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Acute exposure of ozone induced pulmonary injury and the protective role of vitamin E through Nrf2 pathway in Balb/c mice

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Running title: *Protective mechanism of VE in ozone induced pulmonary injury*

ABSTRACT

Ozone (O_3) in the lower atmosphere is generally derived from sources of human activity. It has become a major air pollutant in China and has been shown to adversely affect the health of humans and animals. We undertook a study to ascertain the molecular mechanism of ozone induced lung injury in mice and tried to illuminate the protective mechanism of Vitamin E. In this study, mice were exposed to clean air and three different concentrations of ozone. Oxidative stress (reactive oxygen species and malondialdehyde) and Th cytokines in lung, serum IgE, as well as histopathological examination and airway hyper-responsiveness (AHR) test were used to reflect inflammation and damage to the lungs of ozone-exposed mice. We then chose an effective concentration of ozone and combined treatment with vitamin E (VE) to explore the underlying mechanism of ozone-induced lung damage. The results of immunological and inflammatory biomarkers (total-immunoglobulin (Ig) E and Th cytokines) as well as histopathological examination and AHR assessment supported the notion that high doses of ozone (>0.5 ppm) could induce inflammation and lung injury in mice and that this induction was counteracted by concurrent administration of VE. The elimination of oxidative stress, the reduced Th2 responses and Ig production, and the relief of lung damage were proposed to explain the molecular mechanism of ozone induced lung injury. We also showed that VE, an antioxidant that enhanced the expression of Nrf2 and up-regulated the antioxidant genes HO-1 and NQO1, could decrease the levels of oxidative stress and alleviate ozone-induced lung injury.

Keywords: Ozone; oxidative stress; lung inflammation; airway hyper-responsiveness; vitamin E; Nrf2 pathway

INTRODUCTION

Ozone (O_3) is derived from important sources of human activity, such as operating motor vehicles, and the fuel and petrochemical industries. Ozone is a secondary air pollutant generated *via* a series of complicated photochemical reactions involving nitrogen oxides and sunlight (1). In 2008, the U.S. Environmental Protection Agency (EPA) updated the O_3 standard to 75 ppb (0.075 ppm) based on evidence that demonstrated deleterious health effects. On November 25, 2014, the EPA proposed to strengthen the standard of O_3, based on extensive scientific evidence about ozone's health effects. However, today millions of Chinese are exposed to ozone levels exceeding the recommended EPA limit. In Beijing for example, the annual number of non-attainment days for 1h O_3 were 57, 67, 70, and 76, in 2003, 2004, 2010, and 2012 respectively (2). In 2013, China's Environmental Protection Ministry reported that the averaged non-attainment rates for maximum 8h O_3 in 74 cities varied between 20% and 50% during the period of May to September, and that O_3 has become the second major air pollutant in many of China's cities, following PM2.5.

As a strong oxidant, O_3 is able to react with almost any biological tissue and induce adverse health effects in humans. When O_3 is inhaled, it causes airway inflammation which can lead to constriction of the airways, increased bronchial reactivity and decreased lung function, all of which pose a significant threat to respiratory health (3). In addition, exposure to increased levels of ozone has been associated with the worsening of symptoms in patients with obstructive lung disease such as asthma and COPD (chronic obstructive pulmonary disease) (4), and increased morbidity and mortality rates in cardiovascular and respiratory patients (5, 6). It has been estimated that if there were strict adherence to the established 8-hour O_3 standard each year, this would result in 800 fewer premature deaths, 900,000 fewer school absences and 4,500 fewer hospital admissions which in turn would save an estimated $5 billion annually (7).

However, while it is known that O_3 pollution poses potential health risks, the molecular mechanisms behind ozone-induced lung resistance and inflammation are still unclear. Evidence has suggested that oxidative stress in the lung in response to ozone-induced injury contributes to inflammation (8). When O_3 is inhaled, it becomes toxic by inducing peroxidation of polyunsaturated

fatty acids in membrane lipids and in lung lining fluid, giving rise to the generation of reactive oxygen species (ROS), and a mixture of lipid ozonation products (9). These products are capable of disturbing the redox balance and inducing inflammation, and hence leading to lung function damage (10). Thymic stromal lymphopoietin (TSLP) is an epithelial cell-derived cytokine that has a strong influence on the polarization of dendritic cells (DCs) that drive T helper (Th) 2 cytokines production, and also plays an important role in regulating Th1/Th2 immune balance (11). In addition, ROS orchestrated Th2 responses by inducing oxidized lipids that triggered the induction of TSLP by epithelial cells mediated by Toll-like receptor 4 (TLR4) and the adaptor protein TRIF (12).

Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a redox-sensitive transcription factor that plays an essential role in the promoter region of genes encoding antioxidant and/or detoxifying enzymes and related stress-responsive proteins (13). The small antioxidant enzymes, including NADPH, quinone oxidoreductase-1 (NQO-1), heme oxygenase-1 (HO-1) and γ-glutamate cysteine ligase (GCL), are regulated by Nrf2, responding to attenuate inflammatory damage and neutralize ROS of cells/tissues from inflammatory injuries (14). VE is a potent ROS-scavenging and chain-breaking antioxidant that may prevent the development of respiratory disease (15). Although vitamin E has previously been investigated in pulmonary disease, the lack of evidence for protection mechanism in previous studies limits conclusions concerning its role.

We hypothesized that O_3 causes the increase of ROS in lung, and then modulates Th-2 cell differentiation and lung inflammation, which eventually leads to lung tissue injury and lung function decline. To test our hypothesis, we first used three different exposure concentrations of ozone and analyzed ozone-induced lung injury by measuring the levels of oxidative stress and Th cytokines in the lung, the serum IgE, as well as performing a histopathological examination and an AHR (airway hyperresponsiveness) test. We next chose an appropriate exposure concentration of O_3 and combined treatment with VE in order to explore the molecular mechanism of ozone-induced lung damage. Furthermore, by detecting the expression of Nrf2 and the antioxidant genes HO-1 and NQO1 in lung tissue, we tried to explore the reason VE plays an antioxidant role and attenuates the effect of ozone-induced lung damage.

MATERIALS AND METHODS

The experimental protocols were approved by the Institutional Animal Care and Use Committee of Central China Normal University on March 1, 2012 (Ratification ID: CCNU-IACUC-2012-011).

Animals

Male Balb/c mice (5-6 weeks; approximately 20 g) were purchased from the Hubei Province Experimental Animal Center (Wuhan, China). They were maintained in pathogen-free cages at 24℃-26 ℃ and 55%-75% humidity with a 12h light-dark cycle. Mice were kept 5 mice/cage and provided *ad libitum* access to a commercial diet (Hubei Province Experimental Animal Center) and filtered water. The cages we used were independent ventilation cages (IVC); each cage being equipped with separate air inlet and outlet systems.

Main reagents and kits

Ozone was generated using a KTB portable ozone generator (Guangzhou, China) from ambient air with an ozone calibrator source. All other chemicals used were of analytical grade. Mouse enzyme-linked immunosorbent assay (ELISA) kits for measuring total IgE, IL-4, interferon (IFN)-γ, IL-5 and TSLP were from eBioscience (San Diego, CA, USA). To determine total protein, a Modified BCA protein assay kit was purchased from Sangon Biotech (Shanghai, China).

Experimental Design and Animal Exposure

To determine the effect of ozone exposure on mice, the experiment included 6 groups (n=5): (A) Control group, (B) VE (100 mg VE/kg) control group (intraperitoneal injection), (C) 0.1 ppm ozone exposure group, (D) 0.5 ppm ozone exposure group, (E) 1.0 ppm ozone exposure group, (F) 1 ppm ozone exposure and VE group. Mice were exposed to clean air or ozone (0.1ppm, 0.5 ppm, 1.0 ppm)

for 3 h/day, for seven days. VE was administered as an antioxidant 3 h after exposure to ozone (1 ppm). The detailed protocols are shown in Figure 1.

Figure 1

Experimental error in ascertaining the concentrations of oxidative stress-related biomarkers as well as Th cytokines and IgE concentrations due to the influence of MCH used in AHR assessments was avoided by conducting two rounds of this experiment. In the first round, after 7 days of ozone exposure, the 30 mice were used to measure Th cytokine concentrations and oxidative stress-related biomarkers in lung homogenates and serum IgE production. In the second round, another 30 mice were treated with the same protocol and then used directly for AHR tests and lung histological assays.

First round of testing

The aims of the first round were to ascertain: (i) if ozone exposure affects serum IgE production and lung inflammation in mice; (ii) if ozone exposure can cause oxidative stress and increase the TSLP level in the mouse lung; and (iii) to determine any antioxidant effect of VE on these biomarkers.

Quantitative analyses of total serum IgE

In the first round of experiments, 24 h after final exposure, the mice were anaesthetized with pentobarbital sodium (100 mg/kg bw, intraperitoneally). Serum samples were then collected from heart blood by centrifugation (3000 rpm for 10 min at room temperature). Serum concentration of Total-IgE was measured using ELISA kits according to the manufacturer's instructions.

Tissue sample preparation and ELISA

After serum collection, mice were killed by cervical dislocation and the whole lung was removed using medical scissors and rinsed in ice-cold phosphate-buffered saline (PBS). Lung tissue

was homogenized in a glass homogenizer on ice using 10 mL/g of ice-cold PBS at pH 7.5 to produce a 10% tissue homogenate. Then, the homogenate was centrifuged at 10,000 rpm for 10 min at 4 °C and the supernatant was collected for later assessment of levels of lung cytokines (IFN-γ, IL-4 and IL-5) and TSLP using ELISA kits according to the manufacturer's instructions.

Measurement of GSH and MDA content

The GSH test kits were provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All the operations were performed according to manufacturer's instructions. The MDA concentration in the lung tissue homogenate was measured using a previously described procedure (16). The protein concentration was determined using the Modified BCA Protein Assay Kit (Sangon Biotech, China).

Second round of testing

The aims of the second round were to ascertain: (i) if ozone exposure causes histological lung damage in mice; (ii) if ozone exposure can change AHR and (iii) to determine any protective effect of VE in lung histological assays and the AHR test.

Measurement of AHR

In the second round of experiments, 24 h after the final exposure, the other 30 mice were tested for AHR using the AniRes2005 Lung Function System (Bestlab ver2.0; Beijing, China) according to a previously described procedure (17).

Lung histological assay

After testing for AHR, the left lung was removed for preparation of histopathology slices. Samples were fixed in 10% formalin solution for 24 h at room temperature and cut into 5 μm slices of the distal pieces for H&E staining. The section was observed using the DM4000B microscope (Leica Microsystems GmbH, Wetzlar, Germany). The numbers of inflammatory cells in each sample

were counted using Image-Pro Plus software (Image-Pro Plus 6.0, Media Cybernetics). The histological scores were assessed by a pathologist who knew nothing of the origin of the sample.

Immunohistochemistry for IL-13, Nrf2, HO-1, NQO-1 and Mast Cell Tryptase

The sections of lung tissue were deparaffinized, rehydrated and subjected to antigen retrieval, then incubated with 0.3% hydrogen peroxide and blocked by appropriate normal serum. Immunohistochemical detection of IL-13, Nrf2, HO-1, NQO-1 and mast cell tryptase were performed using primary antibodies anti-IL-13 (1:100, Boster, Wuhan, China), anti-Nrf2 (1:100, Proteintech, Chicago, USA), anti-HO-1 (1:50, Proteintech, Chicago, USA), anti-NQO-1 (1:200, Proteintech, Chicago, USA) and anti-Mast Cell Tryptase (1:50, Abcam, MA, USA), respectively. After incubation in primary antibodies, the sections were sequentially incubated in an appropriate biotinylated immunoglobulins and avidin-biotin peroxidase complex. The reaction product was visualized using hydrogen peroxide (3%) and diaminobenzidine tetrahydrochloride (DAB, 5 mg/10 ml) (Sigma-Aldrich, St. Louis, MO, USA) as chromogen. The immunohistochemical control was obtained by omitting the primary antibody. All sections were finally counterstained with hematoxylin, dehydrated and mounted in DPX (Sigma-Aldrich).

Statistical Analyses

Data are presented as mean ± SEM. Statistical graphs were generated using GraphPad Prism 5.0 (San Diego, CA, USA). A one-way ANOVA combined with Fisher's protected t-test was used to determine the significance of differences between groups. $p<0.05$ was considered significant and $p<0.01$ was considered extremely significant. Data analyses were carried out using SPSS ver13 (SPSS, Chicago, IL, USA).

RESULTS

Effect of ozone exposure on serum levels of IgE and degranulation of mast cells

To evaluate the effect of ozone exposure on serum, we measured total-IgE in serum of mice. Figure 2 shows the total serum IgE data after 7 days ozone exposure. There were three important findings: (i) exposure to VE and 0.1 ppm O_3 did not cause changes in total serum IgE and degranulation of mast cells; (ii) the total IgE levels for the 0.5 ppm O_3, 1.0 ppm O_3 and 1.0 ppm O_3+VE exposure groups were significantly increased compared to those of the control group (Fig. 2A, $p<0.01$); Furthermore, 1.0 ppm O_3 exposure groups were significantly increased the degree of mast cell degranulation (Fig. 2B and C, $p<0.01$); (iii) compared with the 1.0 ppm O_3 group, the 1.0 ppm O_3+VE group showed a decreased level of total IgE and mast cell degranulation (Fig. 2, $p<0.01$).

Figure 2

Effect of ozone exposure on levels of Th cytokines in the lung

The levels of the Th1 cytokine IFN-γ, the Th2 cytokines IL-4, IL-5 and IL-13 were assessed in lung tissue samples (Fig. 3A, B, C and Fig. 4). Administration of VE did not result in significant changes in IFN-γ, IL-4, IL-5 and IL-13 levels. Exposure to O_3 (0.1 ppm O_3, 0.5 ppm O_3, 1.0 ppm O_3 and 1.0 ppm O_3+VE groups) lead to rapid drops in the levels of IFN-γ compared with the control group (Fig. 3A, $p<0.01$). The protein levels of IL-4, IL-5 and IL-13 in the 1.0 ppm O_3 group were significantly greater than those in the control group (Fig. 3B, C and Fig. 4, $p<0.01$). Administration of VE combined with 1.0 ppm O_3 exposure led to a significant decrease in the levels of IL-4, IL-5 and IL-13 compared with the 1.0 ppm O_3 group (Fig. 3B, $p<0.05$; Fig. 3C and Fig. 4, $p<0.01$).

Given that TSLP is an important cytokine produced by epithelial cells and has been linked to ROS and inflammation, we measured TSLP expression in the lung after ozone exposure (Fig. 3D). The VE and low ozone exposure groups (0.1 and 0.5 ppm) did not show changes in TSLP levels compared with the control group. The TSLP levels in the 1.0 ppm O_3 group were significantly higher than those of the control group ($p<0.05$). The 1.0 ppm O_3 exposure plus VE group showed a strong alleviating effect on the results of TSLP ($p<0.01$).

Figure 3, Figure 4

Ozone effects on oxidative stress in mice lungs and antioxidation of VE

To evaluate the level of oxidative stress after ozone exposure, we measured GSH and MDA in the lung. In the 1.0 ppm O_3 group, the levels of GSH and MDA were significantly increased compared to those of the control group (Fig. 5A, $p<0.05$; 5B, $p<0.01$); Compared with the 1.0 ppm O_3 group, the 1 ppm O_3+VE group showed significantly decreased levels of GSH and MDA (Fig. 5, $p<0.01$).

Figure 5

Effect of ozone exposure on histological changes

To evaluate histological changes, we carried out hematoxylin and eosin (H&E) stains 24 h after the final O_3 exposure (Fig. 6). Exposure to VE and 0.1 ppm O_3 did not lead to significant pathologic alterations. The 0.5 and 1.0 ppm O_3 groups caused inflammatory cell infiltration, bronchial remodeling (Fig. 6 A4 and A5; B, $p<0.05$, $p<0.01$). In addition, as the concentration of O_3 increased, the aggravation effect was stronger. Furthermore, the 1.0 ppm O_3 exposure plus VE group showed significant attenuated these adverse effects (Fig. 6 A6; B, $p<0.01$).

Figure 6

Effects of ozone exposure on AHR in mice and the ameliorating effects of VE

Ozone treatment followed by a methacholine (MCH) challenge assay produced an increase in the R-areas of respiratory resistances (Re and Ri, respectively) and a decrease in dynamic lung compliance (Cldyn) (Fig. 7A-C), which supported the validity of this mouse model. Continuous upward shifts of the Ri and Re curves and downward shifts of the Cldyn curves were detected as the O_3 concentration increased from 0.1 to 1.0 ppm, suggesting that O_3 (especially 1.0 ppm O_3) adversely affected both large and small airways of the lung. Treatment with VE dramatically reduced Ri and Re values, and increased Cldyn in the 1.0 ppm O_3 group.

Figure 7

Vitamin E up-regulating the expression of Nrf2 and antioxidant genes HO-1 and NQO1

To investigate the protective mechanism of VE in ozone induced airway inflammation, the expression of Nrf2 and the antioxidant genes HO-1 and NQO1 were detected using immunohistochemistry (Fig. 8). It was observed that the expression of transcription factor Nrf2 was enhanced in 1.0 ppm O_3 group when VE was applied (Fig. 8A and 8D). The up-regulation of antioxidant genes HO-1 (Fig. 8B and 8E) and NQO1 (Fig. 8C and 8F) is shown, by comparing the 1.0 ppm O_3 + VE group with 1.0 ppm O_3 group.

Figure 8

DISCUSSION

This study shows that high doses of ozone (>0.5 ppm) can induce inflammation and injury in the lungs of mice. This lung inflammation was concomitant with production of IgE and increased Th2 cytokines such as IL-4, IL-5 and IL-13. Also, a histopathological examination and an AHR test showed that there was lung damage in mice after high doses of ozone exposure. Furthermore, combining treatment with VE, we demonstrated that oxidative stress is an important mechanism behind ozone-induced lung injury, and activating Nrf2 pathway is probable to be a potential protective mechanism of VE (Fig. 9).

Figure 9

With increasing ozone exposure levels being experienced, the potential toxicity of this chemical is receiving increasing attention. In this study, we have investigated the ozone exposure mouse model in detail. In previous studies, mice were largely exposed to extremely high doses of ozone (usually 2.0 ppm for 3 h) (18-21), which were beyond our normal environmental exposure levels. In contrast to other experiments, our study exposed the mice to three different ozone concentrations (0.1 ppm, 0.5 ppm and 1.0 ppm) for 7 days based on actual environmental levels so as to simulate real environmental exposure.

Numerous studies have shown the connection between ozone exposure and lung inflammation (21-24). However, the precise mechanisms involved in ozone-induced inflammation remain elusive. We measured levels of IFN-γ, IL-4, IL-5 and IL-13 in lung tissue and IgE in serum to show levels of inflammation in this mouse model after ozone exposure. IFN-γ is a key biomarker for Th1, and IL-4 IL-5 and IL-13 represent the Th2 immune response and up-regulates IgE production to fight extracellular organisms (25, 26). In this study, the O_3 exposure groups displayed a decrease in IFN-γ (p<0.01) and an increase in IL-4, IL-5 and IL-13levels in comparison to the control group (Figure 3B, C and Fig .4, p<0.01). These data suggest that the Th1/Th2 balance was broken and that the Th2 response had a dominant role in the high dose ozone (>0.5 ppm) exposure mice.

It is worth mentioning that airway remodeling and AHR, the consequence of airway inflammation, are well-established features of lung injury (27, 28). H&E staining can be used to examine the pathological features of airway inflammation and structural alteration, including leucocyte infiltration in the surrounding peribronchiolar areas, epithelial folding and thickened subepithelial cell layers. In this study, airway remodeling was clearly observed in the 0.5 and 1 ppm ozone exposure groups. Mouse AHR was evaluated by measuring inspiratory resistance (Ri), expiratory resistance (Re) and dynamic lung compliance (Cldyn) using methacholine (MCH) challenge tests. The R-areas of respiratory resistances (Ri and Re) and a change in Cldyn represent variation of the large airways and the state of the small airways, respectively (29). Upward shifts of Ri (Figure 7A) and Re (Figure 7B) curves and downward shifts of Cldyn curves (Figure 7C) were seen as the ozone concentration increased from 0.1 to 1.0 ppm (especially in 1 ppm), suggesting that high doses of ozone adversely affects the large and small airways of the lung.

Oxidative stress is the result of an imbalance in the production of antioxidants and free radicals. It is thought to play an important part in the pathogenesis of various types of lung inflammation (30). Ozone is a highly reactive oxidant that induces lung injury and impairs pulmonary function (1). ROS are generated by oxidative stress and have an important role in the redox-dependent regulation of signal transduction processes. However, high-levels of ROS are associated with damage to a wide variety of cellular constituents and can induce apoptosis or necrosis, which may ultimately result in

pathological changes and lead to organ dysfunction or cancers (31, 32). One primary result of increasing ROS is lipid peroxidation. Malondialdehyde (MDA) is a metabolite of the lipid peroxidation of membranes and usually signifies ROS damage to lipids.

Ozone can induce toxicity *via* a ROS-dependent pathway (33). Therefore, we hypothesized that ozone could generate oxidative stress to induce inflammation and damage to lung tissue and consequently exacerbate AHR. To verify this hypothesis, we first measured levels of GSH and MDA in the lung after ozone exposure. Exposure to 1.0 ppm ozone led to depletion of GSH and increases in the level of MDA indicating that oxidative stress had occurred (Figure 4). We then administered VE, an antioxidative reagent, to relieve ozone-caused oxidative stress in mice in the 1.0 ppm ozone exposure group. Interestingly, the combined administration of VE with 1.0 ppm ozone not only caused an increasing of GSH and reduction in the levels of MDA, but also antagonized inflammation effects and lung injury compared with 1.0 ppm ozone only exposure group. Based on these results, we speculated that the adverse effects of ozone on inflammation and damage to the lung could account (at least in part) for the increased oxidative stress.

TSLP is a novel IL-7 like cytokine, which can activate dendritic cells (DCs) to induce Th2 inflammatory responses (34). When TSLP-DCs are used to stimulate naive allogeneic CD4+ T cells *in vitro*, they induce a unique type of Th2 cell that produces the classical Th2 cytokines IL-4, IL-5, and IL-13 and large amounts of TNF, but little or no IL-10 (35). This study demonstrated that the growth trend of TSLP was coincident with the IL-4 and IL-5 cytokine data, which suggested that the activation of TSLP promoted the polarization of Th2 cells. And with TSLP increasing, the IFN-γ decreased sharply, which demonstrated that the activation of TSLP inhibited the polarization of Th1 cells. Previous studies showed that ROS orchestrated Th2 responses by inducing oxidized lipids that triggered the induction of TSLP by epithelial cells (12). In this study, the level of TSLP leading to Th2 immune responses and lung injury was shown to be counteracted by the concurrent administration of vitamin E. These findings are important as they suggest a potential role of TSLP signaling in ozone-induced oxidative stress and inflammatory responses to lung tissue injury. Meanwhile, other ways of measure TSLP expression (ie western blot and PCR) and *in vivo* model (ie

TSLP knock-out mouse) should be used for the sake of further determine the role of TSLP in ozone-induced lung tissue injury. Therefore, more studies are needed for understanding the molecular mechanism on ozone-induced pulmonary injury in order to effectively prevent related health problems in the future.

The transcription factor Nrf2 is a powerful redox sensor to oxidative stress and an essential element in the regulation of many antioxidant genes (36, 37). Here we propose that VE could activate Nrf2 pathway after ozone exposure. This promoted the up-regulation of antioxidant genes NQO1 and HO-1, which inhibited the accumulations of ROS. These results illuminate why VE plays an antioxidant role and attenuates the adverse effect of ozone. The results in the present study agree well with those reported in an investigation that Nrf2 expression in human asthmatics can be rescued by vitamin E *in vivo* (38). In this experiment we suppose that the expression of Nrf2 is not increased after VE alone treatment due to the redox balance *in vivo*. Once the redox balance is broken by environmental pollutants, such as ozone, administration of VE can exhibit antioxidation by activating Nrf2 pathway.

CONCLUSION

By detecting oxidative stress and using vitamin E, we demonstrated that oxidative stress might be a mechanism of ozone-induced inflammation and lung injury, and activating Nrf2 pathway is probable to be a potential protective mechanism. This result could help to provide effective prevention strategies against ozone induced injury of the respiratory system.

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Figure and Figure Legends

Figure 1 Experimental design and animal exposure

Figure 2 Total Serum IgE levels and immunohistochemistry with Tryptase. (A) Total IgE concentrations (ng/ml). **: $p<0.01$, compared with the control group; ##: $p<0.01$, compared with 1.0 ppm O_3 exposure group. (B) Immunohistochemistry with Tryptase in lung tissue. B1-B4 represent different exposure groups (control, VE, 1.0 ppm O_3, 1.0 ppm O_3+VE), Scale bars=50 μm. (C) The scores of degranulation were calculated from the expression levels of tryptase. ** $p<0.01$, compared with control group; ## $p<0.01$, compared 1.0 ppm O_3 group with 1.0 ppm O_3+VE group.

Figure 3 Effects of ozone on cytokine concentrations in the lung. (A) Levels of IFN-γ. (B) Levels of IL-4. (C) Levels of IL-5. (D) Levels of TSLP. *: $p<0.05$, **: $p<0.01$, compared with the control group; #: $p<0.05$, ##: $p<0.01$, compared with 1.0 ppm O_3 exposure group.

Figure 4 Immunohistochemical analyses of IL-13 in lung. Panel: (A1) Control group, (A2) VE (100 mg VE/kg) protected group (ip), (A3) 0.1 ppm ozone exposure group, (A4) 0.5 ppm ozone exposure group, (A5) 1.0 ppm ozone exposure group, (A6) 1.0 ppm ozone exposure and VE group. Scale bars=50 μm. (B) Analyses of IL-13 expression levels according to average optical density. Animal groups (in all panels): n = 3 mice per group. **: $p<0.01$, compared with the control group; ##: $p<0.01$, compared with 1.0 ppm O_3 exposure group.

Figure 5 Ozone effects on oxidative stress and the antioxidant effect of VE. Concentrations of GSH (A) and MDA (B) in the lung. *: $p<0.05$; **: $p<0.01$, compared with the control group; ##: $p<0.01$, compared with 1.0 ppm O_3 exposure group.

Figure 6 Effects of ozone treatment visualised as histopathological lung changes. (A) H&E staining. (B) Number of inflammatory cells infiltrating. Panel: (A1) Control group, (A2) VE (100 mg VE/kg) protected group (ip), (A3) 0.1 ppm ozone exposure group, (A4) 0.5 ppm ozone exposure group, (A5) 1.0 ppm ozone exposure group, (A6) 1 ppm ozone exposure and VE group. Black arrow: bronchial remodeling. *: $p<0.05$; **: $p<0.01$, compared with the control group; ##: $p<0.01$, compared with 1.0 ppm O_3 exposure group.

Figure 7 Effects of ozone treatment on AHR in mice. (A-C), Ri, Re, and Cldyn values, respectively, of control, 0.1 ppm O_3, 0.5 ppm O_3, 1.0 ppm O_3 groups, 1.0 ppm O_3 +VE groups; *: $p<0.05$; **: $p<0.01$, compared with the control group; #: $p<0.05$, ##: $p<0.01$, compared with 1.0 ppm O_3 exposure group.

Figure 8 Vitamin E up-regulating the expression of Nrf2 and antioxidant genes NQO1 and HO-1. (A-C), Immunohistochemistry with Nrf2, NQO1 and HO-1 in lung tissue, respectively. Panel: (A1, B1 and C1) Control group, (A2, B2 and C2) VE (100 mg VE/kg) protected group (ip), (A3, B3 and C3) 1.0 ppm ozone exposure group, (A4, B4 and C4) 1.0 ppm ozone exposure and VE group. Scale bars=50 μm. (D-F), The optical density of immunohistochemistry for Nrf2, NQO1 and HO-1, respectively. * $p<0.05$, ** $p<0.01$, compared with control group; ## $p<0.01$, compared 1.0 ppm O_3 group with 1.0 ppm O_3+VE group.

Figure 9 The mechanisms of ozone-induced lung injury and protect effect of VE.

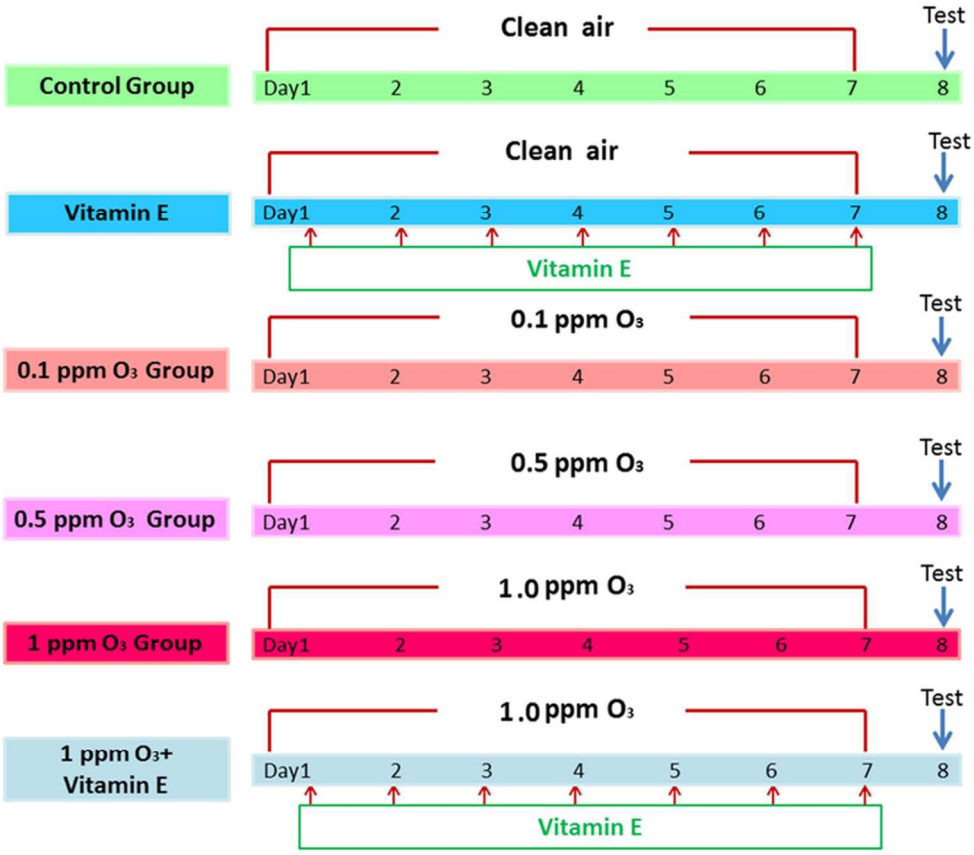

Figure 1 Experimental design and animal exposure

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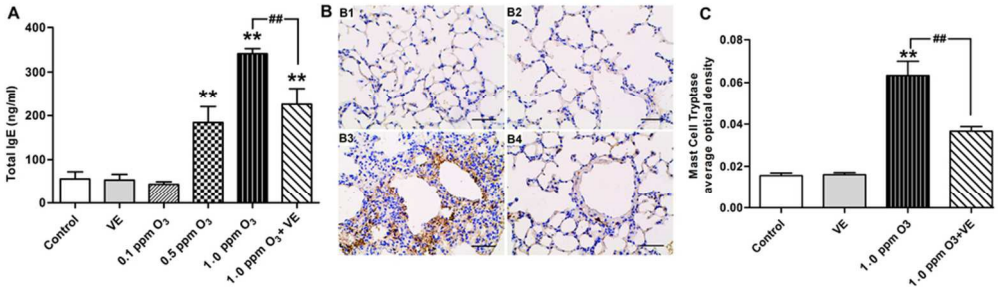

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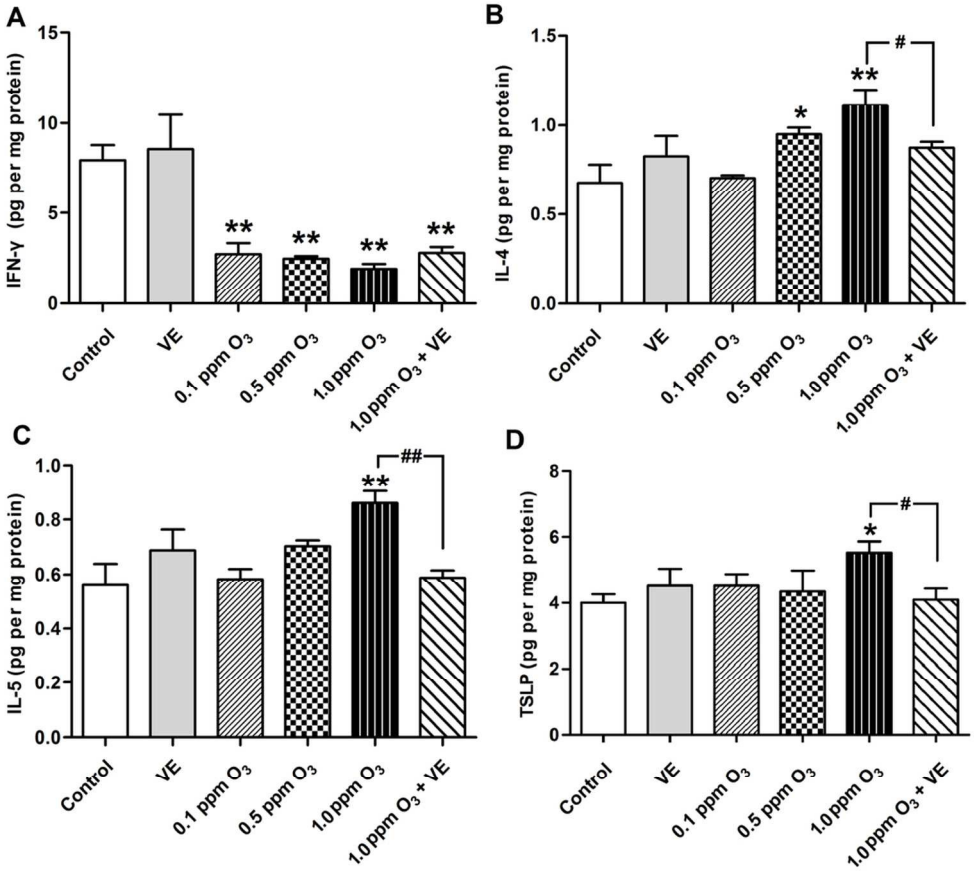

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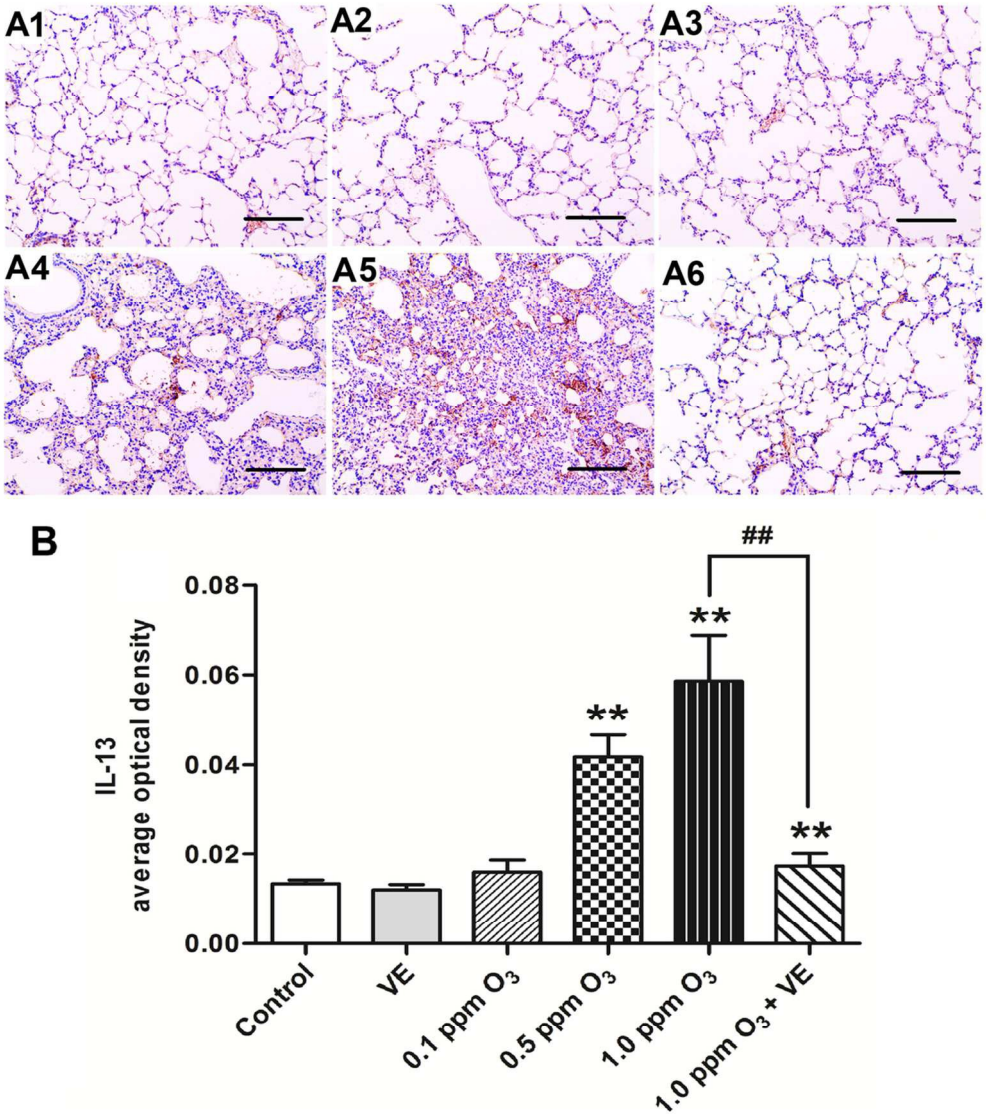

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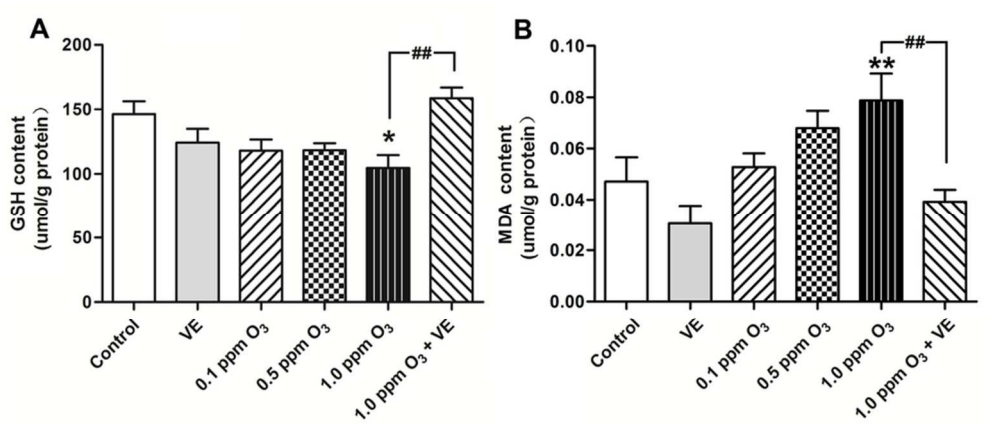

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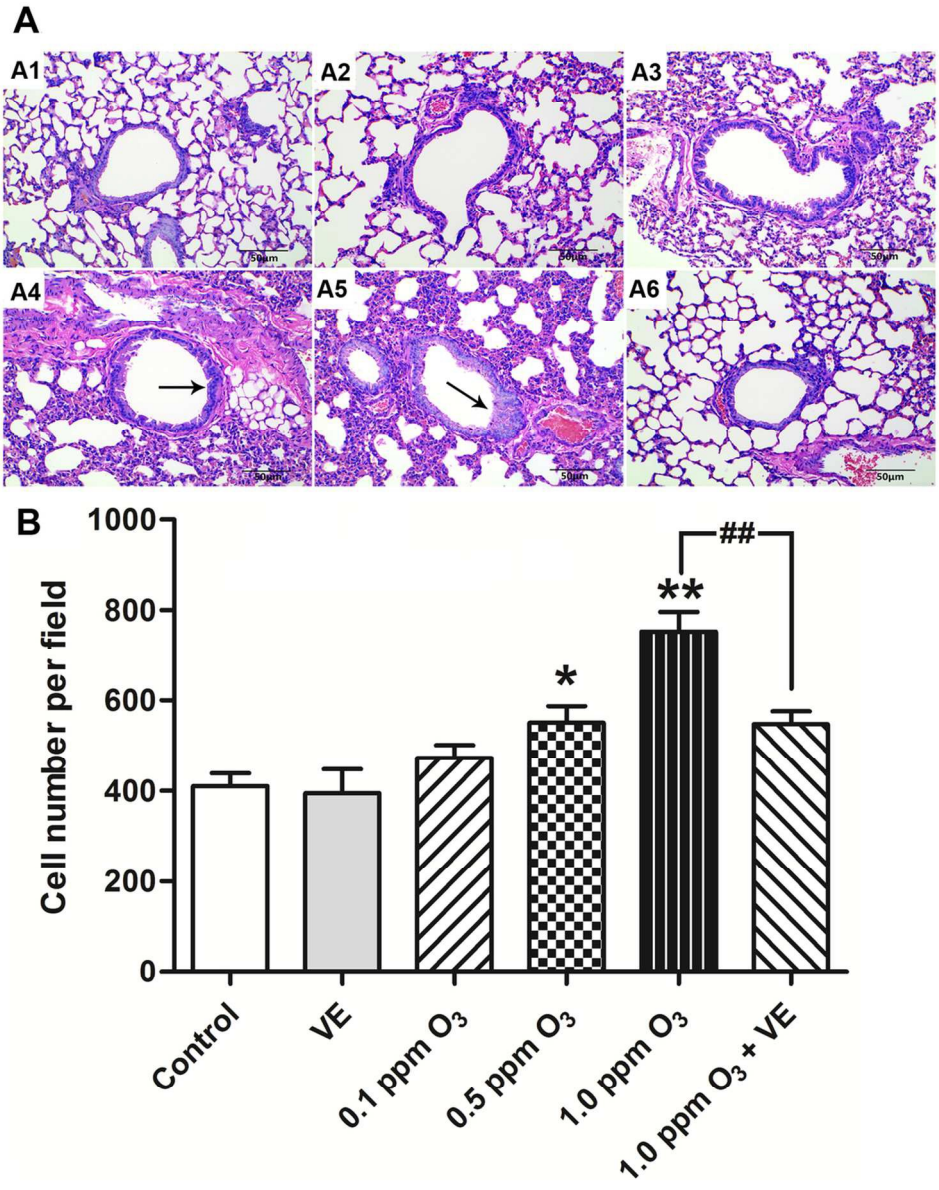

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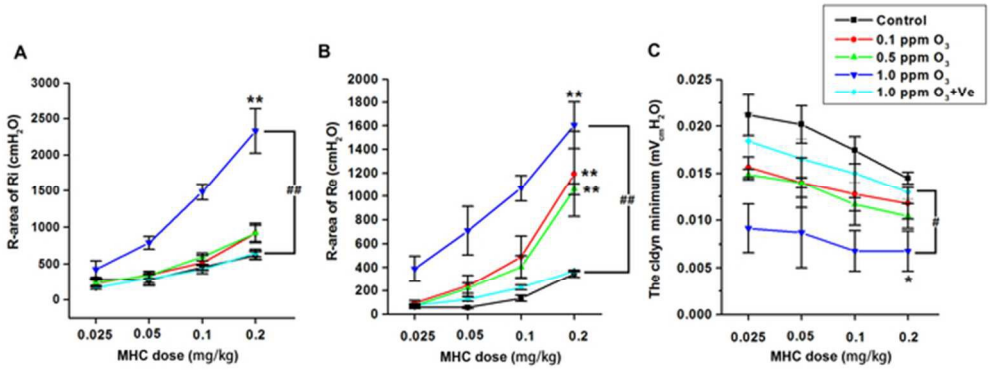

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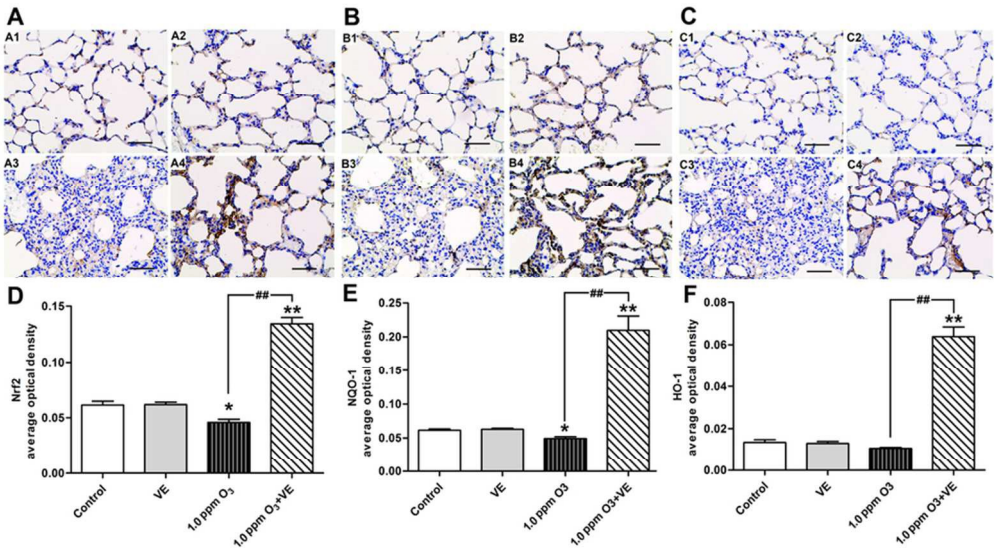

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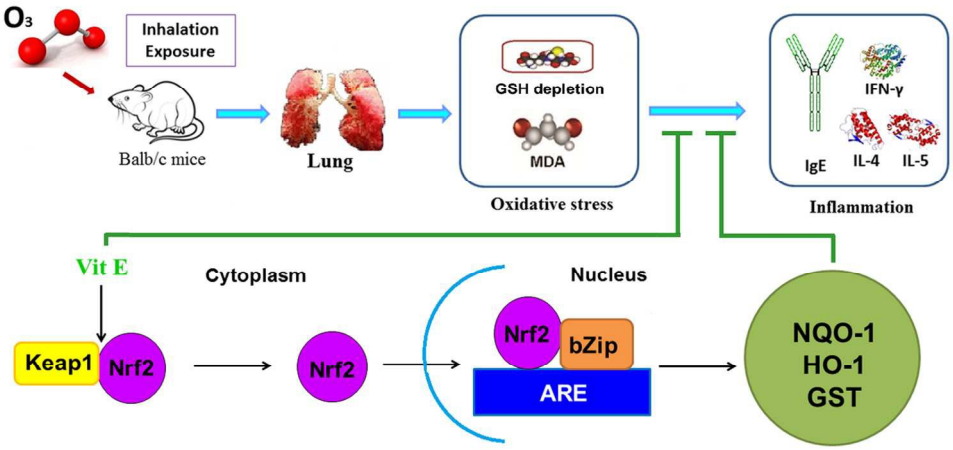

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