted to be high. Policy makers' efforts to reduce air pollution and to improve air quality are also necessary for prevention of asthmatic exacerbations.

Author contributions

Wuping Bao and Xin Zhou conceived and designed entire study.

Wuping Bao, Yingying Zhang, and Min Zhang performed the experiments.

Xia Fei and Xue Zhang contributed to cell counts and data collection.

Aihua Bao was involved in interpreting bronchial provocation tests and histological data.

Wuping Bao and Yingying Zhang wrote the manuscript supervised by Xin Zhou.

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Conflict of interest

All authors declare that there are no conflicts of interest.

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