Original research

Streamer discharge reduces pollen-induced inflammatory responses and injury in human airway epithelial cells.

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Running title：Streamer discharge reduces pollen-induced inflammatory responses

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Abstract

Although epidemiologic studies have demonstrated that cedar pollen influences respiratory health, effective method for inactivating cedar pollen has not been established. Streamer discharge is a type of plasma discharge in which high-speed electrons collide with oxygen and nitrogen molecules. It reportedly has the ability to eliminate bacteria, mould, chemical substances and allergens. The present study investigated the influence of pollen on BEAS-2B cell line, derived from human airway epithelial cells, as well as the efficiency of streamer discharge on pollen-induced health effects. Airway epithelial cells were exposed to non-treated pollen and streamer-discharged pollen at doses of 100 and 1000 μg/mL for 6 or 24 h. Non-treated pollen at a dose of 1000 μg/mL significantly decreased cell viability and induced both mRNA and protein expression of IL-6, whereas streamer-discharged pollen showed the attenuated changes as compared to non-treated pollen. Further, scanning electron micrographs showed that streamer discharge caused the fine structural changes of pollen. These results provide the first experimental evidence that pollen at a high dose affects cell viability and inflammatory responses, and streamer discharge technology attenuates their influences by decomposing pollen.

Keywords: BEAS-2B cells; cedar pollen; inflammation; injury; streamer discharge

Introduction

Pollinosis is characterized by allergic symptoms such as rhinitis and conjunctivitis, and is caused by some species of plants including grass, ragweed, cedar, birch, mugwort and olive around the world.[1, 2] Cedar pollinosis occurs during spring in Japan where cedar forest covers.[3, 4] Recently, not only adults but also children suffer from Japanese cedar pollinosis, and cedar pollen allergy is one of the major public health problems in Japan because of its high prevalence.[5, 6] The rapid rise of Japanese cedar pollen allergy involves various factors including changes in lifestyle, sanitary condition, diet and environmental pollutants.[7, 8] Moreover, it has been suggested that exposure to pollen rise a risk for development of allergic asthma.[9] Bronchial asthma has been recognized as a chronic type of allergic airway inflammation with hyperresponsiveness to foreign allergens. The presence of mucus hypersecretion also contributes to airflow obstruction and bronchial reactivity. Clinical studies have provided evidence for the association between cedar pollen and asthma.[10, 11] Although cedar pollen itself is too large to reach the lower airways, crushed pollen may contribute to immune response.[12] Recently, Wang et al.[13, 14] have suggested that air polluted rainfall increase the small sized cedar pollen allergens (<1.1 μm). However, main mechanism of allergic inflammation in asthma by cedar pollen remains unclear.

“Streamer discharge” is a type of plasma discharge in which high-speed electrons collide with oxygen and nitrogen molecules, which is capable of oxidative decomposition of bacteria and mould as well as chemical substances and allergens. The decomposition mechanism

of streamer technology is that massive streamer discharge hits the xenobiotics, decomposing their surface proteins, leading to the destruction through oxidation. The discharge range of streamer is wider than that of standard plasma discharge (glow discharge). This technology can disseminate high-velocity electrons, which have the highest oxidation activity. After the reaction, streamer discharge breaks down high-velocity electrons into safe nitrogen, oxygen atoms and water molecules.[15, 16]

In the present study, we focused on airway epithelial cells which are the primary site of entry for inhaled xenobiotic in the respiratory system to elucidate the effects of pollen on airway inflammation and damage. Accordingly, we investigated whether pollen enhances inflammatory responses and damages on airway epithelial cells. In addition, the effects of streamer discharge on pollen were also determined.

Materials and Methods

Cell culture

The BEAS-2B cell line, derived from human bronchial epithelial cells transformed by an adenovirus 12-SV40 hybrid virus, was purchased from European Collection of Cell Cultures (Salisbury, Wiltshire, UK). Airway epithelial cells were seeded in 96 well or 12 well collagen I coated plates and incubated for 72 h to reach semi-confluence in serum free-medium LHC-9 (Life

technologies, Carlsbad, CA, USA) at 37°C in a humidified atmosphere of 5% CO_2.

Treatment of pollen with streamer discharge

Crude cedar pollen (Cosmo Bio Co. ltd., Tokyo, Japan) on the plastic dish was placed at 10 cm from electrode in the chamber (520 mm × 520 mm × 430 mm), and was treated with active species generated by streamer discharge (5.6 kV × 55 μA) for 0, 2, 4, and 8 days.

Experimental protocol

After airway epithelial cells were grown to semi-confluence in LHC-9, cells were exposed to pollen at a dose of 100 or 1000 μg/mL for 6 or 24 h. The cell viability, gene expression, cytokine production, structure of pollen and endotoxin levels were examined by WST-1 assay, real-time RT-PCR, enzyme-linked immunosorbentassay (ELISA), electron microscope images and diazo-coupling method, respectively.

Cell viability

After exposure to pollen for 24 h, cell viability was measured by WST-1 assay using Premix WST-1 Cell Proliferation Assay System (TaKaRa Bio Inc., Shiga, Japan). In brief, 7 μl of WST-1 reagent was added to each well of 96 well plate (the volume of WST-1 reagent was

1/10 of original culture medium) and mixed well by gently rocking the plate. Airway epithelial cells were incubated with WST-1 reagent at 37°C for 3 h. After incubation, absorbance was measured on iMarkMicroplate Absorbance Reader (Bio-Rad Laboratories, Hercules, CA, USA) with wavelength at 450 nm and a reference wavelength at 630 nm. Results were expressed as the percentage of viable cells compared with untreated cells (0 μg/mL).

Extraction of RNA and gene expression

After exposure to pollen for 6 h, mRNA levels of interleukin (IL)-6 in the airway epithelial cells were measured by real-time RT-PCR. Total RNA was extracted with RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. This RNA was treated with DNase by RNase-Free DNase Set (Qiagen). Total RNA was reverse transcribed to cDNA using High Capacity RNA-to-cDNA kit according to the manufacturer's instructions (Life technologies). In brief, reverse transcription was carried out at 37°C for 60 min and at 95°C for 5 min in 20 μL reaction of RT Buffer Mix and RT Enzyme Mix. The mixture was then cooled to 4°C and stored at -20°C until use. The quantitation of mRNA expression was carried out using the ABI Prism 7000 Sequence Detection System (Life technologies). The PCR amplification was performed as Yanagisawa et al[17]. The PCR amplification performed with 50 μL final reaction mixture consisting of 25 μL TaqMan Gene Expression Master Mix (Life technologies), 2.5 μL TaqMan Gene Expression Assay contained with TaqMan probe and pair primers (Life

technologies), 20.5 μL DNase/RNase-Free Distilled Water (Life technologies) and 2 μL cDNA. The cDNA was amplified according to the thermal profile of 50°C for 2 min and then 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The relative intensity was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control gene. TaqMan probes and pair primers for IL-6 and GAPDH were designed by Life technologies, which did not disclose these sequences.

Cytokines in the culture supernatants

After exposure to pollen for 24 h, medium was harvested and centrifuged at 400 *g* for 5 min to remove floating cells. The final supernatants were stored at −80°C until analysis. The levels of IL-6 (Thermo Scientific, Waltham, MA, USA) in the culture supernatants were measured by ELISA according to the manufacturer's instructions. Absorbance was measured on iMarkMicroplate Absorbance Reader (Bio-Rad Laboratories) with wavelength at 450 nm and a reference wavelength at 550 nm. The detection limits of IL-6 were less than 1 pg/mL.

Electron microscope images of pollen

The pollen grain was mounted onto specimen holders, and put under a discharge reactor. After streamer discharging for 0 to 8 days, the specimens were coated with 10 nm gold palladium

in a vacuum sputter, and were examined in a Hitachi S-5000S scanning electron microscope (Hitachi High Technologies, Tokyo, Japan) at 5 kV.

Endotoxin in pollen

Endotoxin in pollen was extracted using a modified protocol of Spaan et al.[18] In brief, 5 mg pollen was immersed in 5 mL of pyrogen free water (1000 μg/mL) with 0.05% Tween 20 for 1 h at room temperature on a shaker. After 15 min of centrifugation at 1,000 *g*, 1 mL supernatant per sample was collected and stored at -80°C until analysis. Levels of endotoxin were measured by Pyrochrome with Glucashield Buffer, Control Standard Endotoxin and PyroColorDiazo Reagents DIA150-MP (Seikagaku Biobusiness, Tokyo, Japan) according to the manufacturer's instructions. Absorbance was measured on iMarkMicroplate Absorbance Reader (Bio-Rad Laboratories) with wavelength at 550 nm and a reference wavelength at 630 nm.

Statistical analysis

Data were represented as mean ± standard deviation (SD) for each experimental group (n=4). The significance of variation among different groups was determined by two-way ANOVA analysis. Differences among groups were analyzed using Tukey multiple comparison test (Excel Statistics 2010, Social Survey Research Information Co. Ltd., Tokyo, Japan). A p value<0.05 was

considered to indicate a significant difference.

Results

Effects of non-treated pollen and streamer-discharged pollen on viability of airway epithelial cells

We investigated the effect of pollen on the viability of airway epithelial cells after exposure to pollen for 24 h. Non-treated pollen at a dose of 1000 μg/mL significantly decreased the viability of airway epithelial cells. Exposure to pollen treated with streamer discharge for 2, 4 and 8 days significantly increased viability compared to that to non-treated pollen (Fig. 1).

Effects of non-treated pollen and streamer-discharged pollen on gene expression and cytokine production of airway epithelial cells

To evaluate whether pollen has pro-inflammatory effects on airway epithelial cells, we examined both mRNA and protein expression of IL-6 after exposure to pollen for 6 and 24 h, respectively. Exposure to non-treated pollen at a dose of 1000 μg/mL resulted in a significant increase in IL-6 mRNA and protein release as compared to that at a dose of 0 μg/mL. Treatment with streamer discharge for 2, 4, and 8 days significantly attenuated the IL-6 mRNA and protein release caused by pollen (Figs. 2A and B).

Effects of non-treated pollen and streamer-discharged pollen on morphological features

A grain of cedar pollen was composed of a mushroom-like structure, 30 μm in diameter and 16 μm high, with many orbicles, 0.5 μm in diameter (Figs. 3A and B). After streamer discharging for 2 days, shrinkage and dents were observed on the surface of a grain (Fig. 3C), and sharp spikes on the surface of an orbicle became dull (Fig. 3D). Some of orbicles contacted and fused together. These fine structural changes became more apparent with the length of streamer discharge.

Endotoxin in non-treated pollen and streamer-discharged pollen

We evaluated the contents of endotoxin in non-treated pollen and streamer-discharged pollen at a dose of 1000 μg/mL to understand exposure to ambient endotoxin. As shown in Fig. 4, the highest levels of endotoxin were observed in non-treated pollen (21.3 pg/mL). On the other hand, streamer-discharged pollen showed lower levels than non-treated pollen (the levels detected in streamer-discharged pollen for 2, 4 and 8 days were 1.5, 3.1, 4.2 pg/mL, respectively).

Discussion

In the present study, we determined the effects of pollen on respiratory health, as well

as the influence of streamer discharge on the pollen-induced effects. We demonstrated that pollen exposure at a high dose exacerbated cell viability and elevated IL-6 release in airway epithelial cells, furthermore, streamer discharge technology attenuated their influences by decomposing pollen.

It has been reported that proteases released from pollen might be able to injure the airway epithelilal cells.[19] Additionally, Aguilera-Aguirre et al.[20] have suggested that ragweed pollen extract leads mitochondrial dysfunction, which can be caused by oxidative damage to mitochondrial respiratory chain-complex and associated proteins using type II pneumocyte cell line (A549). We also observed that non-treated cedar pollen at a high dose decreased cell viability as evidenced by the result of WST-1 assay based on activity of mitochondrial dehydrogenase. However, it is unlikely that they induced apparent cell death via membrane permeabilization as evidenced by the results of LDH release assay (data not shown). Thus, *in vitro* study, pollen can lead to damages on airway epithelial cells.

Airway epithelial cells are source of cytokines such as IL-6 which is an inflammatory cytokine induced in response to environmental insults and plays an important role in inflammation of respiratory system by stimulating lymphocytes and up-regulating mucin secretion[21, 22, 23]. Indeed, various chemicals and allergens, including diesel exhaust particles, polycyclic aromatic hydrocarbons and dust mite stimulate IL-6 production in airway epithelial cells.[24-26] It has been

reported that pollen allergen from grass and pollen extract of parietaria officinalis activates A549 by analysis of IL-6, IL-8, IL-4, IL-5 and IL-13 release.[27, 28] Also in our present study, cedar pollen significantly elevated IL-6 mRNA and protein release. The present results support pollen at a high dose caused cell damage and inflammation directly on airway epithelial cells, which can induce disruption and weakness of defense against invading xenobiotics such as inhaled allergen in respiratory tract, and might stimulate subsequent mucin production and immune responses including uptake of allergen by dendritic cells and lymphocyte activation. Accordingly, pollen exposure may exacerbate or trigger allergic responses or allergic/respiratory diseases. On the other hand, Österlund et al.[29] have shown that recombinant pollen allergens from birch and timothy do not activate airway epithelial cells. There is possibility that unknown substances adhering to crude pollen generate bad effects or it depends on pollen species.

Endotoxin is known to induce IL-6 production in airway epithelial cells. Previous reports have indicated that treatment with lipopolysaccharide at concentrations more than 1 μg/mL activates the product.[30] To understand exposure to ambient endotoxin, the concentration of endotoxin in pollen were measured in the present study, and the level was very low (equal or less than 21.3 pg/mL). Therefore, our result and previous reports suggested that activating capacity of airway epithelial cells did not result from ambient endotoxin, although streamer discharge further decreased endotoxin even at extremely low levels in pollen. The pollen at the

high concentration may have been sufficient to elevate IL-6 release as a xenobiotic.

We first found approximate 15% increase in cellular viability and 40% reduction in IL-6 protein level after streamer discharge. Electron micrographs clearly showed different shapes between non-treated and streamer-discharged pollen. Streamer discharge caused shrinkage and dents on the surface of pollen and dull-surfaced and fused orbicles. It is possible that the changes in substances adhered to surfaces of crude pollen except for endotoxin and physical stimulation caused by the shape of crude pollen may contribute to cell damage and inflammation in airway epithelial cells (Fig.5). Several studies have reported methods of xenobiotic reduction using corona discharge, ions generated by plasma discharge, colloidal silica and hypochlorous acid.[31-35] Additionally, we suggest usefulness of streamer discharge as one of xenobiotic reduction technologies.

In conclusion, exposure of airway epithelial cells to cedar pollen facilitated cellular damage and inflammatory responses, at least via the expression of IL-6. Furthermore, streamer discharge inhibits the influence. These results provide evidence that cedar pollen might contribute to exacerbation of respiratory/immunological health and streamer discharge has function to suppress the health effects of cedar pollen. Further investigation is necessary to elucidate its benefits.

Author contributions:

All authors participate in the design, interpretation of the studies and analysis of the data and review of the manuscript. AH, RM, KT, YM, EK and NS conducted the experiments. YO supplied non-treated and streamer-discharged pollen. AH and NS wrote the manuscript. HT assisted in data analysis and editing the manuscript.

Figure legend

Figure 1 Effect of non-treated pollen and streamer-discharged pollen on the viability of airway epithelial cells. Cells were exposed to the indicated concentrations of pollen for 24 h. Cell viability was assessed by WST-1 assays. Data are presented as the percentage of the viability of the control. Data are mean ± SD of four individual cultures. $^{*}p<0.05$ versus 0 μg/mL. $^{\#}p<0.05$ versus non-treated pollen.

Figure 2 IL-6 production from airway epithelial cells in response to non-treated pollen and streamer-discharged pollen. IL-6 mRNA level in airway epithelial cell after exposure to pollen for 6 h (A) and the protein level in the culture supernatant after exposure to pollen for 24 h (B) were measured by real-time RT-PCR and ELISA, respectively. Data are mean ± SD of four individual cultures. $^{*}p<0.05$ versus 0 μg/mL. $^{\#}p<0.05$ versus non-treated pollen.

Figure 3 Scanning electron micrographs of non-treated and streamer-discharged pollen. Mushroom-like pollen (A) and many orbicles on their surface (B) are observed in non-treated pollen. In streamer-discharged pollen for 2 days, shrinkage of pollen grains (C) and dull-surfaced and fused orbicles (D) are apparent. Bar 10 μm (A, C); 0.5 μm (B, D)

Figure 4 Endotoxin levels in cedar pollen. The contents of endotoxin non-treated pollen and streamer-discharged pollen were determined by diazo-coupling method. Data are mean of duplicate samples.

Figure 5 The scheme of pollen-induced inflammatory responses and injury in human airway epithelial cells, and the effects of streamer discharge on the health.

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Fig.1

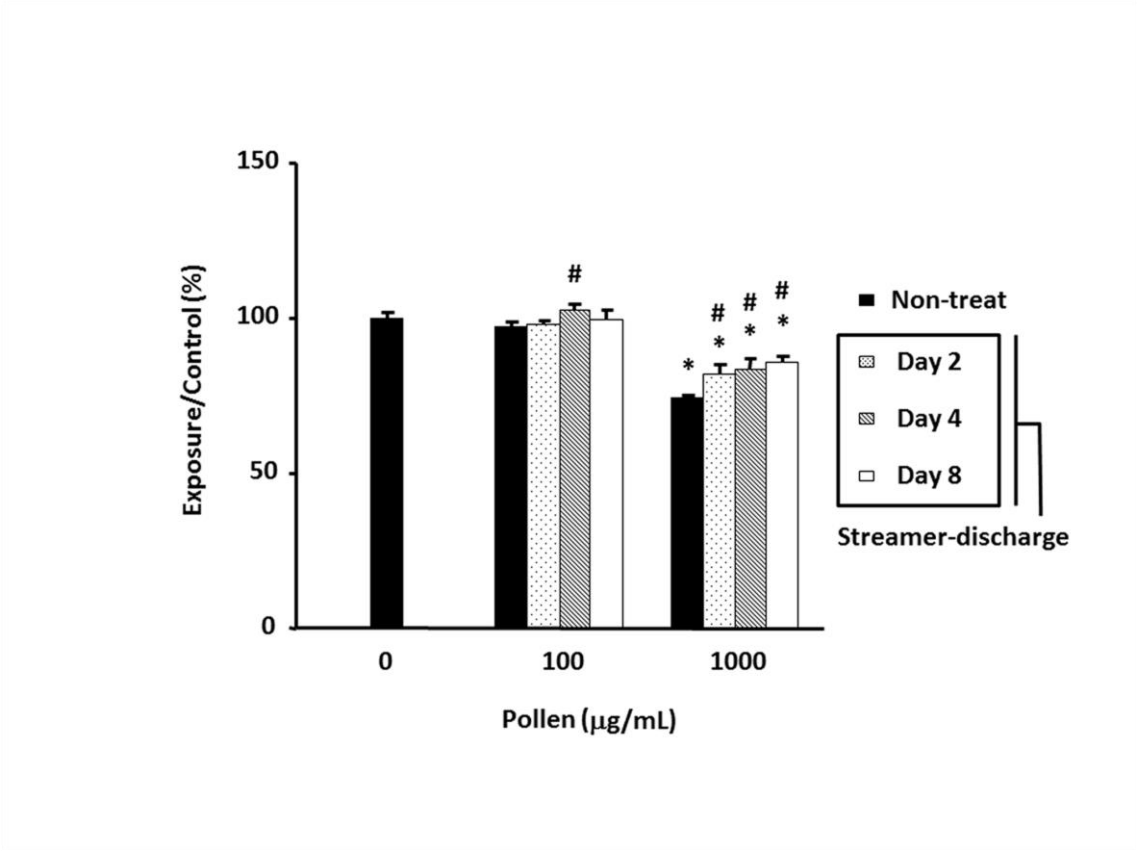

150
100
50
0
Exposure/Control (%)
0
100
1000
Pollen (μg/mL)
Non-treat
Day 2
Day 4
Day 8
Streamer-discharge

Fig.2

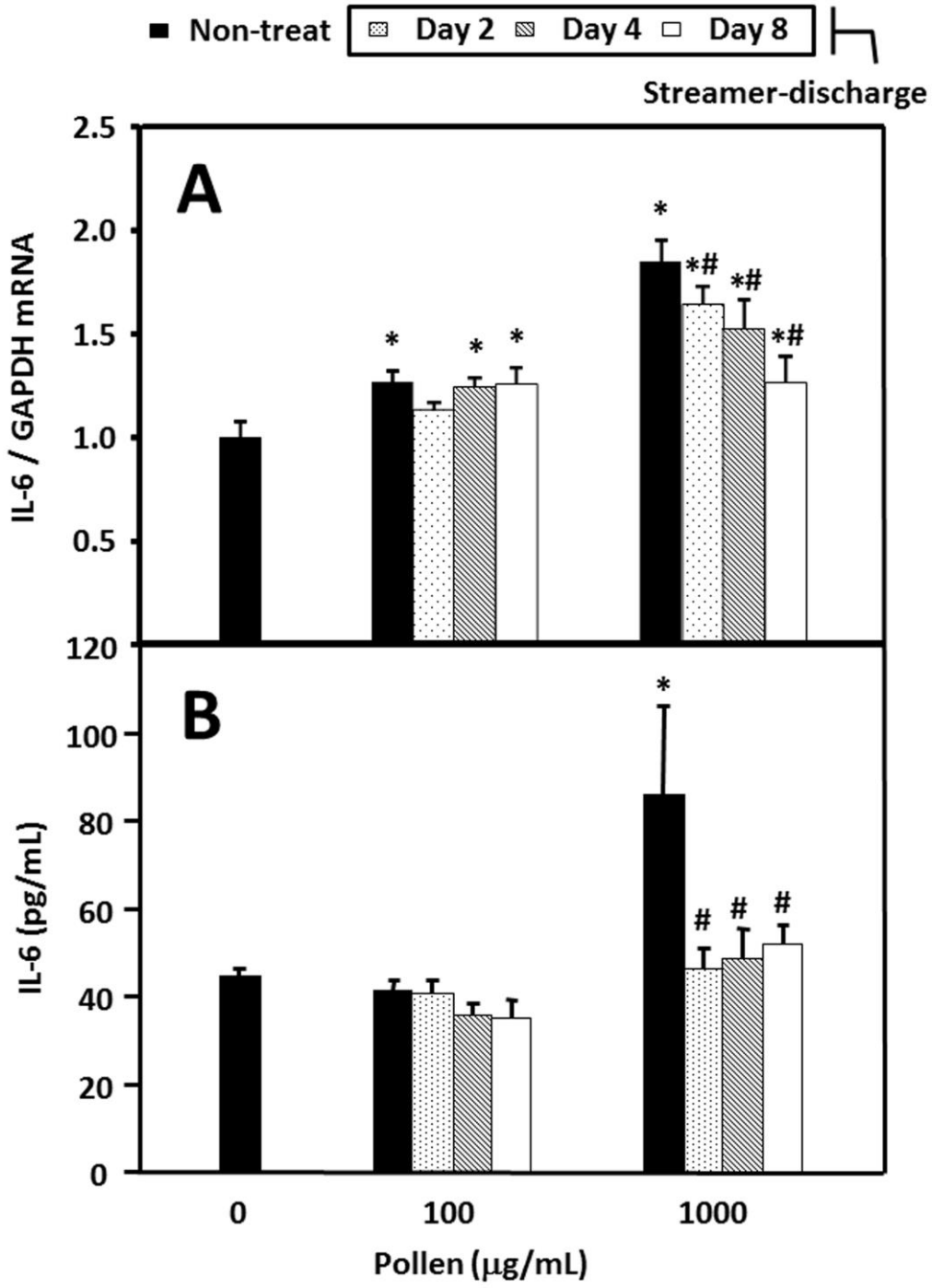

Non-treat
Day 2
Day 4
Day 8
Streamer-discharge
A
B
IL-6 / GAPDH mRNA
IL-6 (pg/mL)
Pollen (μg/mL)
0
100
1000

Fig.3

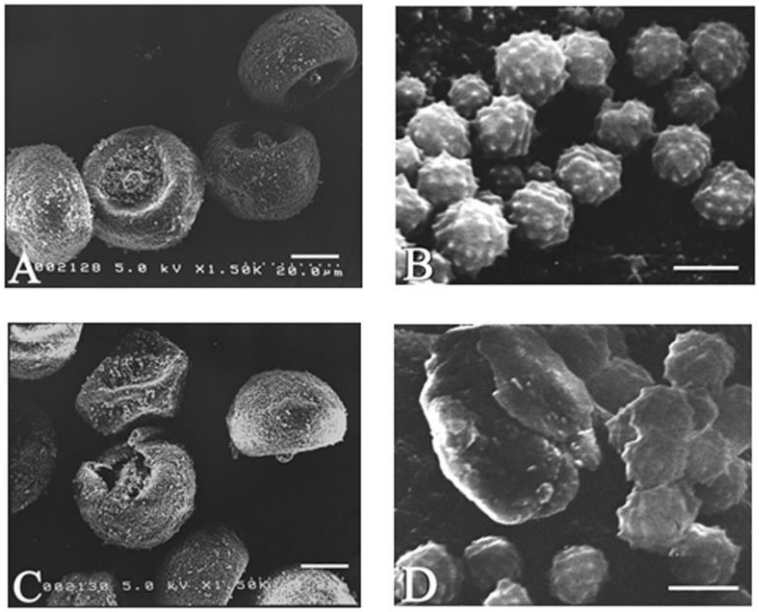


Fig.4

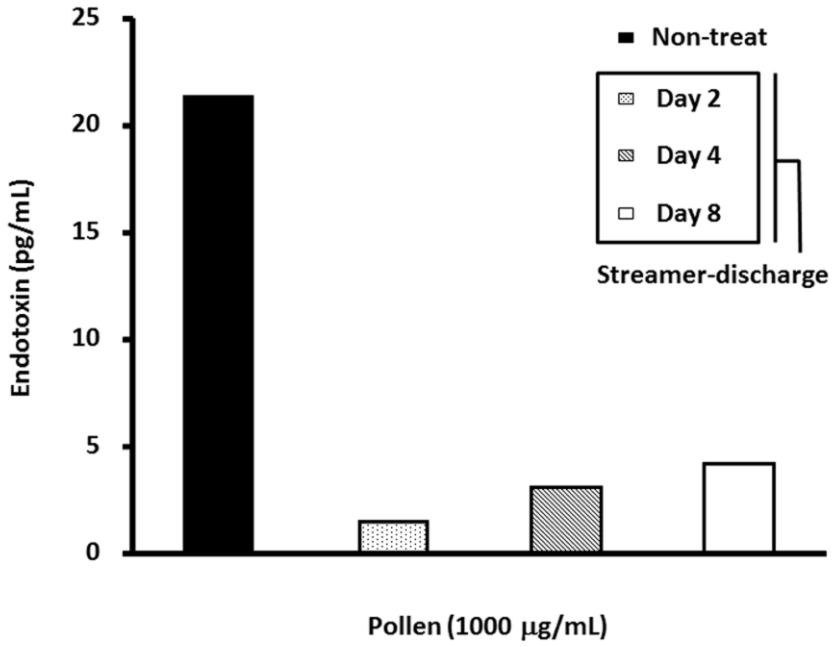
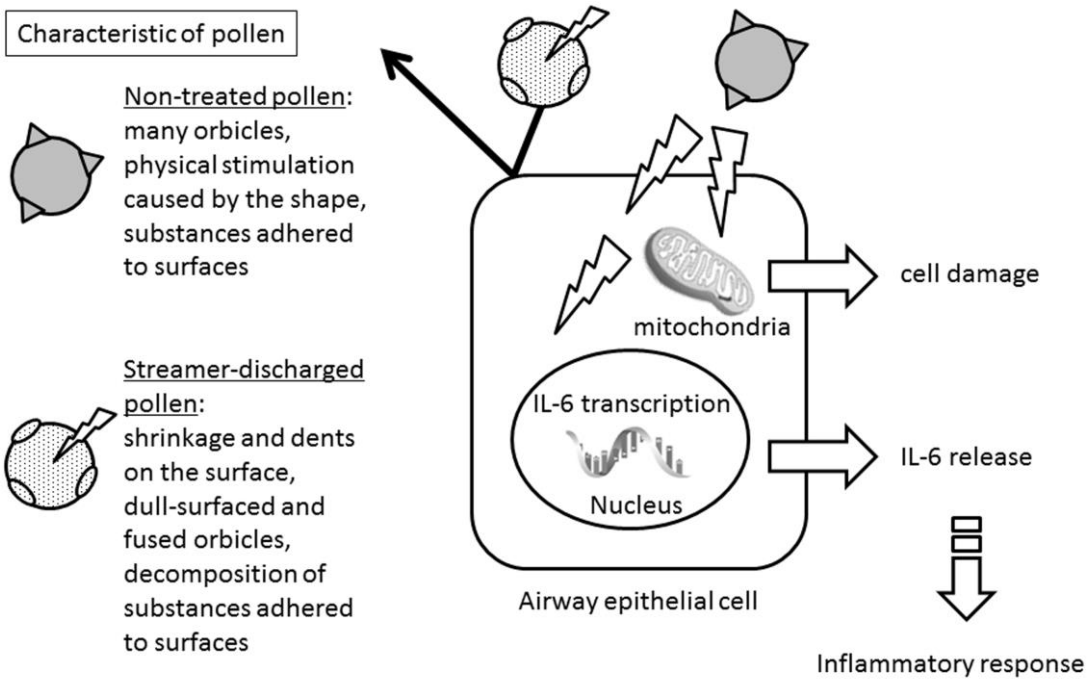

25
20
15
10
5
0
Endotoxin (pg/mL)
Non-treat
Day 2
Day 4
Day 8
Streamer-discharge
Pollen (1000 μg/mL)

Fig.5


Characteristic of pollen
Non-treated pollen: many orbicles, physical stimulation caused by the shape, substances adhered to surfaces
Streamer-discharged pollen: shrinkage and dents on the surface, dull-surfaced and fused orbicles, decomposition of substances adhered to surfaces
mitochondria
cell damage
IL-6 transcription
Nucleus
IL-6 release
Airway epithelial cell
Inflammatory response