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An Acceptable Concentration (0.1 ppm) of Ozone Exposure Exacerbates Lung Injury in a Mouse Model

To the Editor:

Ozone, which is present in the atmosphere, has a strong oxidation effect and has been used for disinfection and sterilization. Ozone generators have increasingly been installed at medical institutions, where they have frequently been used for infection control against coronavirus disease (COVID-19). However, an acceptable concentration of ozone (0.1 ppm) exposure has been defined on the basis of its effects on healthy people (1). Some epidemiological studies suggest that ozone exposure may be an environmental risk factor for acute respiratory distress syndrome (2), but its toxic effects in patients with respiratory dysfunction remain uncertain. The aim of this study was to compare the toxic effects of ozone exposure on an untreated mouse with its effects on a mouse with acute lung injury (ALI).

In this study, we compared the development of ALI symptoms in untreated mice with their development in a mouse model of ALI with and without exposure to ozone (0.1 ppm). Eight-week-old female BALB/c mice (Japan SLC, Inc.) were used, and ALI was developed by intranasal administration of LPS (from *Escherichia coli* O111:B4; Sigma-Aldrich) 48 hours before killing the animals (3–5). Mice were exposed to ozone 5 consecutive days before being killed. An acceptable concentration of ozone in Japan and the United States

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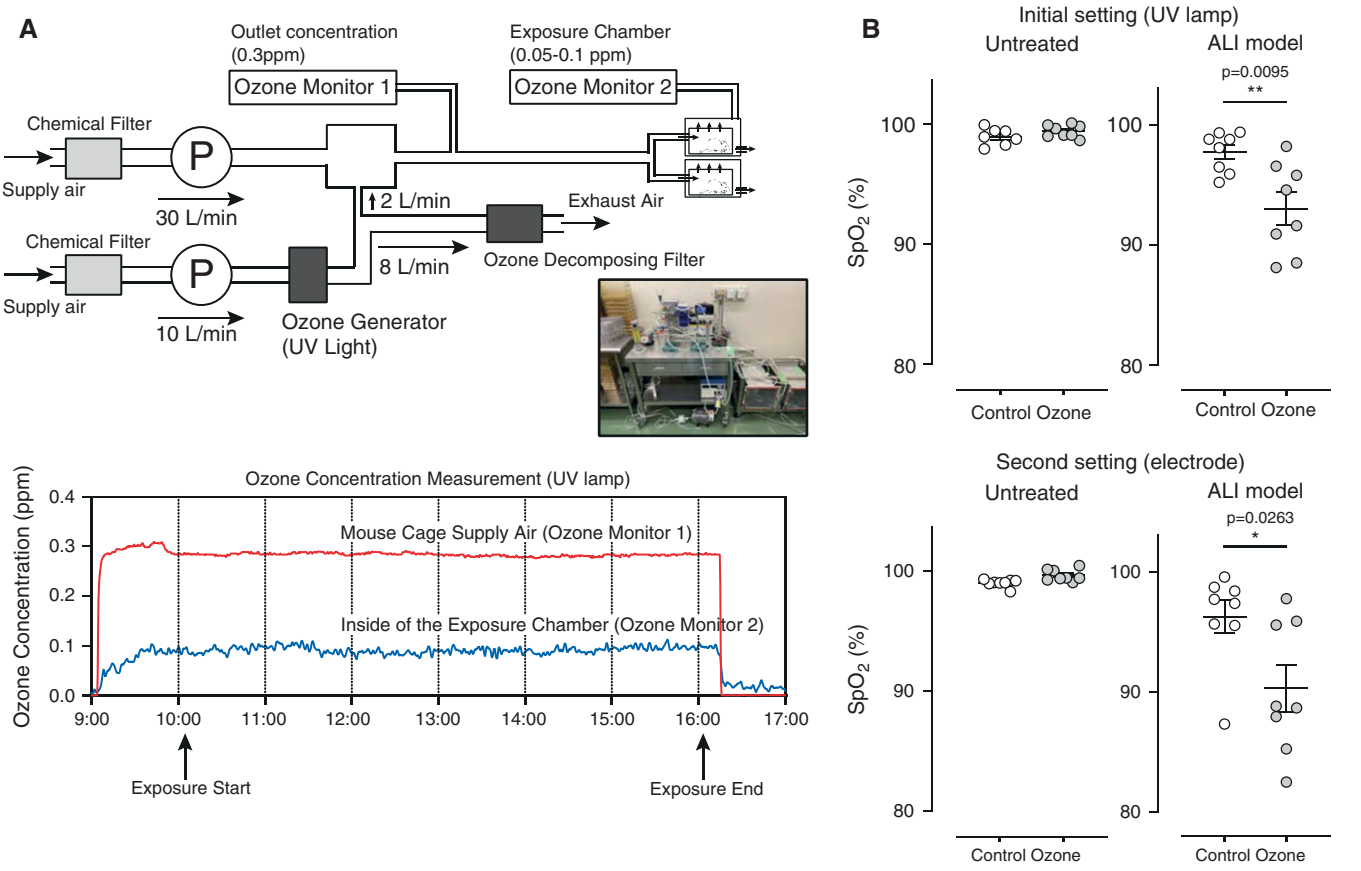

Figure 1. The ozone exposure system that was used in this study is shown (*A*). Ozone concentrations were stably controlled at nearly 0.1 ppm using an initial setting (*B*). A significant downregulation in Sp_{O_2} (percentage) is shown in the ALI group after ozone exposure, whereas no significant changes are observable in untreated mice (*C* and *D*). Each result is presented as the mean ± SEM. Seven to eight mice were assessed per group (*$P < 0.05$ and **$P < 0.01$; unpaired *t* test with Welch's correction vs. the filtered-air control group). ALI = acute lung injury; Sp_{O_2} = oxygen saturation as measured by pulse oximetry; UV = ultraviolet.

(0.1 ppm) was produced either by ultraviolet (UV) light or by an electrode (second setting; Tamura Teco Co. Ltd.), and the concentration of ozone, temperature, and humidity inside the exposure chamber were fully monitored throughout ozone exposure. Temperature and humidity inside the exposure chamber were equivalent to the conditions observed in the mouse room. When initial UV light settings were used, the inner chamber ozone concentration was stably controlled at nearly 0.1 ppm (Figure 1A). However, a bid deviation occurred when the electrode was used for ozone generation (Figure E1A in the data supplement). Nevertheless, the outcome of the mouse study was consistent, and reproducibility was sufficiently ensured because similar results were produced using both settings. All aspects of the mouse study were conducted in accordance with the Animal Care and Use Program of Azabu University (Approval No. 2010223).

To confirm whether an acceptable concentration of ozone (0.1 ppm) influence the clinical condition of the respiratory system, we first measured percutaneous oxygen saturation using a pulse oximeter (Sp_{O_2}) (MouseOx PLUS, Starr Life Sciences Corp.). In this study, all data are expressed as the mean ± SEM. Differences between the control (without ozone) and ozone exposure groups were assessed using an unpaired *t* test with Welch's correction (Prism 9, GraphPad Software). Ozone exposure significantly reduced the amount of Sp_{O_2} in the ALI mouse model when both initial and second settings were used, whereas ozone exposure had no effect on Sp_{O_2} in healthy mice (Figure 1B).

Next, a histological evaluation of the lung was performed. A semiquantitative histopathological evaluation of the lung was performed using hematoxylin and eosin staining to assess bronchial inflammation, alveolar inflammation, alveolar necrosis, and thickened alveolar wall. The assessment was performed in a blinded fashion, and the severity of each pathological feature was determined using the following grading system: 0, within normal limits; 1, mild; 2, moderate; and 3, severe. The total score of each lesion was used for statistical evaluation. Our findings indicated that there was no difference between the control and ozone exposure groups in healthy mice. This was consistent with prior Sp_{O_2} level findings. In contrast, ozone exposure significantly aggravated alveolar inflammation and alveolar necrosis and thickened the alveolar wall in the ALI model when either an electrode or UV light was used to produce ozone (Figures 2A and 2B).

Finally, inflammatory responses observed in the ALI model mice that were exposed to ozone were confirmed via molecular

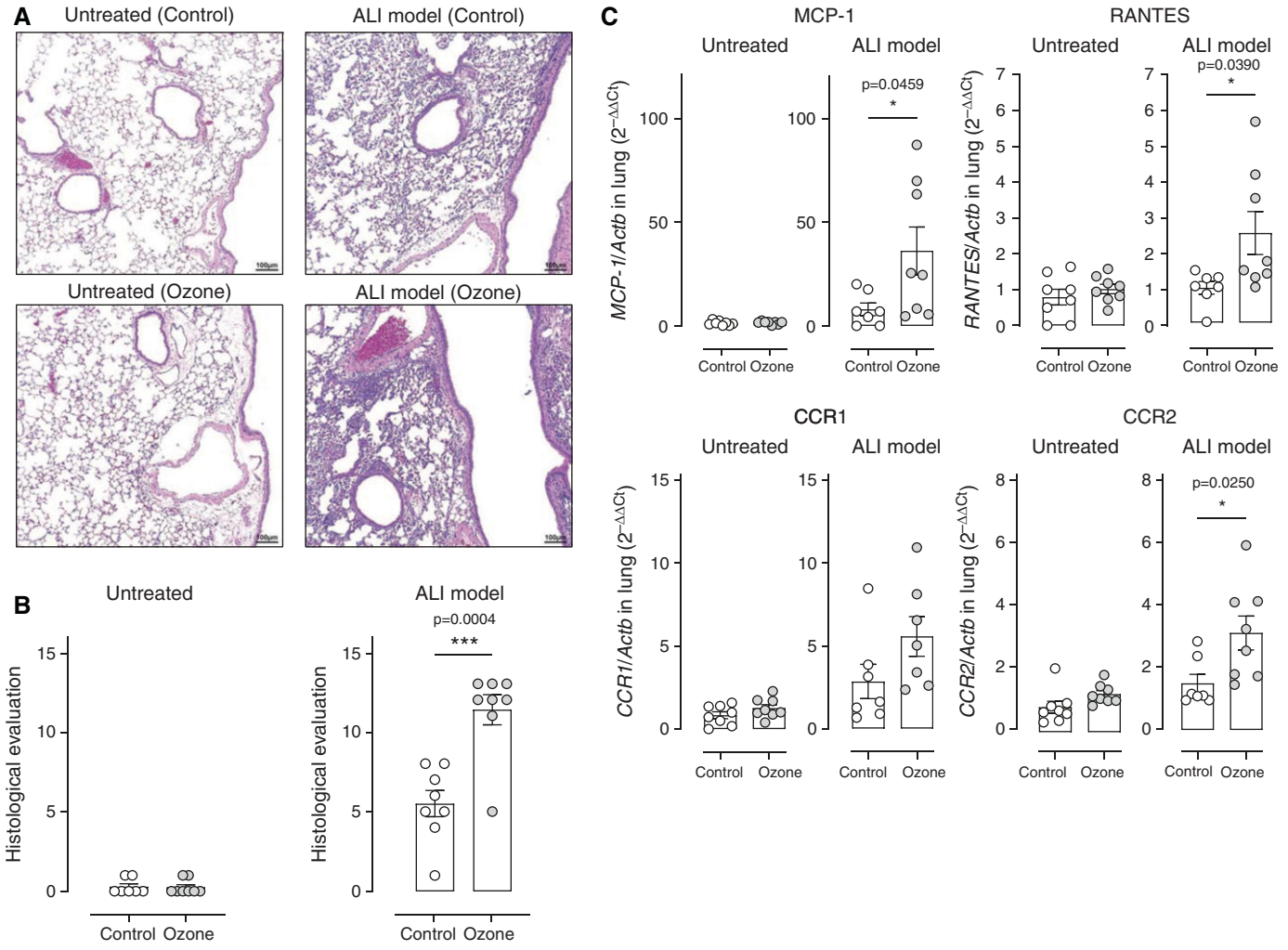

Figure 2. Histological evaluation and chemokine expression after ozone exposure. (*A*) Representative microscopic features of the lung. Scale bars, 100 μm. The total score for each lesion as determined by a semiquantitative evaluation was enhanced by ozone exposure in the ALI model compared with the filtered-air group, whereas no impact of ozone was observed on untreated mice (*B*). Significant upregulation of gene expression levels of MCP-1, RANTES, and CCR2 (values are normalized to β-actin) after ozone exposure were observed in the ALI group; however, no significant changes were observed in untreated groups and CCR1 gene expression in the ALI group (*C*). Scale bar, 100 μm. Each result is presented as the mean ± SEM. Seven to eight mice per group were assessed (*$P < 0.05$ and ***$P < 0.001$; unpaired *t* test with Welch's correction vs. the filtered-air control group). MCP-1 = monocyte chemoattract protein-1.

biological analyses of lung tissue and BAL fluid. Expression levels of chemokine genes, including CCL2/MCP-1 and CCL5/RANTES, were assessed using specific primers (Takara Bio Inc.) and a qPCR system (ABI PRISM 7,000 Sequence Detection System, Thermo Fisher Scientific K.K.). Expression levels of the genes were significantly upregulated by ozone exposure in ALI mice. However, ozone exposure did not impact the healthy mice. A significant enhancement of MCP-1 and RANTES levels in BAL fluid by ozone exposure in ALI mice was observed via an enzyme-linked immunosorbent assay (R&D Systems) when the second setting, but not the initial setting, was used (Figure E1B). This finding indicated that a bit of symptom onset lag occurred on initial and second settings. Gene-specific primers were used to determine expression levels of CCR1 and CCR2 (Takara Bio Inc.), receptors of MCP-1 and RANTES. Interestingly, findings indicated that an upward trend was shown in *CCR1* gene expression, and gene expression of *CCR2* was significantly upregulated by ozone exposure in ALI groups (Figure 2C). These findings suggest that ozone exposure induces the expression of chemokine receptors as well as enhances the secretion of their ligands.

A previous study by Hollingsworth and colleagues demonstrated that extremely high-concentration (2 ppm) ozone exposure increased both the pulmonary and the systemic biological response to inhaled LPS by priming the innate immune system (6). However, 2-ppm ozone also affected the pulmonary and immune functions of the healthy subject, and the influence of low concentrations (acceptable concentrations) of ozone was still unclear. Our findings indicate that currently acceptable concentrations of ozone likely adversely affect patients with ALI.

In summary, our results suggest that controlling low-concentration chemicals such as ozone may improve the treatment of respiratory diseases such as ALI or acute respiratory distress syndrome. Furthermore, the use of ozone generators in rooms without concentration control may increase the risk of severe lung injury for patients with COVID-19 receiving home care or persons at risk of lung disease. The limitation of our study was that all experiments were conducted on animals. Thus, prospective studies in humans to explore this research question would be necessary for future work. ■

Author disclosures are available with the text of this letter at www.atsjournals.org.

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