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Abbreviations:

AR: Allergic Rhinitis

EEC: Environmental Exposure Chamber

TNSS: Total Nasal Symptom Score

Abstract

Background: A variety of different environmental exposure chambers (EECs) have been used to evaluate treatments for allergic rhinitis.

Objective: To describe and test a system for a homogenous distribution of grass pollen, *Phleum Pratense*, in an EEC to be used for controlled pollen exposure studies in allergic participants.

Methods: A chamber made of stainless steel with completely rounded corners, seating four individuals at a time, was used. Room pressure, temperature, humidity and the air change rate were kept constant throughout the study period.

A rotating pipette dispensed a uniform supply of pollen into a turntable's v-shaped grooves. A stainless-steel capillary tube sucked the pollens into a venturi throat at which time, the pollens were mixed with a high-pressure airstream of compressed HEPA filtered air and then transported to a spreading plate inside the EEC.

To achieve uniform concentrations in the EEC, the turntable's rotating speed was continuously adjusted using information from video-coupled feedback and feed forward mechanisms. Pollen levels were detected using standard volumetric air samplers and laser particle counters. The target pollen exposure level was 1,000 pollens/m³. 21 participants were exposed to pollens in the EEC twice for 210 minutes. Participants evaluated their symptoms every 30 minutes using a total nasal symptom score (TNSS) consisting of blocked nose, runny nose, nasal itching and sneezing.

Results: Across fifteen study days the average pollen level was 982 pollens/m³ (SD, 102 pollens/m³). On average, participants experienced a 10% difference in overall pollen levels between their two visits to the EEC. The mean TNSSs rose throughout the exposure period, with a low at baseline of 0.43 (SD, .68) to a high of 4.71 (SD, 2.43) just before exiting the EEC.

Conclusion and Clinical Relevance: This EEC provides a reproducible, precise and homogenous distribution of pollens making it suitable for single-centre allergy clinical trials.

Introduction

Allergic rhinitis (AR) is estimated to affect roughly 500 million people worldwide (1). Among other elements, AR has been linked to a general impairment in quality of life and loss of productivity (2–4). Although many different treatment options exist, some patients are still under-treated or bothered by drug-related side effects (5,6). Thus, a continuous need for evaluating new allergy treatments exist. As a possible setting for conducting randomized clinical trials on new allergy treatments, environmental exposure chambers (EECs) are generally recognised as safe, effective and suitable for single-centre studies (1,7). EECs are clean and highly controlled facilities dedicated to (human) experimental environmental exposure studies. While a few dedicated allergen challenge chambers exist, most EECs are used for a variety of environmental exposures. The US Food and Drug Administration authorities have endorsed EECs for AR trials and find EECs particularly suited for investigating onset and duration of action as well as short-term effects of novel treatments (8). Other benefits of EECs that contribute to the reduction of confounding factors are the control of exposure levels compared with outdoor trials, the opportunity to conduct studies outside the normal allergy season and the possibility of reproducing study settings (9–11). Since their conception in the 1980s, more than forty peer-reviewed AR trials from EECs have been published (7,12).

However, even though a number of different facilities for AR trials exist, for example ceiling-based pollen feeding systems (7,13,14) or pollen dispersion via a laminar air stream (15), to date, no international standards have been established for validating such facilities (16). Thus, in order to compare research methods and results from different EECs, detailed information on each EEC's system is needed (7,13).

Therefore, this study sought to describe and evaluate a new system for distributing pollen in an EEC in a reproducible, homogenous and precise manner.

Material and Methods

A detailed methodology regarding the clinical trial is reported in a previous article (17). Briefly, the EEC system was used for a single-centre, randomized (1:1), double-blind, placebo-controlled crossover trial (clinicaltrials.gov ID: NCT01699165) conducted in Denmark from December 2012 to February 2013, thus during the grass allergy off-season. The objective was to assess the efficacy of a nasal filter for alleviating or preventing allergic rhinitis. Twenty-four adult participants with confirmed grass pollen-induced allergic rhinitis were included, of these twenty-one were included in the analyses (17). Main inclusion criteria were positive skin prick test response to *P pratense* (wheal diameter of at least 3 mm) and specific IgE for timothy grass pollen of at least 3.5 kU/L. Among other exclusion criteria, subjects were excluded if they had perennial AR unless symptom free, medication-requiring AR caused by other allergens unless symptom free or asthma unless mild and only occurring in relation to the grass pollen season. For all inclusion and exclusion criteria see previous article (17). Written informed consent was obtained from participants. The regional ethics committee approved all relevant trial material. The trial was conducted in accordance with the Helsinki Declaration, the International Conference on Harmonisation Tripartite Guideline for Good Clinical Practice, the European standard "Clinical investigation of medical devices for human participants – good clinical

practice (ISO 14155:2011),” and the requirements of the Danish Health and Medicines Authority, as well as the Danish Data Protection Agency. Participants visited the EEC twice, separated by at least 16 days between each visit. No run-in or priming period was used. Participants were exposed to grass pollens for 210 minutes (minute 0 to 210). They assessed their nasal symptoms at baseline (before entering the EEC), every 30 minutes while in the EEC, shortly after exiting the EEC (minute 220) and three hours later (minute 390) using the total nasal symptom score (TNSS). TNSS is a composite of the symptoms blocked nose, runny nose, sneezing and nasal itching; each evaluated on a 0-3 scale (no symptoms to severe). For this study, only TNSS results from the day the participants wore the placebo device are presented. Participants wore disposable coveralls, shoe covers and nurse caps while in the EEC to avoid contamination. All material that entered the EEC was cleaned before and after exposure either by vacuuming it or wiping it clean. Following each study day, the EEC was vacuumed and washed in distilled water. Visual inspection and baseline measurements (see below) were used to check the cleanliness of the EEC.

Trial settings:

This trial took place in an EEC at the Section of Environment, Occupation and Health, Department of Public Health, Aarhus University, Aarhus, Denmark. This EEC is a 4.15 x 2.9 x 2.7 (length, width, height in meters) room made of stainless steel with completely rounded corners. Four chairs (numbered 1-4) were placed centrally in the room around a table at optimum positions in regard to securing a homogenous distribution of pollen for each participant. The participants sat in the same position on both study days.

General conditions in this EEC:

Pressure was kept constant between 0 and -10 Pascal relative to the ambient pressure, temperature was set at 22 degrees Celsius (SD: 0.5C), humidity was set at 35 % (SD: 5%) and the air change rate was 8.5 times/hour which corresponded to 280 m³ air/hour. The EEC conditions were

controlled with a HVAC system (Schneider Electric SE, Rueil-Malmaison, France) and temperature and humidity recorded with measuring systems from Campbell Scientific Inc. (Logan, UT, USA).

Distributing pollens in an Environmental Exposure Chamber:

The dispersion of pollen was done using a modified small-scale powder dispenser model 3433 (TSI Inc., Shoreview, MN, US) and a TSI turntable with 3 concentric v-shaped grooves (part number 103772). The turntable rotated one round every 40 minutes. Using a rotating modified serological 5 ml pipette (Sarstedt AG & Co, Nürnbrecht, Germany) a uniform supply of pollen was dispensed into the outermost v-shaped groove of the turntable and transported to the dispenser's suction system. The pipette was modified by adjusting the diameter of its opening so that it matched the width of the v-shaped groove. The groove had a width of one mm and a depth of one mm. A miniature DC-powered motor powered the pipette's rotation. The supply of pollens was inspected visually and using dedicated measuring equipment, see below. A stainless-steel capillary tube positioned in the same plane as the top of the v-shaped groove sucked the pollen grains into a venturi throat with the help of a sheath flow that created a clean air supply. The supply and suction points of the turntable were monitored using USB Webcams (König, De Tweeling, Netherlands) directly linked to a live video feed on a nearby PC. The turntable's rotating speed determined the amount of pollen delivered to the dispenser's suction system. Using online particle measurements and 30-minute pollen measurements (both feedback information) and using the USB webcam surveillance system (feed forward information), the rotating speed was continuously adjusted manually to secure a steady pollen level.

The TSI powder dispenser was coupled to a high-pressure injector that mixed pollen grains with compressed HEPA filtered air. The mixture was transported in a specialised tube (Lighthouse Worldwide Solutions, Fremont, CA, USA) that reduced electrostatic attraction. The high-pressure air stream moved the pollen particles to a spreading plate inside the EEC. The spreading plate was placed 12 centimetres above a standard three-blade ceiling ventilator with a diameter of 1 m. The ceiling ventilator's rotating direction was reversed which resulted in an upward moving airstream. This

served two main functions; 1) it increased the dispersion of pollen in the chamber and 2) it reduced the participants' exposure to draughts.

The purpose of the spreading plate was to spread the particles equally around 360 degrees perimeter. The pressurized air propelled the particles a small distance from the spreading plate and into the ventilator blades' jet stream. The ventilator's suction was upward going which helped in keeping the pollen grains in the air for the longest possible time. An exhaust system was established to avoid accumulation of pollens on the floor when they had settled. At the opposite end to the exhaust system, a slot inlet ventilation system provided a cool air stream (cooler than the ambient room temperature) at floor level (up to a height of about 40 cm) that was directed toward the exhaust system. Using a Coanda effect, the air stream ran parallel to the floor from the inlet at one side of the room to the exhaust duct that removed settled/surplus pollen. This method allowed pollens to be removed while avoiding draft conditions above 40 cm. The slot inlet ventilation system also provided an air stream that was sent upwards and across the overhead surface of the room. This air stream was also cooler than the ambient room temperature. This caused the air stream to settle and mix with the air in the room thus allowing for CO₂ ventilation and a more comfortable temperature and humidity for study participants.

The acetone-defatted grass pollens (Phleum Pratense, ALK Abello, Hørsholm, Denmark) were kept at -18 degrees Celsius in a sealed container until use. A 3rd party (Biopol Laboratory Inc., Spokane Wa, US) tested the pollens microscopically for purity. Purity was confirmed when the pollens contained less than .01% foreign pollen, no more than .03% mold spores, and no more than 2% plant parts. The pollens were not tested for endotoxins. The target pollen exposure level was 1,000 pollens/m³.

Method for detecting pollen levels:

Three personal volumetric air samplers (Burkard Manufacturing Co. Ltd, Hertfordshire, England) were used to collect particles onto glass slides. The battery-powered samplers were fully recharged prior to each study day. The samplers had an impaction orifice in the vertical plane, a nominal 10 L/min throughput and an on-off switch at its base. The throughput was assessed once

before study commencement and it was found that all three samplers delivered at 10 L/min. The samplers were set to run continuously and were placed close to the participants' breathing zones. The samplers were placed in such a way that the participants' exhalations would not affect the samplers' particle collection. The glass slides were prepared by applying them with grease. The grease was made using 1 part Vaseline to every 5 parts alpha-hexan. The mixture became liquidated when warmed to 85 degrees Celsius. The glass slides were warmed at 37 degrees Celsius. This helped in applying an even distribution of grease. A Samco Transfer Pipette (Thermo Fisher Scientific, Waltham MA, USA) was used to apply the warmed grease to the glass slides. Glass slides with grease were numbered and colour-coded to uniquely identify study day, interval and sampling position in the EEC.

On every study day baseline pollen levels were recorded for 15 minutes prior to the participants' entry into the EEC. Pollen recordings ran from exposure start and for 30 minutes. Recordings were made 21 times during the 3½ hours of exposure, seven recordings for each of the three air samplers. Every 30 minutes an operator entered the EEC, remove the glass slides from the samplers and inserted new ones for the recordings of the next 30 minutes. The pollens were then counted without colouring at 10x zoom on a standard light microscope (Nikon Eclipse E400, Nikon Corp., Tokyo, Japan). To verify the counted number, the glass slides were coloured using fuchsin dye. The dye was placed in a boiling water bath for approximately 10 minutes following which a transfer pipette was used to transfer two drops of dye to a 24x24mm cover slip (Menzel-Gläser, Gerhard Menzel GmbH, Braunschweig, Germany). The drops were distributed on the cover slip with the tip of the pipette and promptly thereafter placed carefully on the glass slides on top of the visible pollen line.

A Solair 3100 airborne particle counter (Lighthouse Worldwide Solutions, Fremont, CA, USA) counted particles and grouped them into 8 size fractions. Two size fractions, >7.0 to 10.0 µm and larger than 10 µm were counted. The particle counter was not able to distinguish pollens from e.g. dirt and was used solely to give a rough online second-by-second indication of particle levels (as a proxy for pollen levels) and the cleanliness of the EEC. Sample runs of 3.5 minutes were both printed

and logged directly onto a hard drive. Minute-to-minute levels of fine particles (20nm to 1 μm) were recorded with a P-trak Ultrafine Particle Counter (TSI Instruments Ltd., High Wycombe, UK).

Following the completion of the randomized clinical trial, and to further validate a homogenous distribution of pollens in all seating positions, extra tests were carried out. Using the three personal volumetric air samplers, eight iterations of 20-26 minutes were run. The samplers were placed in different sequences at the seating positions. These tests were done without any participants in the EEC.

Simple descriptive statistics were used to summarize data. Time-point specific differences in TNSSs between participants randomized to a placebo device on day 1 versus those randomized to a placebo device on day 2 were evaluated using Wilcoxon rank-sum tests. A P value of less than .05 was used to measure significance. All graphic work was done with Prism 6.0g software (Graphpad Software, La Jolla, Calif).

Results

The 21 participants consisted of 10 women and 11 men. The mean age was 24.9 years (SD, 3.8 years). The mean wheal diameter for the Phleum pratense response was 12.4 mm (SD, 3.8 mm), and mean specific IgE was 39.3 kU/L (SD, 34.3 kU/L). Across the fifteen study days, the mean pollen level during the exposure period was 982 pollens/ m^3 (SD, 102 pollens/ m^3). On average, participants experienced a 10% difference in overall pollen levels between their two visits to the EEC. Time point specific pollen levels are presented in Fig 1. The samplers' recordings were on average within 6% of each other. Also, the mean baseline pollen level was 21 pollens/ m^3 (SD, 20), or less than 2.5% of the mean pollen level during the exposure period.

The mean pre-exposure particle level for particles larger than 10 μm was 181 particles/ m^3 (SD, 85 particles/ m^3). During the exposure period this rose to a mean of 2979 particles/ m^3 (SD, 365 particles/ m^3), see Fig 2.

The mean TNSSs rose throughout the exposure period, see Figs 2 and 3. The lowest score was at baseline with a mean TNSS of .43 (SD, .68), the highest was at minute 180 (the last rating performed in the EEC) with a mean TNSS of 4.71 (SD, 2.43). At minute 220, ten minutes after having ended the pollen exposure, the mean TNSS was 3.81 (SD, 2.84) and three hours after ending the exposure, mean TNSS was 1.33 (SD, 1.46). Non-significant differences in mean TNSSs between participants who received a placebo device on their first visit to the EEC and the participants who received a placebo device on their second visit are shown in Fig 3. Efficacy and safety results related to the evaluation of the nasal filter have been reported in an earlier study (1).

Figs 4 and 5 show time-point specific comparisons of the symptom severity in the EEC with placebo groups from a similarly designed two-day in-season park study (18).

The mean exposure level for ultrafine particles was 118 particles/cm³ (SD, 15 particles/cm³, see Fig 6.

The post-trial validation of exposure levels at each of the seating positions showed an average difference of 9% between the position with the highest pollen level and the position with the lowest level.

Discussion

The objective of this study was to describe and evaluate a new system for exposing patients with AR to a homogenous distribution of airborne grass pollens while being in an EEC. Results of this study demonstrated that the system was able to deliver an average pollen level of 982 pollens/m³ (SD, 102 pollens/m³), close to the pre-study target of 1,000 pollens/m³. The variation was within what is generally accepted in other EEC studies (7,11,13,15,19). The spatial distribution of pollens varied by 9%, similar to what has previously been found (20). All of this made it possible to reproduce typical symptoms of seasonal AR out-of-season during the Danish winter. Consistent with findings from other EECs (16,21), the participants' symptoms rose throughout the exposure period.

This EEC utilized a ceiling-based pollen feeding system that is somewhat comparable to other EECs (7,13,14). It would be interesting to compare this approach with an approach where fans at one end of the room provide a laminar air stream for pollen dispersion (15), for example a comparison of each system's ability to eliminate draughts and achieve control over pollen dispersion. Also, this EEC was kept at a slightly negative pressure to minimize pollen loss. This is known from other EECs (13,15), although some prefer a positive pressure as this should reduce introduction of outside contaminants with the opening of doors (14). As can be seen from Fig 2, the particle count of $>10\ \mu\text{m}$ particles did indeed spike every thirty minutes, corresponding with the time the EEC door was opened and the operator entered the room to remove and replace the glass slides for the pollen samplers. This suggests that other particles were introduced to the EEC at this time or that the increased activity in the room caused more particles to become airborne resulting in spikes that were measurable with the laser counter.

This study had some key differences compared with other studies. Firstly, the target exposure level was low compared with most other EEC studies (7,15,16,21). The level was chosen primarily as a safety precaution as the study team had insufficient information regarding symptom responses when participants were exposed to *P pratense*. The level had been calculated based on a height-to-ground ratio from a Danish setting (22) and on seasonal pollen reports from the Danish Meteorological Institute. When using this ratio, the level in the EEC should thus resemble pollen levels found during the main grass pollen season in Denmark. Previous studies have shown a dose-dependent relationship between symptom severity and pollen levels (13,16) leading some to argue that pollen levels in EECs should be in or above the upper range of naturally occurring grass pollen (7). Also, a recent study using rye grass (*Lolium Perenne*) suggested that 3500 ± 500 grains/ m^3 be considered optimal in eliciting sufficient symptoms when using grass pollens in EECs (16). At 3500 pollens/ m^3 and after three hours of exposure, a mean TNSS of 9.7 was seen (16), the TNSS maximum is 12. At 2500 pollens/ m^3 the mean was TNSS of 7.6 (16). However, an earlier study using a different grass pollen (*Dactylis glomerata*) and a concentration of 4000 ± 300 pollens/ m^3 found a mean TNSS of 4.5 after four

hours of exposure (23), quite similar to this current study's finding of a mean TNSS of 4.71 at 3½ hours. The reason for the discrepancies between exposure levels and symptom severity remain unexplained. All studies were done out-of-season on seemingly similar participants with no priming period or screening exposures to remove low-responders. It may be that the participants had different sensitivities to the pollens. Unlike this current study (17), the other studies did not exclude participants with on-going concomitant allergies (e.g., dust mite allergies) (16,23). This could potentially make participants more reactive in the EEC (11). Variation in allergenic properties of the pollens could perhaps also explain the differences or it could simply be considered a result of random variation. It would, however, be interesting to study in greater detail, as more knowledge on this would increase the transferability of results between different EEC facilities and increase the transferability of results from studies done with different allergens.

Secondly, no run-in priming period was used even though this is typically recommended to increase the participants' sensitivity and decrease inter-participant symptom variability (7,24). A study found that participants can be categorized into groups based on the rate at which they experience symptoms and the degree of their symptom severity in an EEC (11). It was argued that those who were slow/low responders would need more run-in priming to maximize their allergic responsiveness compared with rapid/high responders who might achieve adequate symptoms at only one or two priming visits (11). Further, while one study found little correlation between symptoms measured out-of-season in an EEC with those found outdoors in the season (23), another study found only slightly higher symptoms within the season compared with an out-of-season priming-free EEC study (25). With all of this in mind, it still seems fair to assume that this study's choice of exposure level, its limited length, its lack of priming, its lack of screening EEC visits to remove low-responders and the out-of-season timing all contributed to the generally low symptom severity.

It should also be noted that this study's use of protective clothing could potentially promote a feeling of being in a clinical setting compared with a situation where participants are free to wear their own clothes (17). For this particular study, we used protective clothing to make sure that, once participants had left the EEC and had the nasal filter/placebo removed, they were no longer exposed to pollens. If they had been, it could have affected the outcome of the study and obscured a treatment difference at the post-exposure ratings.

A recent article called for more insight into correlations between controlled allergen challenges in EECs and natural exposures (20). As can be seen in Fig 4, when comparing the time-point specific symptom severity found in this EEC trial with the symptom severity found in a two-day in-season outdoor park study evaluating the same nasal filter (18), the EEC's constant high pollen exposure resulted in a faster and steadier build-up of symptoms. Symptom development on day two in the park was comparable to the development in the EEC if looking solely from minute 210 and onwards, corresponding to the time that pollen levels started rising above 100 pollens/m³. Mean pollen levels in the park study, measured at a similar height to the EEC measurements, were 56.12 pollens/m³ for day one and 140.19 pollens/m³ for day two. Thus, on both days of the in-season study, pollen levels were considerably lower than what was used in the EEC. The second day in the park followed a relatively stable period of high pollen levels whereas day one at the beginning of the season had followed a period with lower pollen levels (18). Reaching similar symptom severities with less than half the exposure could at least partly be explained by the effect of seasonal priming. To our knowledge, no full explanation has been provided regarding the discrepancies between pollen levels in EECs and in-season natural exposures. This supports the need for more knowledge on the transferability of out-of-season EEC studies to in-season natural exposure studies.

Important strengths of this system were the use of both feedback and feed forward mechanisms thus allowing for a continuous fine-tuning of the pollen levels. Also, as can be seen from Figs 2 and 5, the room was clean both pre-exposure and during exposure. In regard to

ultrafine particles, indoor or outdoor concentrations in Scandinavian urban and rural settings have been shown to typically exceed 2000 particles/cm³ (26). During the exposures in this EEC, the mean was 118 particles/cm³, thus considerably lower.

A washout period of at least 16 days was chosen to minimize any priming effect between visits. In a crossover design it is important that disease severity is consistent between visits (10) and it has been shown that priming is reversible within days to weeks (27). The fact that the treatment order did not affect symptoms in any meaningful way, see Fig 3, further underlines that no priming between visits occurred.

In summary, the study confirmed that this EEC setup was able to replicate exposure settings from day to day and secure a homogenous distribution of pollens on each study day. Thus, this EEC provides a reproducible, precise and homogenous distribution of pollens making it suitable for single-centre allergy clinical trials.

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Disclosure of potential conflict of interest:

P. Kenney is inventor of the Rhinix nasal filters and part owner of Rhinix ApS. The rest of the authors declare that they have no relevant conflicts of interest.

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Figure legends:

Figure 1. Mean pollen exposure levels with SEMs as error bars for the three volumetric air samplers (n=15). For some time points, the number of sessions deviated. Symptom severity in the placebo group presented as mean TNSSs with SEMs as error bars. Note that exposure in the EEC was from time point 0 to 210 minutes.

Figure 2. Exposure levels for all particles larger than $10 \mu\text{m}/\text{m}^3$. Data collected every 3.5 minutes. Data presented as means with SEMs as error bars. Symptom severity in the placebo group presented as mean TNSSs with SEMs as error bars. Note that exposure in the EEC was from time point 0 to 210 minutes.

Figure 3. Mean TNSSs with SEMs as error bars, shown by day of wearing a placebo device. Note that exposure in the EEC was from time point 0 to 210 minutes. Symptoms at baseline and 220 minutes and 390 minutes were recorded outside of exposure.

Figure 4. Comparison of symptom severity development between placebo in the EEC with placebo groups in a similarly designed in-season park study (18). Shown as mean TNSSs with SEMs as error bars. Park study participants include those with baseline TNSSs of 0 or 1 (Day 1, N=18 and Day 2, N=9). **A**, Diurnal changes in grass pollen concentration for the 2 park study days, courtesy of (18).

Figure 5. Comparison of symptom severity development between placebo in the EEC with placebo groups in a similarly designed in-season park study (18). Shown as mean TNSSs with SEMs as error bars. Park study participants include all placebo users on each day regardless of their baseline values (Day 1, N=34 and Day 2, N=31).

Figure 6. Minute-by-minute counts of ultrafine particles (20 nm to 1 μ m) per cm^3 during the exposure period. Data presented as means with SEMs as error bars.







