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Communication

Inactivation of Indoor Environmental Allergy-Related Substances by Ozone Gas in a Small Chamber

Yoshinobu Murakami [1,2,*], Yukio Tobi [3], Kota Emura [3], Kimiye Baba [2], Masahiko Taniguchi [2] and Fukumi Furukawa [4,5]

[1] Department of Aesthetics and Health Sciences, Wakayama Medical University, 811-1 Kimiidera, Wakayama 641-8509, Japan

[2] Department of Natural Products Research, Faculty of Pharmacy, Osaka Medical and Pharmaceutical University, 4-20-1 Nasahara, Takatsuki, Osaka 569-1094, Japan

[3] Haier Asia R&D Co., Ltd., 404-2 Oyabu-cho, Kuze, Minami-ku, Kyoto 601-8206, Japan

[4] Takatsuki Red Cross Hospital, 1-1-1 Abuno, Takatsuki, Osaka 569-1096, Japan

[5] Department of Forensic Medicine, Wakayama Medical University, 811-1 Kimiidera, Wakayama 641-8509, Japan

[*] Correspondence: ymurakam@wakayama-med.ac.jp

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Abstract: Allergic diseases are increasingly recognized as a worldwide public health problem, affecting countries at all levels of economic growth. In recent years, it has been proposed that allergic diseases result from excessive type 2 inflammation, which is driven by cooperative interactions between the innate and acquired immune systems. In this model, allergens, pathogen-associated molecular patterns (PAMPs), and proteases would all be considered allergen-associated substances. In this study, the Japanese ceder allergen Cry j1 and the house dust mite (HDM) allergen Der f1 were selected as representative allergens; lipoteichoic acid (LTA) from *Staphylococcus aureus* as a PAMP; and V8 protease (V8) from *S. aureus*, fungal *Alternaria* extract (Alt), and HDM fecal extract Dff as proteases. The effects of ozone gas on these substances were investigated in terms of allergenicity, proinflammatory activity via innate immunity, and protease activity. Ozone gas inactivated the allergenicity of both Cry j1 and Der f1, and the protease activities of V8, Alt, and Dff, in a CT value (the product of concentration [C] and exposure time [T])-dependent manner. The proinflammatory activity of LTA via innate immunity was significantly inactivated after ozone exposure (301 ppm·min). Although this study was carried out in a small chamber at the basic research level, the results suggest that ozone gas can inactivate indoor allergy-related substances and may help alleviate allergic symptoms. With appropriate safety measures, such as using it in a closed system, this technology has great potential for practical application to allergy management.

Keywords: Allergen; Protease; Pathogen-Associated Molecular Patterns (PAMPs); CT-Value

1. Introduction

Allergic diseases are now recognized as a worldwide public health problem, with a significant impact in countries at all levels of economic growth [1]. The worldwide prevalence of allergic diseases is estimated at 262 million for asthma [2], 171 million for atopic dermatitis [2], and 400 million for allergic rhinitis [3]. Concerning etiology, allergic diseases have traditionally been attributed to activation of the acquired immune system. However, more

recently it has been proposed that they are driven by excessive type 2 inflammation resulting from cooperative interactions between the innate and acquired immune systems [4]. Specifically, allergic diseases would involve activation of acquired immunity, excessive activation of innate immunity, and disruption of epithelial barrier function in the new model, meaning that allergens, pathogen-associated molecular patterns (PAMPs) [5], and proteases [6] would all be considered allergen-related substances.

Although medication is the most common treatment modality for allergic diseases, it is also recommended that allergy sufferers avoid exposure to allergy-related substances in daily life [7]. In the indoor environment, this is generally achieved by physical removal through cleaning and laundering. However, the harmful properties of allergy-related substances can also be inactivated through denaturation or decomposition.

Because of its strong oxidizing power, ozone gas is used to decompose malodorous substances [8] and harmful substances such as agrochemicals [9], bacteria [10], and viruses [11], meaning that ozone gas contributes to various aspects of daily life. Regarding the clinical use of ozone, ozonated water has been used in dentistry and oral medicine for many years [12]. About the improvement of allergies, there are only animal experiments [13] and a clinical study [14] on the improvement of atopic dermatitis (AD) using ozonated water. These reports focus on the bactericidal effect on live *Staphylococcus* species or the restoration of microbial diversity. Even *in vitro* experiments, there has been little research on the inactivation of allergy-related substances by ozone, and our recent research on the inactivation of these substances by ozonated water [15] is the first attempt in this field. Furthermore, the effects of ozone gas on these substances have not been studied.

The concentration-time (CT) value, which is defined as the product of concentration (C) in ppm and exposure time (T) in min, is a common index used to evaluate the strength of ozone disinfection [16]. The CT value of disinfectants originates from the Chick-Watson model [17], which was originally developed for disinfection in the liquid phase. However, it is also applied to disinfection of air and surfaces [18]. The CT value is widely used as an indicator of inactivation by ozone gas [19,20].

Based on this background, we here investigated the possibility of harnessing the oxidizing power of ozone gas to inactivate allergy-related substances in indoor environments, using the CT value as an indicator.

2. Materials and Methods

2.1. Allergy-Related Substances

The Japanese cedar pollen allergen Cry j1 was purchased from BioDynamics Laboratory Inc. (Tokyo). The recombinant Der f1, the major allergen 1 of *Dermatophagoides farina*, was purchased from Asahi Food and Healthcare (Tokyo). Lipoteichoic acid (LTA) from *Staphylococcus aureus*, an agonist of Toll-like receptor 2 (TLR2), was purchased from InvivoGen (San Diego, CA). V8 protease (V8) from *S. aureus* was purchased from Fujifilm Wako Pure Chemicals (Osaka, Japan). *Dermatophagoides farinae* feces extract (Dff) was purchased from Biostir (Osaka, Japan), and *Alternaria* extract (Alt) was purchased from the Institute of Tokyo Environmental Allergy (Tokyo).

2.2. Cell Line and Cell Culture Reagents

Cells of the human epithelial keratinocyte cell line HaCaT were obtained from CosmoBio (Tokyo). Dulbecco's modified Eagle's medium (DMEM) (high glucose) and penicillin-streptomycin solution were obtained from Fujifilm Wako Pure Chemicals. Fetal bovine serum (FBS) was obtained from Biowest (Nuaillé, France).

2.3. Ozone Gas Exposure to Allergy-Related Substances

Allergy-related substances were exposed to ozone gas in a chamber, as shown in **Figure 1**. The chamber was made of acrylic resin and had a volume of approximately 12 L (internal dimensions: 26x18x26 cm). Ozone gas was supplied from a silent discharge ozone generator (SFG1210M-F; Ornit, Okayama, Japan). Ozone concentration was recorded with an ozone monitor (model EG-3000G; Ebatens, Tokyo) and a data logger (model GL840; Graphtec, Kanagawa, Japan). Allergy-related substances were placed on petri dishes and exposed to ozone gas (average concentration: 5 ppm) in the chamber at room temperature. The same substances were placed in a chamber without ozone gas as a control. The amounts of Cry j1, Der f1, and LTA were 1 μg/spot, while V8, Alt, and Dff were each added to the chamber in three different amounts: 5, 10, and 20 μg/spot.

The temperature and humidity in the chamber during the ozone gas exposure test were as follows. Cry j1: 12.0–

16.0 °C, 23.8–43.8%. Der f1: 14.0–20.3 °C, 37.7–67.9%. LTA: 19.4–20.2 °C, 57.7–67.7%. V8: 5.5–20.9 °C, 22.1–31.7%. Alt: 15.0–19.4 °C, 21.2–33.1%. Dff: 15.3–19.5%, 22.1–26.4%.

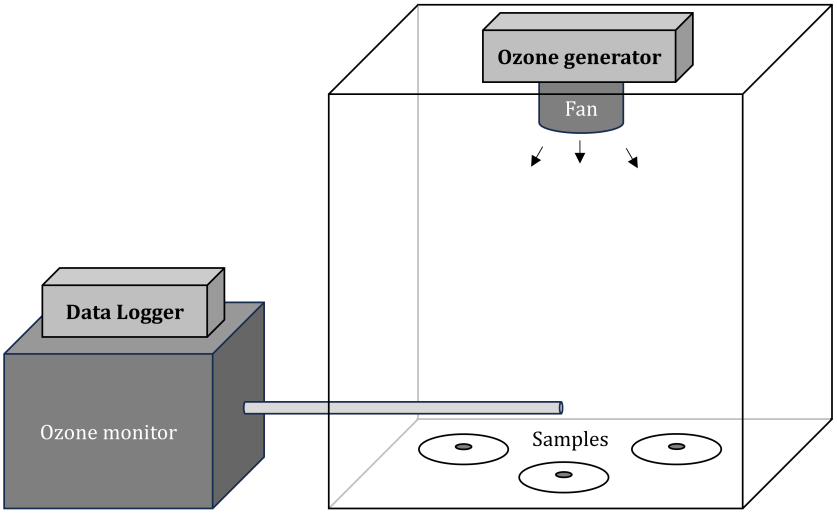

Figure 1. Schematic diagram of the chamber for ozone gas exposure.

Note: The chamber is made of acrylic resin with a volume of approximately 12 L (internal dimensions: 26 x 18 x 26 cm). Ozone gas is supplied from the top and dispersed with a fan mounted on the chamber ceiling. The open end of the ozone concentration measurement tube is placed near the petri dishes containing the samples. The average ozone concentration in the chamber is approximately 5 ppm.

2.4. Measurement of Antigenicity by ELISA

The antigenicity of Cry j1 and Der f1 was evaluated with commercial enzyme-linked immunosorbent assay (ELISA) kits (Institute of Tokyo Environmental Allergy) following the respective manufacturer's instructions. The absorbance was read at 450 nm using a microplate reader (model 680XR; Bio-Rad, Hercules, CA). Antigenicity at each CT value was calculated as a percentage of that in the corresponding control experiment (set as 100%) conducted under the same experimental conditions but without ozone.

2.5. Cell Culture and Cell-Stimulation Experiments

HaCaT cells were maintained in DMEM containing high glucose (4500 mg/L), sodium pyruvate (110 mg/L), and L-glutamine (584 mg/L), supplemented with 10% FBS, 100 units/mL of penicillin, and 100 μg/mL of streptomycin. The cells were incubated at 37 °C with 5% CO_2 in a humidified atmosphere.

In the cell-stimulation experiments, cells were incubated in DMEM supplemented as described above, but with 1% rather than 10% FBS [15]. The cells were seeded in 24-well plates at a density of 3.0×10^5 cells/well, pre-incubated at 37 °C for 24 h, and then stimulated with LTA for an additional 24 h. After stimulation, the culture supernatants were collected and stored at −20 °C until assayed.

2.6. Cell Viability Assay

For the measurements of cell viability, we used a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) in accordance with the manufacturer's instructions. The absorbance was read at 450 nm using a 680XR microplate reader. The cell viability ratio was calculated by the following formula: Cell viability (%) = (average of treated group/average of control group) × 100.

2.7. Measurements of the Levels of IL-8 and MCP-1 in the Culture Supernatants

ELISA kits (BioLegend, San Diego, CA) were used to measure the protein levels of interleukin (IL)-8 and monocyte chemoattractant protein-1 (MCP-1) in the culture supernatants. The assays were performed according to the manufacturer's instructions. The absorbance was read at 450 nm using the 680XR microplate reader.

2.8. Measurement of Proteolytic Activity Using Peptidyl–MCA Substrate

Protease activity was measured as described by Takai et al. [21] with minor modifications. Briefly, 50 µL of enzyme solution (V8, Dff, or Alt) was incubated with or without 50 µL of 2 mM dithiothreitol (DTT) (Fujifilm Wako Pure Chemicals) for 5 min at 37 °C. Then 50 µL of 100 µM enzyme substrate in reaction buffer was added to bring the total volume to 150 µL, and the mixture was further incubated for 30 min at 37 °C. The reaction was carried out in a 96-well microplate (Sumilon MS-8496K; Sumitomo Bakelite, Tokyo). The enzyme substrates used were benzyloxycarbonyl-Leu-Leu-Glu-MCA (Z-LLE) for V8 and butyloxycarbonyl-Gln-Ala-Arg-MCA (Boc-QAR-MCA) for Dff and Alt. All enzyme substrates were obtained from the Peptide Institute (Osaka, Japan). The fluorescence of 7-amino-4-methylcoumarin (AMC) released from the peptidyl–MCA substrates was measured at 365 nm excitation and 465 nm emission on a fluorometer (Multimode Detector DTX 800; Beckman Coulter, Brea, CA). The protease activity at each CT value was expressed as a percentage of that in the corresponding control experiment (set as 100%) conducted under the same experimental conditions but without ozone.

2.9. Statistical Analysis

The results were examined using Student's *t*-test after confirming equal variance using the F-test, and are presented as the mean ± standard deviation (SD) (n = 4). Statistical significance is denoted as $*p < 0.05$ or $**p < 0.01$.

3. Results

3.1. Inactivation of Allergens by Ozone Gas

The effects of ozone gas exposure on the antigenicity of cedar pollen allergen Cry j1 or HDM allergen Der f1 (both at 1 µg/spot) are illustrated in **Figure 2**. The allergenicity of both Cry j1 and Der f1 decreased in a CT value-dependent manner. The CT values required to halve allergenicity were 124 ppm·min for Cry j1 (**Figure 2a**) and 132 ppm·min for Der f1 (**Figure 2b**), respectively.

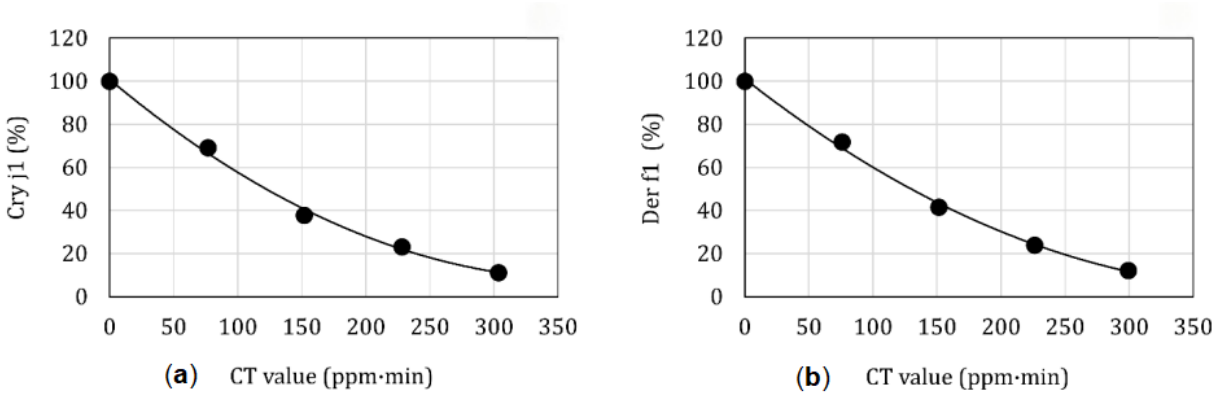

Figure 2. Inactivation of allergens by ozone gas.

Note: Cry j1 (**a**) or Der f1 (**b**) was exposed to ozone gas in the chamber at room temperature. In the control experiments, both substances were added to an identical chamber under the same conditions but without ozone gas. The amount of both allergens was 1 µg/spot in both the ozone gas exposure and control groups. Antigenicity at each CT value was calculated as a percentage of that in the corresponding control experiment (set as 100%) conducted under the same experimental conditions but without ozone. Three independent experiments were carried out. Both samples kept in chambers without ozone gas maintained more than 95% of their initial activity (at 0 min). Results are presented as mean ± standard deviation (SD) (n = 3).

3.2. Inactivation of Lipoteichoic Acid (LTA)-Induced Innate Immune Activation by Ozone Gas

Figure 3 shows the effects of ozone gas exposure on the innate immune activation induced by *S. aureus*-derived LTA. The amount of LTA during ozone gas exposure was 1 µg/spot, and the CT value was 301 ppm·min. The activation of innate immunity by LTA was examined using cells of the human skin keratinocyte cell line HaCaT. The concentrations of IL-8 and MCP-1 in the culture supernatants were evaluated as indices of innate immune activation. The LTA concentrations in culture medium during the cell-stimulation experiments were 1 µg/mL.

When the cell viability in the absence of LTA was set as 100%, the cell viability of the air-exposed-LTA group

was 102.9 ± 3.0% and that of the ozone-exposed-LTA group was 89.6 ± 5.6%. The concentrations of IL-8 and MCP-1 were normalized to cell numbers. Evaluations of IL-8 (**Figure 3a**) and MCP-1 (**Figure 3b**) concentrations in culture supernatants showed that ozone gas significantly suppressed the LTA-induced inflammation in HaCaT cells.

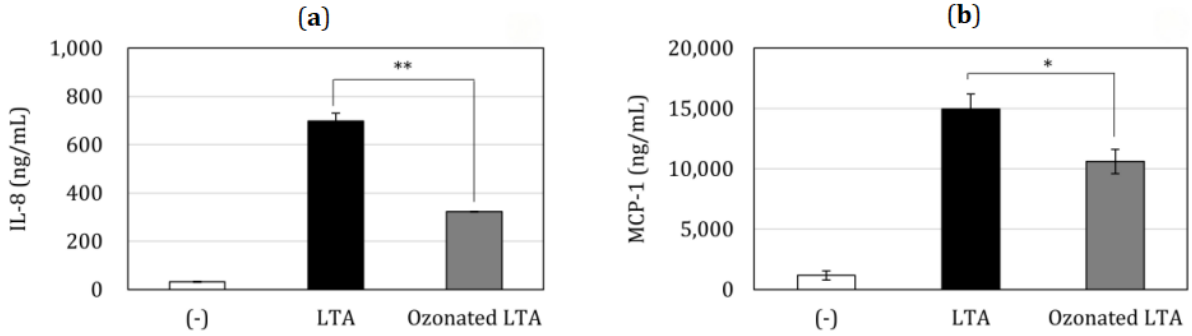

Figure 3. Inactivation of LTA-induced innate immune activation by ozone gas.

Note: LTA was exposed to ozone gas in the chamber at room temperature (Ozonated-LTA: gray column). In the control experiments, LTA was placed in an identical chamber under the same conditions but without ozone gas (LTA: black column). The amount of LTA was 1 μg/spot in both the ozone gas exposure group (CT value: 301 ppm·min) and the control group (CT value: 0 ppm·min). HaCaT cells were stimulated by ozone gas-exposed or air-exposed LTA for 24 h. The LTA concentrations in culture medium were 1 μg/mL. The concentrations of inflammatory mediators such as IL-8 (**a**) and MCP-1 (**b**) in the supernatants were evaluated. These concentrations were normalized by cell numbers. Four independent experiments were carried out. The concentrations of each cytokine stimulated by untreated LTA (preserved in ice) were 722 ± 29.0 ng/ml for IL-8 and 16,753 ± 1,352 ng/ml for MCP-1. Results were presented as mean ± standard deviation (SD) (n = 4). Statistical analyses were calculated using the Student's *t*-test. Statistical significance is denoted as $*p < 0.05$, or $**p < 0.01$ compared to the air-exposed group (CT value: 0 ppm·min).

3.3. Inactivation of Proteases by Ozone Gas

The changes in the protease activity of V8 from *S. aureus*, the fungi extract Alt, and the HDM feces extract Dff after ozone gas exposure are depicted in **Figure 4**. Each protease was added to separate petri dishes at 5, 10, and 20 μg/spot and exposed to ozone gas. The activities of all three proteases in all amounts decreased in a CT value-dependent manner. The CT values required to halve the protease activity at 5, 10, and 20 μg were 114, 120, and 223 ppm·min for V8 (**Figure 4a**) and 199, 269, and 458 ppm·min for Alt (**Figure 4b**), respectively. For Dff, the values at 5 and 10 μg were 377 and 539 ppm·min, but at 20 μg, even 600 ppm·min was not sufficient to halve the activity (**Figure 4c**).

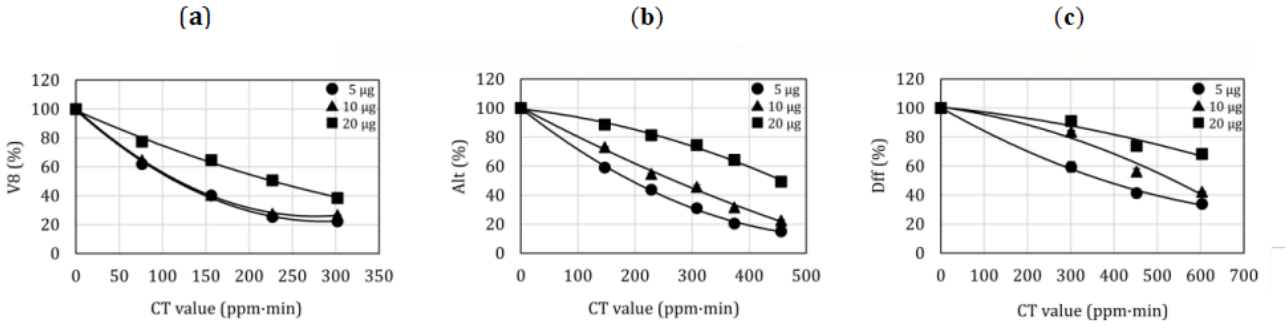

Figure 4. Inactivation of proteases by ozone gas.

Note: V8 (**a**), Alt (**b**), or Dff (**c**) were exposed to ozone gas in the chamber at room temperature. In control experiments, they were kept in an identical chamber under the same conditions but without ozone gas. The amounts of each protease during ozone gas exposure were 5, 10, and 20 μg/spot. The protease activity at each CT value was expressed as a percentage of that in the corresponding control experiment (set as 100%) conducted under the same experimental conditions but without ozone. Three independent experiments were carried out. Both samples kept in chambers without ozone gas maintained more than 95% of their initial activity (at 0 min). Results are presented as mean ± standard deviation (SD) (n = 3).

4. Discussion

Allergic diseases result from the entry of causative agents into the body. Therefore, prevention or alleviation of allergic disease requires targeting the causative agent, the epithelial cells at the interface with the external environ-

ment, and the symptoms. The most common approach to targeting symptoms is medication. However, in addition to pharmacotherapy, we have been exploring other ways to alleviate allergic symptoms in daily life. For example, we have been studying the effects of consuming lactic acid bacteria [22] and *Citrus jabara* peel [23]. In terms of the interface with the external environment, we have also been studying borage oil [24] and topical creams [25]. In the present research, we focused on the third leg of allergic treatment, the targeting of allergy-related substances themselves.

Indoor environments in Japan contain a variety of allergy-related substances, including HDM, mold, bacteria, pet hair, and substances that come in from outdoors, such as pollen, air pollutants, and yellow sand. These allergy-related substances include allergens, PAMPs, and proteases. For this study, we selected the most common allergens among the allergy patients in Japan, such as the Japanese cedar pollen allergen Cry j1 and the HDM allergen Der f1. Another reason for choosing Der f1 is that many global epidemiological studies have used the amount of Der 1 (Der f1 + Der p1) in fine house dust as an indicator of sensitization and asthma risk [26,27]. *S. aureus*-derived LTA was selected among PAMPs because of the close relationship between *S. aureus* and atopic dermatitis [28]. *S. aureus*-derived V8, fungal Alt, HDM fecal extract Dff were selected as proteases, because they are known to be the main sources of protease in indoor environments [29,30]. These allergy-related substances were exposed to ozone gas (average concentration: 5 ppm) in a small chamber at room temperature, and the relationships between the CT values and inactivation of antigenicity, proinflammatory activity via innate immunity, and protease activity were examined.

Ozone gas inactivated the allergenicity of both Cry j1 and Der f1 in a CT value-dependent manner (**Figure 2**). The proinflammatory activity of *S. aureus*-derived LTA via innate immunity was significantly inactivated after ozone gas exposure (301 ppm·min) (**Figure 3**). The protease activities of V8 from *S. aureus*, fungi extract Alt, and HDM feces extract Dff were also inactivated by ozone gas in a CT value-dependent manner (**Figure 4**). The purified products were more readily activated by ozone gas compared to the crude products, and lower amounts of allergy-related substances tended to be more readily inactivated compared to higher amounts.

Although this study was carried out in a small chamber at the basic research level, these results suggest that ozone gas can inactivate allergy-related substances in the indoor environment and may alleviate symptoms of allergic diseases. Ozone gas is extremely effective in decomposing and inactivating harmful substances due to its strong oxidizing power. However, its effects on the human body must also be considered when using it. Therefore, ozone gas should be used in a closed system or unoccupied areas.

In practical applications, closed systems are considered suitable for the use of ozone gas. This is because ozone gas exposure in a closed system is not only safe for the human body but also can easily generate hydroxyl radicals, which have stronger oxidizing power than ozone, under humidified and heated conditions. Ozone is a strong oxidant with an oxidant potential of +2.07 V, but in the presence of water, it produces even more powerful hydroxyl radicals (+2.8 V) [19,31]. Experiments on the inactivation of SARS-CoV-2 using ozone gas have shown that the inactivating effect of ozone gas is enhanced under humid conditions [19,32], suggesting that high relative humidity has a positive effect on ozone gas disinfection [18]. Therefore, ozone gas exposure is expected to inactivate allergy-related substances at lower CT values than those shown in this experiment, i.e., at lower ozone concentrations and/or in shorter periods. We are currently developing closed-type household appliances and conducting various studies under conditions that simulate actual usage situations using prototype models. Possible targets for ozone gas exposure include items such as clothing, bedding, and stuffed animals, which tend to be too highly contaminated with allergy-related substances for simple removal by washing or dry cleaning [26,33].

A systematic review has not found strong evidence supporting the effectiveness of indoor allergen reduction in asthma management [34]. However, focusing on studies reporting clinical effectiveness, it appears that thorough removal of allergens is important. Specifically, this includes thorough management of the indoor environment by professional groups [35] and by using highly sensitive allergen detection technology [36]. Conversely, it may be that patients are so sensitive to allergens that standard cleaning methods are insufficient to achieve clinical effectiveness. If this hypothesis is correct, it may be possible to achieve clinical effectiveness by using ozone gas to inactivate allergy-related substances that cannot be completely removed by conventional cleaning methods. However, this is only a hypothesis, and further field tests and clinical studies are needed to verify it. These studies remain as future research topics.

In summary, while medication is the most widely used treatment for allergy symptoms, it is also critical that pa-

tients attempt to remove or inactivate allergy-related substances in their indoor environment. Our present findings may contribute to the practical application of ozone gas for inactivating allergy-related substances and alleviating the symptoms of allergic diseases. However, to achieve clinical efficacy, detailed evaluations of actual use are necessary, and these remain future challenges.

5. Conclusion

Ozone gas treatment in a small chamber was found to inactivate allergens, a PAMP, and proteases that are characteristic of allergy-related substances. These effects of ozone gas may contribute to the alleviation of symptoms and suppression of the onset of allergic diseases. However, this is only a possibility, and to prove it, it is necessary to verify its effectiveness in actual field trials and clinical studies.

Author Contributions

Conceptualization, Y.M., Y.T., K.E., K.B., M.T., and F.F.; methodology, Y.M., Y.T., and K.E.; writing—original draft preparation, Y.M.; writing—review and editing, Y.M., Y.T., K.E., K.B., M.T., and F.F.; supervision, F.F.; project administration, Y.M. and Y.T. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

This study, titled "Inactivation of Indoor Environmental Allergy-Related Substances by Ozone Gas in a Small Chamber", did not involve any experiments on human or animals conducted by the authors. The research does not include any identifiable personal or clinical information. Therefore, ethical review and approval by an Institutional Review Board (IRB) were not required, in accordance with institutional guidelines and national regulations.

Informed Consent Statement

This study did not involve human participants, human data, or human tissue. Therefore, informed consent was not required. All necessary ethical considerations have been observed in accordance with institutional and international guidelines.

Data Availability Statement

The datasets generated and analyzed during this study are available from the corresponding author upon reasonable request.

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Conflicts of Interest

The authors declare no conflict of interest.

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