Original Research Article

Ozone exposure induces cough hypersensitivity in mice

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Abstract

Purpose: *To study the influence of O_3 exposure on cough sensitivity, airway barrier function and airway inflammation in mice.*
Methods: *Cough sensitivity was determined in healthy male C57/BL6 mice (aged 8 - 10 weeks) which were exposed to different concentrations of O_3 (0.5 - 2 ppm) for 3 h daily for 9 days. Hematoxylin and eosin (H&E) staining of lung tissues, collection of BALF, and cell count were carried out. Inflammatory factor levels in pulmonary tissues were determined by enzyme-linked immunosorbent assay (ELISA), while Western blotting was used to assay TRPA1 and Claudin-1 protein expressions in lung tissues.*
Results: *After 9 days of mice exposure to O_3, cough sensitivity increased significantly, and TRPA1 protein was increased in pulmonary tissues, with exposure level of 1 ppm resulting in the highest level of TRPA1 protein expression. Claudin-1 expression in lung tissues of mice decreased after O_3 exposure, especially in the groups exposed to O_3 levels of 0.5 ppm and 2 ppm. The total cell count in alveolar lavage fluid of mice exposed to O_3 was significantly increased (p < 0.05). In addition, O_3 exposure increased IL-1α, IL-6 and TNF-α levels, with the most significant increase in the 0.5 ppm group (p < 0.05). Results from histology revealed that all mice had inflammatory reactions and destruction of lung tissues after O_3 exposure.*
Conclusion: *Exposure to O_3 induces disruption of airway barrier function, infiltration of the airway by inflammatory cells, and increased secretion of inflammatory factors, thereby resulting in enhanced cough sensitivity.*

Keywords: *Ozone, Cough hypersensitivity, Inflammatory cells, Airway barrier, Cytokines*

INTRODUCTION

There is growing evidence that air pollutants such as particulate matter (PM), ozone (O_3), nitrogen dioxide (NO_2), and environmental tobacco smoke (ETS), cause harmful effects on the human respiratory system. The concentration of O_3 pollutants, an atmospheric pollution produced rapidly in polluted air by photochemical oxidation of nitrogen oxides and volatile organic compounds, increases significantly in dry and hot climates. In the last 10 years, during the warmer seasons, the maximum daily 8-hour average concentration of O_3 in China has shown an annual increase of about 5%, which is higher than the corresponding figures in the rest of the world. In Guangdong and Jiangsu Provinces, the population that died from ozone-related respiratory diseases increased by about 41 and 17 % per 100,000, respectively [1-3]. Due to its

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strong oxidizing properties, O_3 inhalation causes airway and lung damage resulting in a severe inflammatory state characterized by accumulation of neutrophils and macrophages in the lungs. Ozone inhalation has been shown to cause lung inflammation, exacerbate lung disease, and increase susceptibility to pathogenic infections [4].

Another major component of urban air pollution is PM. In particular, $PM_{2.5}$ is associated with many respiratory diseases, including bronchitis, chronic cough, asthma, and even lung cancer. Various pro-inflammatory cytokines, i.e., interleukin 1 (IL-1), IL-6, IL-8, and tumor necrosis factor (TNF-α), are transiently or continuously secreted when different cell types are exposed to $PM_{2.5}$ [5]. In addition, $PM_{2.5}$ causes some other cytological effects such as oxidative stress damage, cellular aging and excessive apoptosis [6]. Therefore, $PM_{2.5}$ and O_3 may cause airway inflammation and cellular damage through airway irritation, leading to cough hypersensitivity, which in turn is associated with the development of chronic cough. This research was aimed at investigating the influence of ozone exposure on cough sensitivity, airway barrier function and airway inflammation in mice.

EXPERIMENTAL

Animals

Healthy male C57/BL6 mice at 8 - 10 weeks of age were kept at 20 °C and 40 – 60 % relative humidity in SPF animal housing conditions with *ad libitum* supply of feed and H_2O. All mice had access to sufficient light and sleep. The study received approval from the Animal Ethical Authority of the Affiliated Kunshan Hospital of Jiangsu University, China, *vide* approval no. 201806335. All animal experiments and procedures conformed with the guidelines of the US NIH [7]. The animals were randomly assigned to 4 groups that received different concentrations of ozone (0.5 - 2 ppm): control group, 0.5, 1, and 2 ppm groups (6 mice/group). The mice in different groups were placed in customized containers where ozone was generated by an ozone generator. Ozone concentrations were monitored in real-time using an ozone monitor (Model 205, 2B Corporation, USA). Control group was housed in an environment equipped with an air purifier.

Determination of cough sensitivity

The number of coughs in mice was recorded using the Buxco Small Animal Whole Body Volume Tracing System (DSI, 601-1400-001 Rev13), and it was analyzed using FinePointe software (DSI, 007898-001 Rev03). Citric acid (Sigma, C2404, 0.4 M); capsaicin (Sigma, M2028, 0.01 mM), and saline were used to stimulate cough. A nebulizer (Aerogen Pro) was used to deliver 1 mL of cough provocation solution over 10 min.

### Staining of lung tissue and collection of BALF

After mice were intraperitoneally anesthetized with pentobarbitone (50 mg/kg). The airway was exposed, an in-dwelling needle endotracheal tube was inserted, and the right lung was ligated for H&E staining. To obtain BALF, the left lung was flushed thrice with saline, each time with 0.6 ml. The BALF collected was centrifuged and the supernatant was kept at -80℃. Then, the cells were re-suspended in 300 µL of erythrocyte lysate and left at laboratory temperature for 5 - 10 min. Following centrifugation, the precipitate was solubilized in PBD (1 mL). Then, 10 µL of the cell suspension was subjected to cell counting. An appropriate volume of cell suspension was evenly coated on slides and stained with Giemsa, and the percentages of macrophages, neutrophils and lymphocytes were calculated.

Determination of cytokines in the supernatant of lung tissue homogenates

The mice were anesthetized and sacrificed within 24 h after the end of O_3 exposure, and the lungs were excised. Lung tissue was ground using a high-throughput tissue grinder, and the lung tissue homogenate was subjected to centrifugation. The supernatant was kept in clean EP tubes. The levels of cytokines, i.e., TNF-α, IL-6, IL-1α, IL-25 and IL-33, as well as superoxide dismutase (SOD), and thymic stromal lymphopoietin (TSLP), were measured using the corresponding ELISA kits.

Western blot assay

The expressions of TRPA1 and Claudin-1 in lung tissue were assayed. Total protein was obtained using RIPA lysis buffer tainted with protease inhibitor cocktail. After quantifying the protein levels in the supernatants with BCA procedure, the proteins were subjected to SDS-PAGE, followed by electro-transfer to PVDF membranes which were sealed with 3 % BSA. Thereafter, the membranes were overnight at 4 °C with 1° antibodies, followed by 1 h incubation with HRP-linked 2° immunoglobulin. Blot analysis was done with ECL kit, followed by imaging with CL-XPosure film.
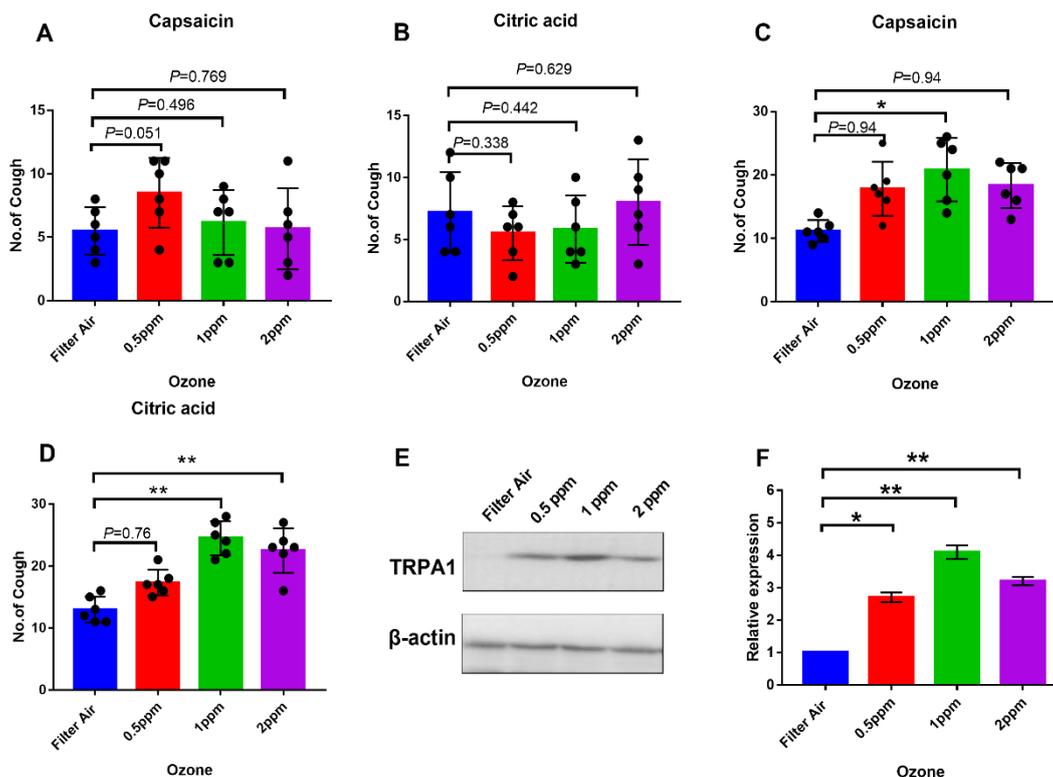
Figure 1: Changes in cough sensitivity of mice after ozone exposure. Mice cough sensitivity induced by capsaicin and citric acid before ozone exposure (A and B). The changes in the cough sensitivity of mice after ozone exposure (C and D). The expression level of TRPA1 in lung tissue (E and F). *$P < 0.05$, **$p < 0.01$, *vs.* filtered air

Immunofluorescence

The tissue sections were cooled in repair reagent at laboratory temperature for 30 min and rinsed thrice in PBS, followed by incubation in goat serum for 1 h at 37 °C. The sections were incubated with primary antibody overnight at 4°C, and then incubated 5 times in PBS, with each incubation lasting for 5 min. The secondary antibody was diluted 1:1000 added to the well plate, and incubated for 1h at room temperature in the dark. Thereafter, DAPI was diluted 1:1000 with PBS. The nuclei were stained for 2 min, and then the well plate was washed 3 times with PBST. Then, the plates were sealed with anti-quenching agent, fixed, and examined under laser confocal microscopy.

Statistical analysis

Data were statistically analyzed using SPSS 23.0. All data are presented as mean ± standard deviation (SD). One-way ANOVA was used for independent comparison between groups, and Tukey's test was applied for multiple comparisons. Statistical significance was fixed at $p < 0.05$.

RESULTS

Effect of acute ozone exposure on cough sensitivity and expression of cough receptors

In this study, capsaicin and citric acid were used as cough stimulants, and the baseline levels of cough sensitivity were assessed in mice in which different cough stimulants were used to induce cough before the mice were exposed to experimental ozone. Cough sensitivity of mice was evaluated in the same manner at the end of ozone exposure. The frequency of cough was significantly increased in the 1 ppm/group when compared to the filtered air group under capsaicin excitation (Figure 1 A and B; $p < 0.05$, filtered air vs. 1 ppm). The frequency of cough tended to increase when the ozone concentration was raised to 2 ppm, but it was not statistically significant (Figure 1 C; $p > 0.05$, filtered air vs. 2 ppm). The classical cough receptor, TRPAI, has citric acid as its ligand, and there was marked accentuation of cough frequency in mice exposed to ozone at doses of 1 and 2 ppm, relative to mice given filtered air (Figure 1 D, $p < 0.05$). Results from Western blot assay showed that different concentrations of ozone exposure produced different expression levels of TRPA1

protein in mouse lung tissues. The highest expression level of TRPA1 protein was seen at ozone dose of 1 ppm, which was statistically significant when compared to the TRPAl expression level in filtered air group (Figures 1 E and F). These data indicate that ozone enhanced the expression of cough receptor TRPA1 in mouse lung tissue, and it elevated cough sensitivity in mice.

Effect of acute ozone exposure on lung tissue barrier function

The airway barrier formed by tight junction proteins is used as the first line of protection in organisms exposed to ozone. Immunofluorescence results showed a decrease in lung tissue Claudin-1 protein expression after ozone exposure (Figure 2 A). Western blot results revealed that Claudin-1 protein expression in pulmonary tissue after ozone exposure was reduced to different degrees, with significant decreases in Claudin-1 protein expression at 0.5 ppm and 2 ppm, relative to mice that received filtered air (Figures 2 B and C, $p < 0.05$). However, Claudin-1 protein levels in the 1 ppm group and the filtered air group were comparable $(p > 0.05)$.

## Effect of ozone on cell counts and cell sorting in BALF of mice after acute exposure

The total number of cells in the alveolar lavage fluid was significantly higher in ozone-exposed mice than in filtered air mice (Figure 3 A; $p <$ 0.05). Cell sorting in the alveolar lavage fluid

showed a marked reduction in population of macrophages in the ozone-exposed mice, relative to the filtered air mice (Figure 3 B; $p <$ 0.05). A sharp increase in population of neutrophils was seen in the ozone-exposed groups, relative to the filtered air group (Figure 3 C; $p < 0.05$). However, the numbers of lymphocytes and eosinophils were comparable amongst the various groups (Figures 3 D and E).
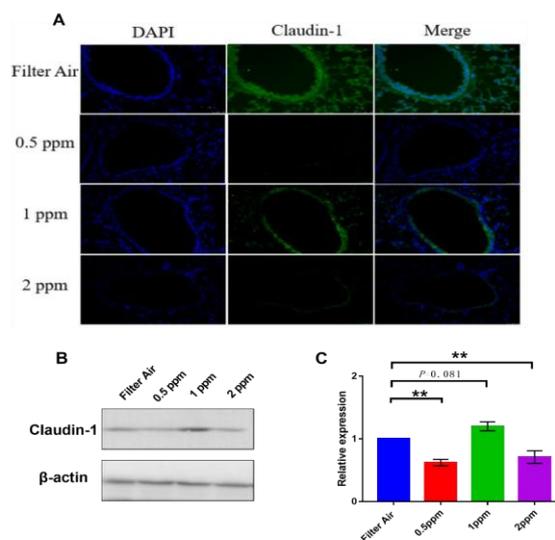
Figure 2: Ozone exposure inhibited Claudin-1 protein expression. Claudin-1 protein expression by immunofluorescence (A). Claudin-1 protein expression by WB (B and C). Scale =200 um. **$P < 0.01$, compared with filtered air
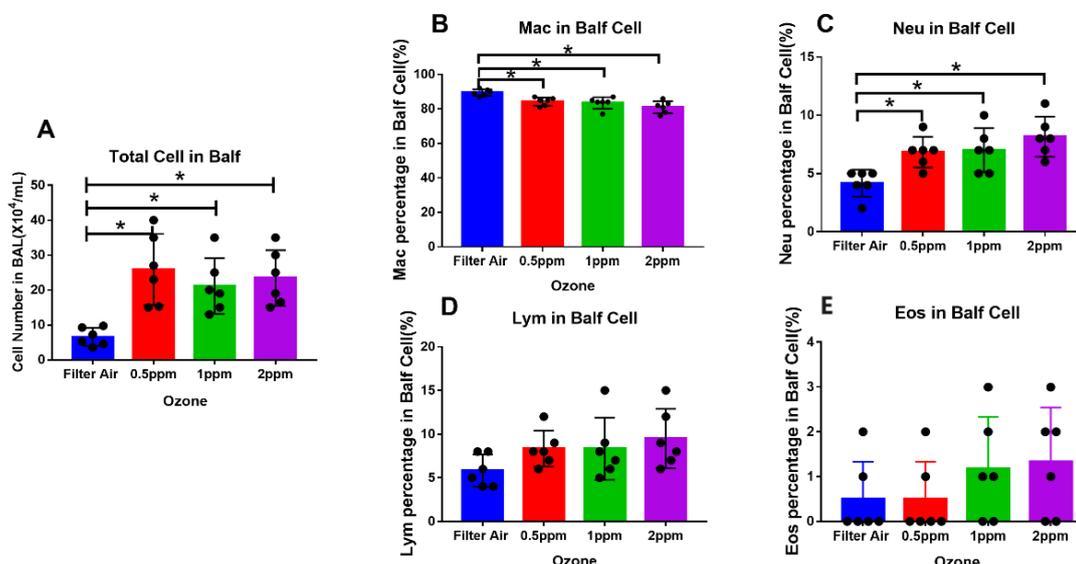
Figure 3: Effect of ozone exposure on inflammatory cells in alveolar lavage fluid. The total number of inflammatory cells in BALF (A). The proportion of macrophages in BALF (B). The proportion of neutrophils in BALF (C). The proportion of lymphocytes in BALF (D). The proportion of eosinophils in BALF (E). Mac = macrophage; Neu = neutrophils; Lym = lymphocyte; Eos = eosinophils. *$P < 0.05$, compared with filtered air
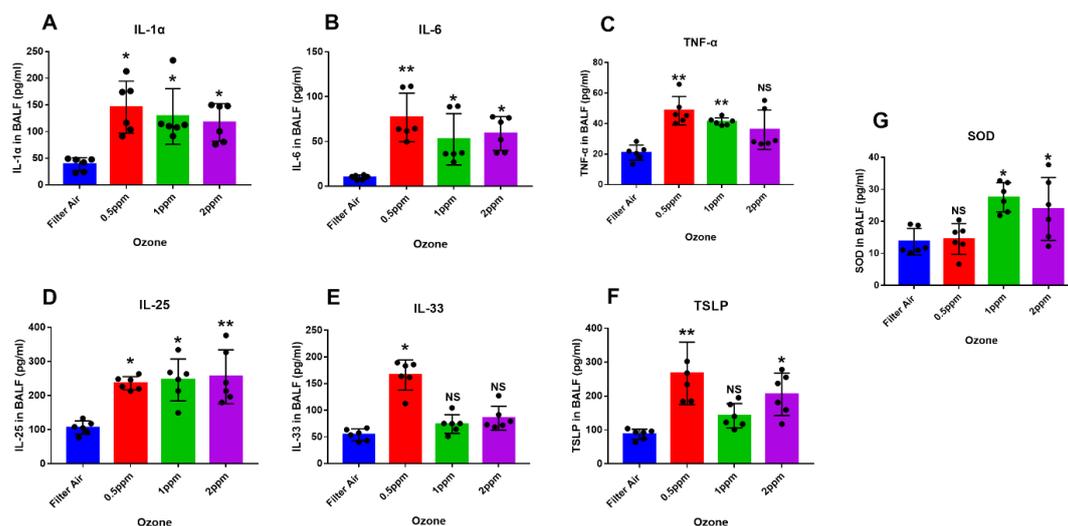
Figure 4: Ozone exposure induced changes of cytokine expression in lung tissue. The levels of IL-1α, IL-6 and TNF-α in lung tissue (A-C). The levels of IL-25, IL-33 and TSLP in lung tissue (D-F). SOD level in lung tissue (G). *$P < 0.05$, **$p < 0.01$, compared with filtered air

Changes in levels of inflammatory factors in mouse lung tissue after ozone exposure

Compared to the filtered air group, exposure to different concentrations of ozone resulted in different levels of elevations in expressions of IL-1α, IL-6 and TNF-α, with the most pronounced elevation in the 0.5 ppm group (Figure 4; $p < 0.01$). Ozone exposure resulted in concentration-dependent increases in IL-25 secretion when compared to filtered air group. There were significant increases in lung tissue secretion of TSLP in the 0.5 and 2 ppm groups when compared to the filtered air group ($p < 0.01$), but no significant difference was seen in relation to the 1 ppm group. The 1 and 2 ppm groups had significant increases in SOD levels, which to some extent, suggest the activation of antioxidant and anti-inflammatory mechanisms in lung tissue.

Inflammatory influence of ozone exposure on mouse lung tissues

Histology results revealed that after O_3 exposure, mice showed lesions in alveolar tissues, relative to filtered air group, as were evident in breakage of the tubular walls of the small airways, damage to the intact structure of the alveoli, and some degrees of inflammatory cell infiltration. These results suggest that the level of inflammatory cell infiltration increased along with damage to the lung tissues of the mice as the level of O_3 exposure increased. These results are presented in Figure 5.
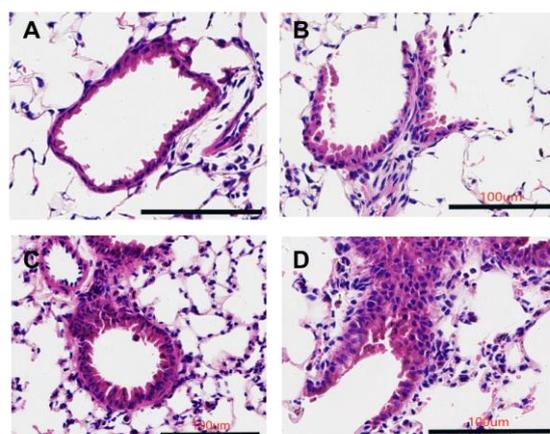
Figure 5: Ozone exposure induced damage in lung tissue and alveolar tissue. (A-D): filtered air, 0.5, 1 and 2 ppm ozone exposure groups of lung tissue, respectively). The small airway and alveoli of the filtered air group were intact and continuous. After ozone exposure, the normal structure of the alveolar tissue was damaged and inflammatory cells were infiltrated

DISCUSSION

The present study has demonstrated that O_3 exposure increased cough sensitivity in mice and also increased the expression level of the cough receptor TRPA1. A prominent characteristic impact of inhaled O_3 is airway hyperresponsiveness (AHR). The crucial role of MAPK/JNK signaling pathway in O_3-mediated mobilization of inflammatory cells, gene expression, and airway hyperresponsiveness has been reported in mice [8]. Moreover, the role of TNF-α receptors in airway hyperresponsiveness

has been reported [9]. Garantziotis *et al* reported that hyaluronic acid is an overall mediator of airway hyperresponsiveness in mice exposed to O_3 [10]. In a study on Rho kinase (ROCK)-mediated airway hyperresponsiveness in patients with allergic asthma, deficiency of ROCK1 or ROCK2 led to a decrease in airway hyperresponsiveness, with no noticeable impact on inflammatory response [11]. In response to oxidative insult, inflammatory conditions and low oxygen tension, activation of transient receptor potential (TRP) channels takes place, and it may be crucial in the etiology of airway disease [12]. It is known that TRPA1 and TRPV1 are mainly expressed in sensory nerves as well as in cells of the airway epithelia and smooth muscles [13]. The main airway stimulus-sensing receptor with the capacity to be activated in both hypoxic and hyperoxia conditions is TRPAI [14]. Acute O_3 exposure directly stimulates TRPA1 channels, which in turn activates broncho-pulmonary C fibers, and it is a vital trigger for inflammatory response in the airway, as well as bronchial hyperresponsiveness (BHR) [15]. Ozone (O_3) exposure increases cough sensitivity by increasing airway hyperresponsiveness, while increased levels of TRPA1 expression further increase airway hyperresponsiveness and enhance airway inflammation, resulting in a further increase in cough sensitivity in mice.

In this research, Claudin-1 protein level was significantly reduced after O_3 exposure, and the integrity of the airway barrier was disrupted to varying degrees in mice. The respiratory airway epithelium is the first line of defense in the formation of physical barrier and mucosal immunity [16]. Tight junctions and adherent junctions, mucus, surface-active proteins and cilia motility are essential for barrier control and intrinsic responses [17]. In addition, exposure to O_3 resulted in increased proliferation of airway epithelial cells, which may be a direct result of oxidative damage to epithelial cells [18]. The disruption of tight junctions and leakage of epithelial cells allow the entry of irritants, pathogens and allergens into the respiratory system, leading to sustained inflammation reactions in the epithelial cells [19]. The first phase of O_3-induced airway epithelial injury involves the direct disruption of the fine bronchial cellular boundary of the epithelium, causing cellular stress, exfoliation and mortality. The second phase of O_3-induced airway epithelial injury immediately follows the first phase for fine bronchial epithelial damage and cell death, including protein leakage and neutrophil aggregation, ROS-expressing myeloid cells, and production of cytokines [20]. Therefore, O_3 causes a biphasic response during epithelial cell injury, i.e., direct damage through ROS and

granulocyte migration, and secondary damage. Indeed, the second phase of damage is attenuated by the production of anti-granulocyte antibodies by neutrophils. Other factors that pollute the air, e.g., NO_2, $-S_2$, particulate matter (PM) and sundry chemicals, have been shown to cause similar damage to the respiratory barrier as O_3, resulting in chronic lung disease, although often with prolonged duration of contact, and at greater levels than O_3 [21]. In addition, this study has shown that IL-33 secretion levels were significantly higher in the group exposed to O_3 at a dose of 0.5 ppm than in the filtered air group. Studies have demonstrated that ZO-1, Cld-4 and epithelial E-cadherin (eE-Cad) expressions were decreased in IL-33 deficiency. Moreover, eE-Cad was also down-regulated in IL-33/ST2 receptor-deprived mouse model, indicating that IL-33 has a protective role. At the transcriptional level, an early increase in Cld-4 or E-cadherin expression has been observed [20]. These results indicate that the IL-33/ST2 signal pathway exerts a defensive role. The IL-33-dependent protective pathway may be a defense mechanism in the early stages of O_3 exposure under physiological conditions.

It was found that the total number of cells in the alveolar lavage fluid after O_3 exposure was significantly higher than that in mice given filtered air: macrophages were severely reduced in number in O_3-exposed groups, while neutrophils were significantly increased. Moreover, various pro-inflammatory cytokines were increased in number to different degrees. These results suggest that O_3 exposure may enhance the development of respiratory inflammation. Acute inflammation helps to confine the damage to the affected site while mobilizing the immune system to accelerate the clearance of harmful stimuli. On the other hand, chronic inflammation 'summons' the immune cells to amplify the inflammatory response, thereby damaging normal tissues [22]. Studies have shown that O_3 exposure leads to impaired macrophage phagocytosis. In the present study, O_3 exposure reduced the number of macrophages in alveolar lavage fluid of mice. Many studies on the mechanism underlying reduced macrophage phagocytosis have focused on the interaction of O_3 with intra-airway fluids consisting of phospholipids, proteins and cholesterol, which are the first points of contact with O_3. Exposure to the reactive gas O_3 leads to oxidation of phospholipids and surfactant proteins, which in turn adversely affects cellular activity and host defense mechanisms [23]. For example, it has been reported that treatment of neutrophils with oxidized phospholipid 4HNE resulted in oxidative rupture and impaired phagocytosis [24]. Thus, O_3 exposure

accentuates airway inflammation, which is also an important mechanism involved in cough hypersensitivity syndrome.

The present experiments have also demonstrated that O_3 exposure increased the levels of cytokine expression in mouse lung tissue. It led to the release of IL-1α from alveolar macrophages, which in turn stimulated the secretion of a neutrophil chemotactic agent, cytokine-induced neutrophil chemotactic agent 1, from alveolar epithelial cells [25]. This effect of neutrophil chemotaxis due to pro-inflammatory cytokines further exacerbated the airway inflammatory response. The pleiotropic cytokine IL-6 is produced by a variety of cell types in response to a broad range of inflammatory stimuli [26]. The epithelium-derived cytokines IL-25 and IL-33 constitute important epithelial responses to foreign stimuli and a crucial defense against airway damage. Superoxide dismutase (SOD) is a vital cytoprotective and antioxidant factor.

This research has demonstrated that in the O_3-exposed group, SOD expression level was significantly increased, most likely as a defense mechanism against oxidative stress, and a strategy for the activation of anti-inflammatory mechanisms. Repeated exposure to low doses of O_3 resulted in a chronic oxidative stress state due to increased levels of ROS and reduced response from antioxidant defense systems.

CONCLUSION

This research has demonstrated that O_3 increases cough sensitivity, enhances TRPA1 expression, and impairs airway barrier function in mice. It causes damage and destruction of lung and alveolar tissues. Moreover, O_3 elevates the levels of inflammatory cell infiltration and stimulates cytokine secretion in mouse lung tissue. This study contributes to the understanding of the mechanisms associated with respiratory diseases induced by air pollution.

DECLARATIONS

Acknowledgements

Ethical approval

The study approved by the Animal Ethical Authority of the Affiliated Kunshan Hospital of Jiangsu University, China (approval no. 201806335).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was performed by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Shu Zhang and Xuemei Liu analyzed the data. Tinglei Li, Tianyuan Xin and Yu Chen interpreted the data and prepared the manuscript for publication. Zhe Chen supervised the data collection, analyzed the data and reviewed the draft of the manuscript. Tinglei Li, Shu Zhang, Xuemei Liu, contributed equally to the work.

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