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ORIGINAL ARTICLE



Extracts of different pollen species and their effect on human tear fluid and an epithelial cell line

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ABSTRACT

Purpose: Hazelnut and birch pollen are known to destroy tear film components and attack ocular surface cells. We investigated further pollen species from different plant families, whether they show similar effects on human tear fluid and an epithelial cell line *in vitro*, to provide a broad basis for further research on pollen reactions affecting the tear film and ocular surface.

Materials and methods: Regional pollen species from different plant families (Adoxaceae, Betulaceae, Fagaceae, Juglandaceae, Malvaceae, Oleaceae, Pinaceae, Plantaginaceae, Poaceae, Salicaceae, Sapindaceae) were collected. Their proteolytic activity was evaluated by Zymography. Human tear fluid and cells of an epithelial cell line were incubated with pollen extracts. Tear fluid was analyzed by Polyacrylamide gel electrophoresis (PAGE). Cytomorphology was assessed microscopically and cell viability by proliferation (MTS), water-soluble tetrazolium (WST-1) assay and the impedance-based xCELLigence real-time analysis (RTCA).

Results: Zymography revealed significant protease activity and PAGE showed the degradation of tear proteins by different pollen species. Cells incubated with pollen extracts presented dose- and time-dependent cytomorphological changes. MTS, WST-1, and RTCA revealed cytostatic as well as cytotoxic effects of pollen extracts.

Conclusions: Pollen species from different plant families exert proteolytic activity and degrade human tear fluid as well as epithelial cells, which may play a crucial role in the pathogenesis of allergic and non-allergic reactions affecting the ocular surface.

ARTICLE HISTORY

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KEYWORDS

Pollen species; tear fluid; epithelial cells; seasonal non-allergic conjunctivitis

Introduction

During the pollen seasons, a lot of people suffer from red-denied, watery, itchy, and sticky eyes. Very often these symptoms can also be observed among proven non-allergy sufferers and patients with ocular surface disease, described as seasonal non-allergic conjunctivitis (SNAC) syndrome¹, resembling the seasonal non-allergic rhinitis (SNAR) syndrome proposed by Eriksson and Wedback^{2,3}.

The SNAC syndrome might occur due to the influence of pollen enzymes, especially proteases, on the tear film and the ocular surface. SNAC might induce a non-classical allergic inflammatory reaction and also affects proven non-allergy sufferers, at least according to today's common allergy tests, e.g. negative RAST and prick test^{1–4}.

It has not been totally understood, how an inflammatory response starts following the exposure to pollen grains. After hydration with body fluids, pollen usually releases different allergens, proteins, lipids, as well as prostaglandin E₂- and leukotriene B₄-like substances, Nicotinamide adenine dinucleotide phosphate oxidases and enzymes, like proteases.

These may facilitate the breakdown of secretions lining mucosal membranes and epithelial barriers and, therefore, act as non-allergic inflammatory mediators^{5–9}.

There are numerous pollen proteases, which are thought to be contributing to the pathogenesis of pollen reactions affecting the human body. Ambrosia, mesquite, and pollen of *Parietaria judaica* have been revealed to release distinctive proteases disabling lung regulatory neuropeptides⁵, degrading the intercellular adhesion proteins from tight junctions and adherence junctions, leading to a detachment of epithelial cells^{5,10}. The incubation of epithelial cell lines with pollen extracts of *Ambrosia trifida*, *Betula*, *Lilium longifolium* and *Poa pratensis* resulted in a loss of immunofluorescence labelling of tight junction proteins⁵.

Hazelnut and birch pollen are already known to destroy tear film components and attack ocular surface cells¹. We investigated further pollen species from different plant families, whether they show similar effects on human tear fluid and an epithelial cell line *in vitro*, to provide a broad basis for further research on pollen reactions affecting the tear film and ocular surface.

Table 1. Pollen collection, zymography and PAGE results of different pollen species.

Species	Clade	Order	Family	Zymographic pattern	PAGE (Pollen)	PAGE (Pollen + Tears)
Birch	Rosids	Fagales	Betulaceae	+	+	^a
Common hazel	Rosids	Fagales	Betulaceae	+	+	^a
Common timothy	Commelinids	Poales	Poaceae	+	+	^a
Corkscrew hazel	Rosids	Fagales	Betulaceae	+	+	^a
Elder	Asterids	Dipsacales	Adoxaceae	+	+	^a
European alder	Rosids	Fagales	Betulaceae	+	+	^a
European ash	Asterids	Lamiales	Oleaceae	+	+	^a
European chestnut	Rosids	Fagales	Fagaceae	+/-	+/-	+/-
European hornbeam	Rosids	Fagales	Betulaceae	+/-	+/-	+/-
European silver fir	Pinopsida	Pinales	Pinaceae	+	+	^a
Golden foxtail grass	Commelinids	Poales	Poaceae	+	+	^a
Greater plantain	Asterids	Lamiales	Plantaginaceae	+	+	^a
Horse chestnut	Rosids	Sapindales	Sapindaceae	+	+	^a
Korean fir	Pinopsida	Pinales	Pinaceae	+	+	^a
Lime tree	Rosids	Malvales	Malvaceae	+	+	^a
Maple	Rosids	Sapindales	Sapindaceae	+/-	+/-	+/-
Orchard grass	Commelinids	Poales	Poaceae	+	+	^a
Scots pine	Pinopsida	Pinales	Pinaceae	+	+	^a
Walnut	Rosids	Fagales	Juglandaceae	+	+	^a
White willow	Rosids	Malpighiales	Salicaceae	+	+	^a

+ clear bands visible, +/- weak bands visible, ^adestruction of tear fluid proteins.

Materials and methods

From 2010 to 2016, we established a collection of regional pollen species at the Department of Ophthalmology of the Medical University of Graz, Austria. Male inflorescences, which contain pollen, were collected from plants in the South East of Styria in Austria. We tried to collect a variety of pollen species from different plant families (Adoxaceae, Betulaceae, Fagaceae, Juglandaceae, Malvaceae, Oleaceae, Pinaceae, Plantaginaceae, Poaceae, Salicaceae, Sapindaceae) in the local area. The male inflorescences were dried, sieved, and stored in paper bags at room temperature as previously described¹.

Table 1 gives an overview of pollen, that have been collected and stored for further analysis.

The different pollen extracts were prepared and analyzed according to established methods, published in our previous study on hazelnut and birch pollen, which may be referred to for further details¹. Additionally, these methods were supplemented by a water-soluble tetrazolium (WST-1) assay and the impedance-based xCELLigence real-time cell analysis (RTCA).

Zymography

Zymography was performed to examine the proteolytic activity of pollen with a 10% zymogram gelatin gel by Invitrogen Life Technologies Corporation, Carlsbad, USA. The zymographic spectra were documented by a Minolta, RD 175 digital-camera¹.

Tear fluid

After approval by the local Institutional Review Board (IRB) of the Medical University of Graz, Austria, tear fluid was collected from five healthy volunteers with normal tear function (median age 57 years, range 25–69 years, 4 females, 1 male), as well as three subjects with known hay fever (median age 41 years, range 31–57 years, 2 females, 1 male). All subjects gave informed consent. The study has been conducted according to the principles expressed in the Declaration of Helsinki.

Fifty microliters of reflex tears were collected from the lower lateral tear meniscus of both eyes with a capillary tube after stimulation with China mint oil applied to the skin around the zygomatic bone. The samples of each subject were pooled into plastic microtubes (Brand GmbH, Wertheim, Germany) and used for the experiments^{1,11}.

Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis of pollen extracts, tear fluid and pollen-tear fluid mixtures were performed after an incubation at 37 °C for 1 h by our established methods with NuPAGE 4–12% Bis-tris gels, 1.0 mm × 10 well (Invitrogen Life Technologies Corporation, Carlsbad, USA) at 200 V, 78 mA, for 35 min. To estimate the molecular weight of the proteins, a protein standard (Novex® Sharp Unstained Protein Standard, Life Technologies, Carlsbad, California, USA), consisting of 12 protein bands in the range of 3.5–260 kDa, was used. The patterns were documented by a Minolta, RD 175 digital-camera and compared to each other¹. All experiments were performed in duplicate and repeated twice.

Pollen extracts for experiments with human epithelial cells

Pollen extracts with a concentration of 5 mg and 25 mg pollen per mL medium (Dulbecco's Modified Eagle Medium by Invitrogen, Carlsbad, California, USA and 1% Penicillin/Streptomycin by Biochrom, Berlin, Germany) were generated for the cell experiments. The concentrations were chosen according to our prior experiments on hazelnut and birch pollen extracts¹.

Human epithelial cells

A human epithelial cell line (CHANG cells, CCL-20.2, clone 1-5c-4m, Wong-Kilbourne derivatives of CHANG conjunctiva by The American Type Culture Collection, ATCC Co., Manassas, VA, USA) was used.

This cell line was originally thought to be derived from normal epithelial conjunctiva but meanwhile was found to

have been established via HeLa cell contamination. According to the International Cell Line Authentication Committee's register of misidentified cell lines, Wong-Kilbourne is not a conjunctival cell line but is contaminated by HeLa (<http://iclac.org/databases/cross-contaminations/>).

The cells are positive for keratin and they grow adherently. As soon as the cells were at least 90% confluent, they were used for the experiments¹. To receive a cell suspension with the desired cell density, cell count was determined by the CASY[®]1 Cell Counter + Analyser System – Model TT from Schärfe System GmbH, Reutlingen, Germany. Cell suspensions with an empiric cell density of 30 000 cells/mL for the MTS¹ and WST-1 assay, as well as 100 000 cells/mL for the RTCA system were created.

Determination of cell viability by MTS assay

Cell viability was determined after 5 and 24 h of incubation with pollen extracts at 37 °C by the Cell Titer 96[®] Aqueous One Solution Cell Proliferation Assay (MTS) from Promega, Madison, WI, USA, a colorimetric method for determination of the number of viable cells in proliferation, cytotoxicity or chemosensitivity assays¹. All experiments were performed at least in triplicate and repeated twice.

Determination of cell viability by WST-1 assay

The WST-1 Cell Proliferation Assay from Roche Applied Science, Roche Diagnostics GmbH, Mannheim, Germany was used. A water-soluble tetrazolium, the sodium salt of 4-[3-(4iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) is cleaved to formazan by cellular enzymes. The amount of formazan dye directly correlates to the number of active cells and was quantified at a wavelength of 450 versus 650 nm reference by a scanning multi-well spectrophotometer¹² (Anthos 2010, ADAP-Software from Anthos Labtec Instruments GmbH, Krefeld, Germany).

The experiments were performed in 96-well-microtiter plates (Becton Dickinson, Franklin Lakes, NJ, USA) according to the manual. All experiments were performed at least in triplicate and repeated twice.

Measurement of cell proliferation by real-time cell analysis (RTCA)

The xCELLigence RTCA from ACEA Biosciences, San Diego, CA, USA provides a label-free, dynamic monitoring of phenotypic changes of cells in real time using electrical impedance data.

The measurements of electrical impedance are carried out via interdigitated micro-electrodes integrated into the bottoms of cell culture E-plates (Omni Life Sciences, Bremen, Germany). The impedance data are displayed as a cell index (CI) value, providing quantitative information on the cells' biological status, including the cell number, viability and morphology. Usually, the real-time monitoring of cell proliferation and viability by electrical impedance measurements is known to correlate well with other cell number and viability assays (e.g. WST-1, MTT)¹³.

For correction of the background absorbance, 100 µL of cell culture medium was pipetted into the 16 wells of the E-Plate. One-hundred-microliter of the cell suspension with a concentration of 100 000 cells/mL, equaling 10 000 cells/well, was added to each well.

At the beginning of the experiments, cells were incubated on the xCELLigence station inside the incubator for 24 h. Every 20 min, electrical impedance was measured to assess cell proliferation and adhesion. On the next day, cell medium was removed and the wells were washed with DPBS once. After that, 100 µL of the pollen extracts with concentrations of 5 mg and 25 mg pollen per mL medium were added accordingly. The incubation of the control cells was performed with DMEM diluted with physiological saline at a 1:1 ratio. The electrical impedance was measured at 20-min intervals for another 48 h. In summary, the electrical impedance of the cells was measured for 72 h on three consecutive days.

Measurement data were displayed as impedance change over time, referred to as CI. The curves were normalized to the point of time before addition of the pollen extracts. All experiments were performed in duplicate and repeated twice.

Statistical analysis

Cell viability of the human epithelial cells was calculated in percent on basis of the respective assay's extinctions. The extinction of the control cells of each microtiter plate were defined as 100% cell viability for the particular microtiterplate¹.

Cell viability is given as mean with standard deviation (SD). Additionally, results for 20 pollen species were pooled into 11 plant families as a biological classification.

To assess the effect of the added pollen of the 11 plant families on the viability of the Chang cells we conducted 4 General Linear Models (GLM) for each of the two exposure time conditions (5 h, 24 h) in the two pollen concentration conditions (5 mg, 25 mg). Dunnett's tests comparing each of the 11 families with the Control condition (no additive) were used as post-hoc tests to detect significant effects of the added pollen.

To test for differences in MTS between the 11 families and the 4 conditions we used a linear mixed effects model (LMM) with family, exposure time (5 h, 24 h) and concentration (5 mg/mL, 25 mg/mL) as fixed factors.

A *p*-Value of less than 0.05 was considered as statistically significant, all post-hoc tests of GLMs and LMM were corrected for multiple comparisons with Dunnett's or Bonferroni correction, respectively.

To compare the means of the MTS and WST-1 assays for the 20 species, the SDs and Coefficients of variation (CV%) were juxtaposed.

Results

Zymography revealed that the pollen used for the experiments contain active proteases, which possess enzymatic activity. The zymographic spectra of the pollen species differed from each other. Figure 1 shows the zymography of birch and orchard grass pollen.



Figure 1. Zymography pattern of birch pollen (lane 1, 2) and orchard grass (lane 3, 4). Both pollen species show proteolytic activity by the presence of visible bands.

PAGE revealed the degradation of tear fluid proteins by pollen species from different plant families. [Table 1](#) gives an overview of the zymography and PAGE results of the different pollen species. The bands at the respective molecular weights were reproducible throughout the experiments. [Figure 2](#) shows the PAGE of different pollen species.

As an example, for the proteolytic activity of pollen, [Figures 3](#) and [4](#) show the electrophoresis-spectra of different pollen extracts, tear fluid, and tear fluid incubated with pollen extract, respectively. Interestingly, after incubation of tear fluid with pollen extracts, single bands of the electrophoresis-spectrum of tear fluid disappeared and a few new bands appeared (arrows). This indicates that pollen proteins exert proteolytic activity destroying tear fluid proteins in allergic as well as non-allergic subjects. Similar results can be obtained using other pollen extracts.

Microscopic evaluation of the cells treated with pollen extracts showed severe structural changes compared to the control. This included cell swelling, the formation of cytoplasmic vacuoles, karyorrhexis, and even the disruption of cell membranes in some of the cells.

MTS and WST-1 assay

The MTS- and WST-1-analysis of pollen revealed that pollen possesses spontaneous dehydrogenase activity. Therefore, the use of two different blanks was necessary to cut out the reagent basic activity and the pollen dehydrogenase activity, respectively. Both blanks were deducted from the corresponding control wells and sample wells.

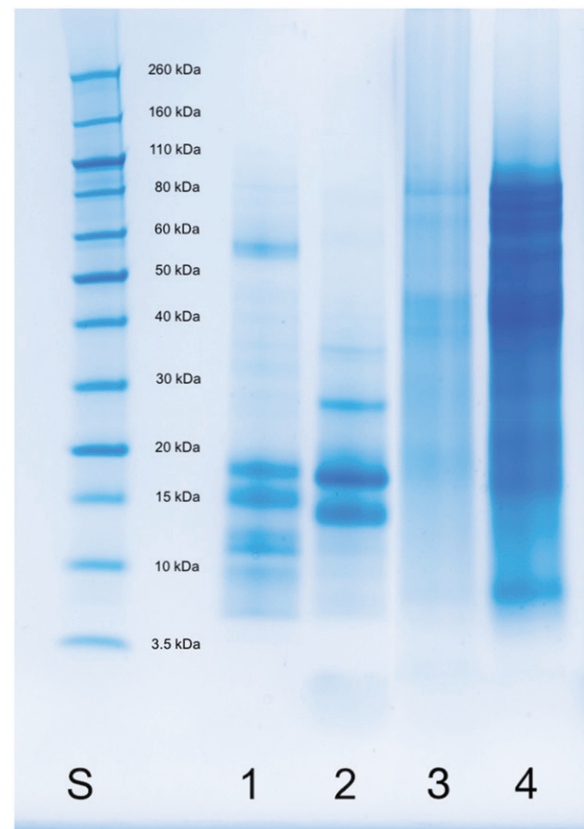


Figure 2. PAGE of Common hazel (1), European alder (2), White willow (3), European ash (4) and Protein Standard (S) to estimate the molecular weight of the proteins by comparison of their visible bands. The electrophoresis spectra of pollen species differ from each other. Pollen species contain different compositions and amounts of proteins.

Changes in cell viability of human epithelial cells incubated with 5 mg/mL or 25 mg/mL of pollen extracts for 5 and 24 h, respectively, could be observed. The extend of the effect depended on the concentration of the pollen extract and incubation time applied.

The results of the MTS and WST-1 assays were comparable, while the WST-1 results for cell viability at a concentration of 25 mg/mL were by trend lower than the MTS results ([Figures 5–8](#)).

At a concentration of 25 mg/mL and after an incubation time of 24 h, a decrease of cell viability compared to control could be observed for all pollen species, except for European silver fir and Korean fir by the MTS assay. Nevertheless, the WST-1 assay showed a reduction in cell viability compared to the control.

The pooled results of the 20 pollen species into their 11 different plant families as a biological classification, revealed that the incubation of human epithelial cells with pollen extracts from Pinaceae ($p = 0.001$), Betulaceae ($p = 0.009$) and Juglandaceae ($p = 0.048$) at a concentration of 5 mg/mL statistically significantly increased cell viability after 5 h of incubation. This effect did not remain significant with an increased pollen concentration of 25 mg/mL or 24 h of incubation.

RTCA

The assessment of cell proliferation via electrical impedance measurements by the xCELLigence RTCA system showed a

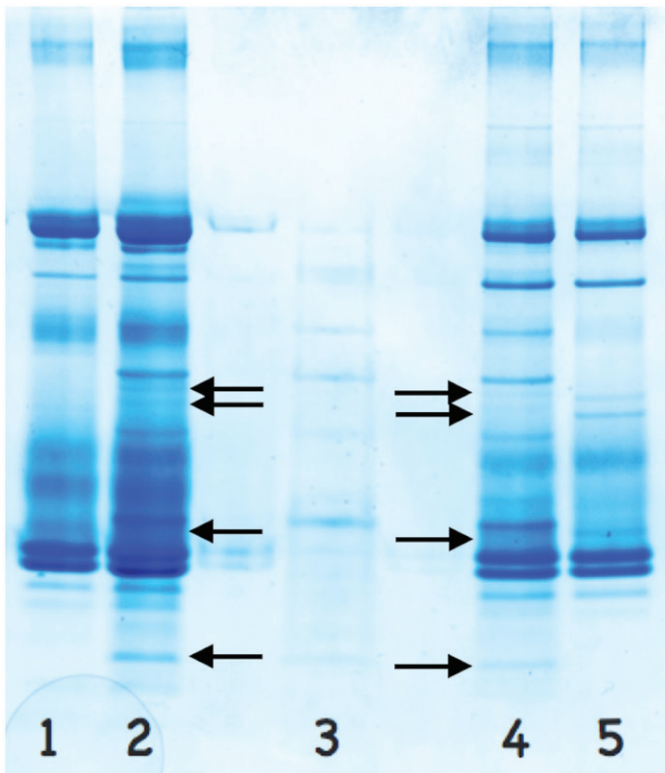


Figure 3. PAGE of Corkscrew hazel. 1 = Tears (Non-Allergic subject), 2 = Tears (Non-Allergic subject)+Pollen, 3 = Pollen, 4 = Tears (Allergic subject)+Pollen, 5 = Tears (Allergic subject). In comparison to the lanes 1 and 5 single bands of the electrophoresis-spectrum of tear fluid disappeared and a few new bands appeared (arrows) within the lanes 2 and 4. This indicates that pollen proteins exert proteolytic activity destroying tear fluid proteins in allergic as well as non-allergic subjects.

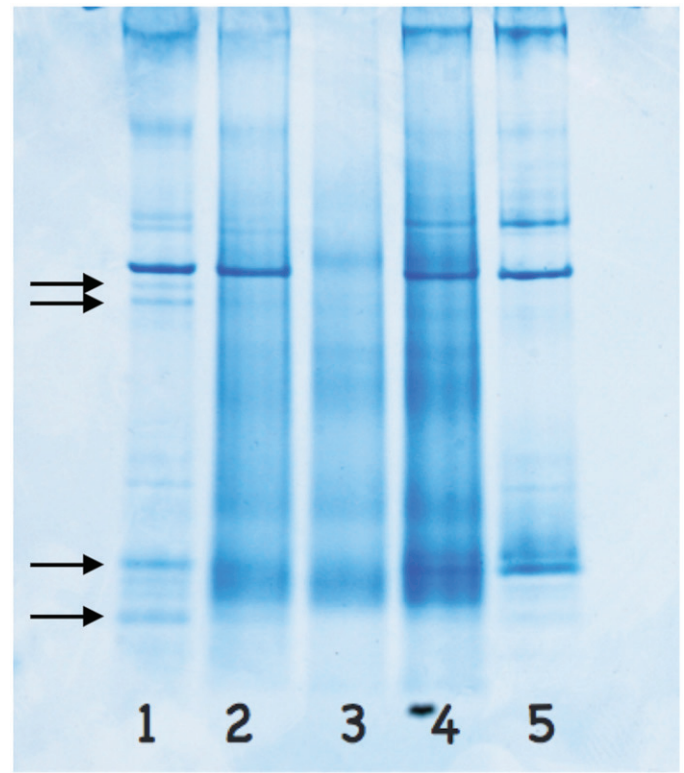


Figure 4. PAGE of Common timothy. 1 = Tears (Non-Allergic subject), 2 = Tears (Non-Allergic subject)+Pollen, 3 = Pollen, 4 = Tears (Allergic subject)+Pollen, 5 = Tears (Allergic subject). In comparison to the lanes 1 and 5 single bands of the electrophoresis-spectrum of tear fluid disappeared and a few new bands appeared (arrows) within the lanes 2 and 4. This indicates that pollen proteins exert proteolytic activity destroying tear fluid proteins in allergic as well as non-allergic subjects.

marked influence of the different pollen extracts on cultivated epithelial cells. The control cells showed a normal proliferation according to the growth curve and CI. In contrast, the incubation of epithelial cells with pollen extracts induced alterations of cell proliferation, adhesion and viability (Figures 9 and 10). Dose depended cytostatic and cytotoxic effects of the different pollen species could be observed. European silver fir pollen showed the weakest, whereas Lime tree pollen showed the strongest effect on the cell growth curves and CI. Table 2 provides a summary of the RTCA results.

Discussion

Pollen is well-known as an important trigger for different kinds of diseases, which have a significant influence on the health care system. It is thought, that pollen-induced reactions represent an important group of diseases affecting approximately 40% of the people, becoming more prevalent during the last decades^{14,15}. Allergy-like symptoms can be observed among allergic as well as non-allergic subjects during pollen seasons. Whether the enzymatic pollen reactions, affecting secretions lining mucosal membranes and mucosal epithelia, represent a separate entity or a subtype of an allergic disease has still to be determined. The number of people suffering from the hypothesized SNAR and SNAC syndromes may be underestimated and mixed forms of allergic and non-allergic reactions might occur as well^{1,4,16}.

From an ophthalmological point of view, pollen reactions primarily affect the tear film and the ocular surface of the human eye. A healthy ocular surface usually forms a protective barrier against environmental influences and also acts as an immune-competent wall against infectious agents by its close connection to the vascular and lymphatic system¹⁷⁻¹⁹. Therefore, it seems only logical, that ocular surface disorders, like dry eye, can be influenced by the presence of pollen. There are distinctive subtypes of dry eye disease due to different pathophysiological pathways^{17,20-24}. Generally, these occur when inadequate tear volume or function (e.g. hyper evaporation) results in an unstable tear film with hyperosmolarity and inflammation, consecutively leading to an ocular surface disease^{17,20-22}. While pollen enzymes could trigger dry eye disease by destroying important tear film components, dry eye disease itself can also facilitate the contact of pollen allergens and enzymes with the ocular surface, when there is little or only nonfunctioning tear film left^{16,23,25,26}. A disruption of the corneal epithelial barrier has been found in dry eye as well, which might make those patients even more susceptible to enzymatic inflammatory reactions caused by pollen²⁷.

Dried pollen getting in contact with any fluid, immediately bloat and release their content through small pores in their outer membrane into the environment. Our biochemical investigation of pollen species from different plant families confirmed the presence of various pollen proteins among

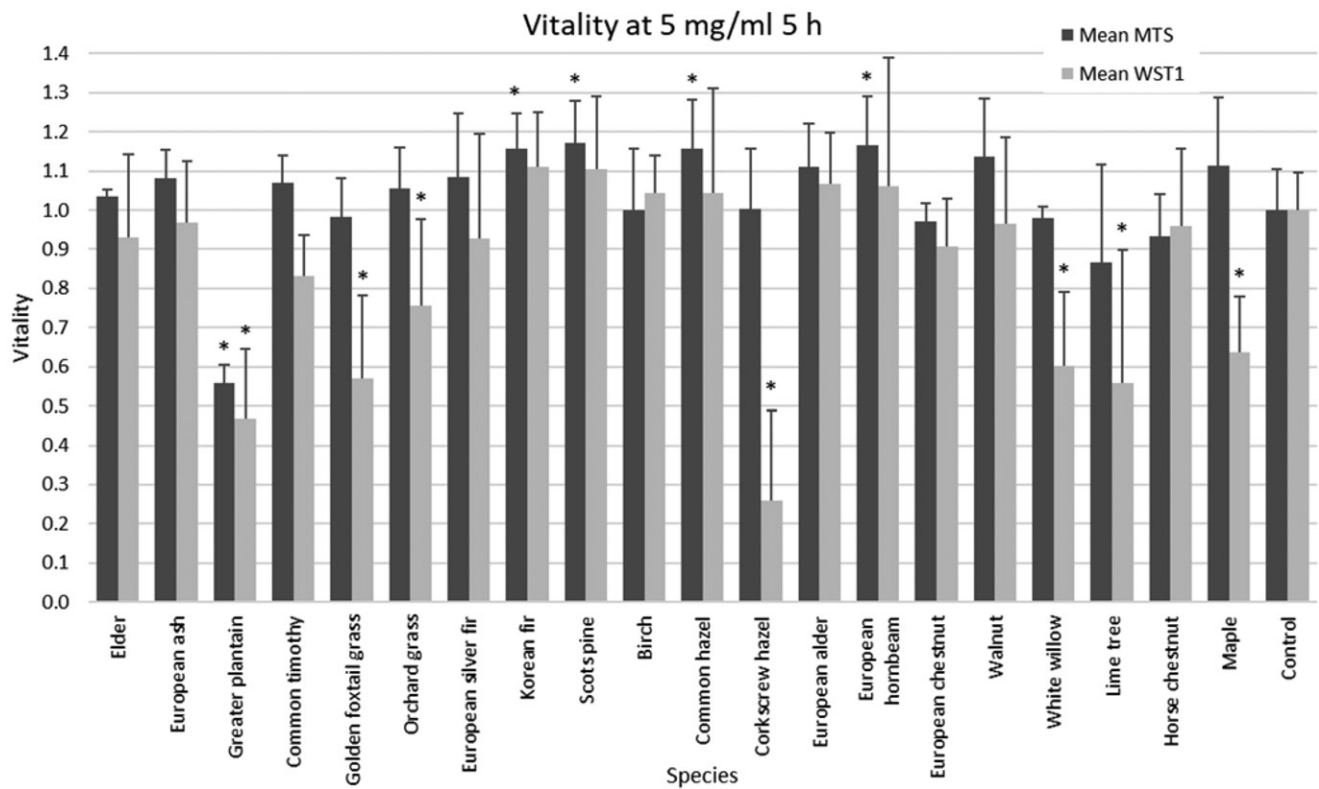


Figure 5. Cell viability after incubation with 5 mg/mL pollen extracts for 5 h. Statistically significant changes compared to the viability of the control are marked with an asterisk.

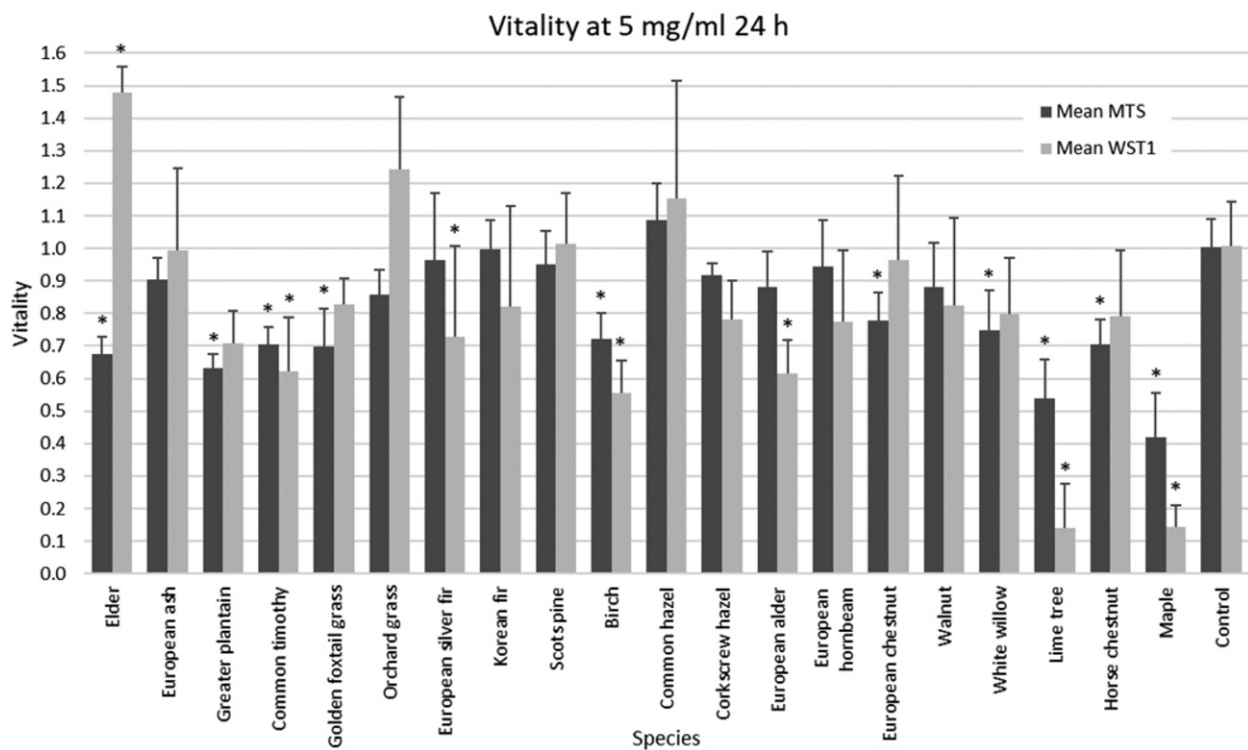


Figure 6. Cell viability after incubation with 5 mg/mL pollen extracts for 24 h. Statistically significant changes compared to the viability of the control are marked with an asterisk.

other substances. Zymography identified some of the proteins as proteolytically active proteases. Depending on the type of proteases, their enzymatic activity varied^{1,16,28–31}. PAGE revealed the degradation of tear fluid proteins by most

of the pollen species from different plant families. Interestingly, there were almost no visible bands in the zymography and PAGE of European chestnut, European hornbeam and Maple. A possible explanation

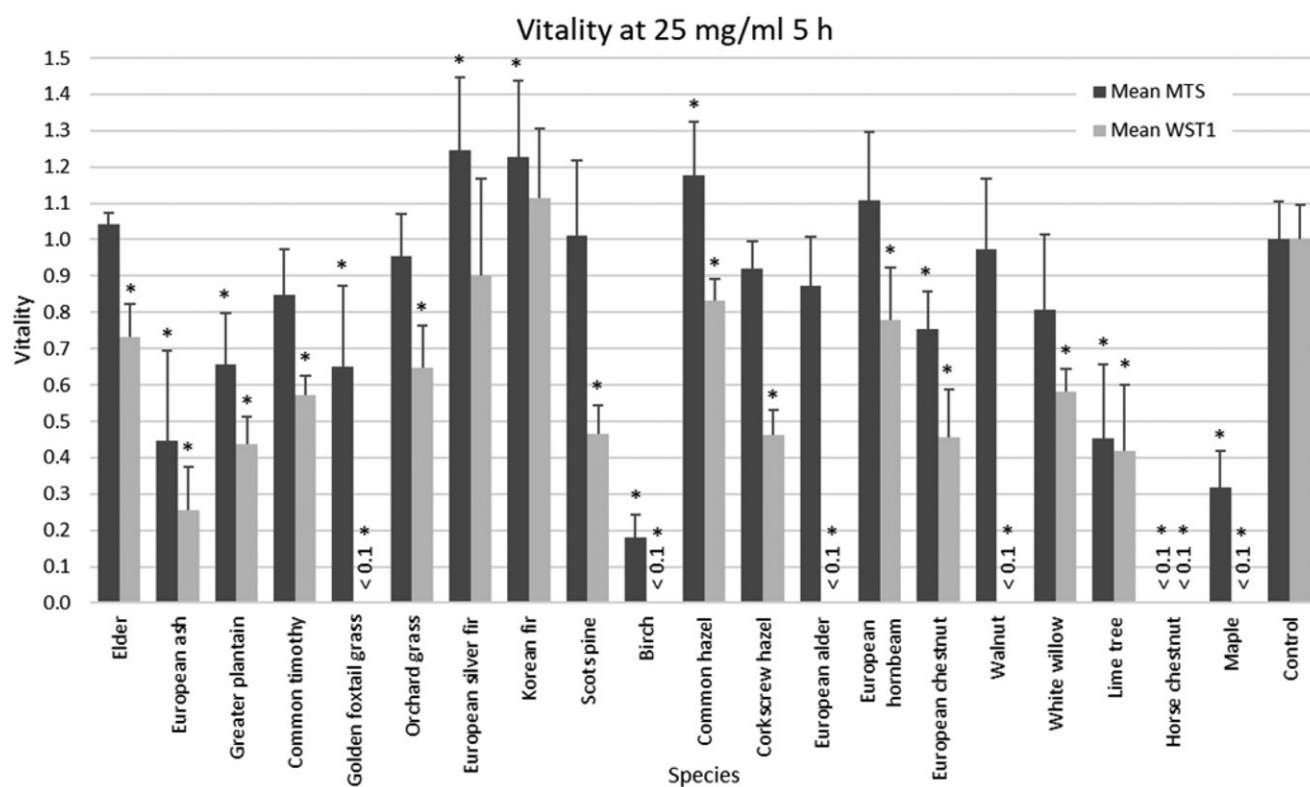


Figure 7. Cell viability after incubation with 25 mg/mL pollen extracts for 5 h. Statistically significant changes compared to the viability of the control are marked with an asterisk.

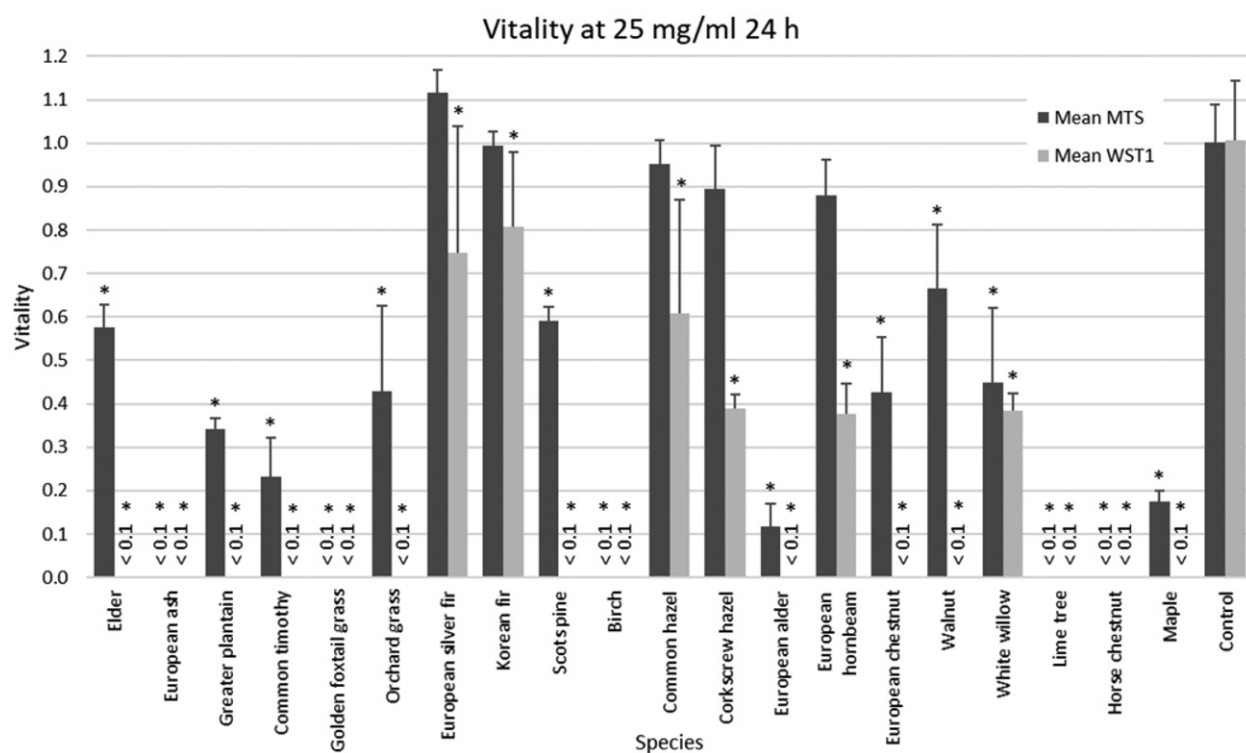


Figure 8. Cell viability after incubation with 25 mg/mL pollen extracts for 24 h. Statistically significant changes compared to the viability of the control are marked with an asterisk.

might be the increased presence of lipids in the coating of some of the pollen species compared to others, thus making it more difficult to obtain the content of the pollen for the generation of pollen extracts. A limitation of our

study is, that only tear fluid from a few subjects has been studied. Especially, for the comparison between allergic and nonallergic subjects a larger sample size is warranted.

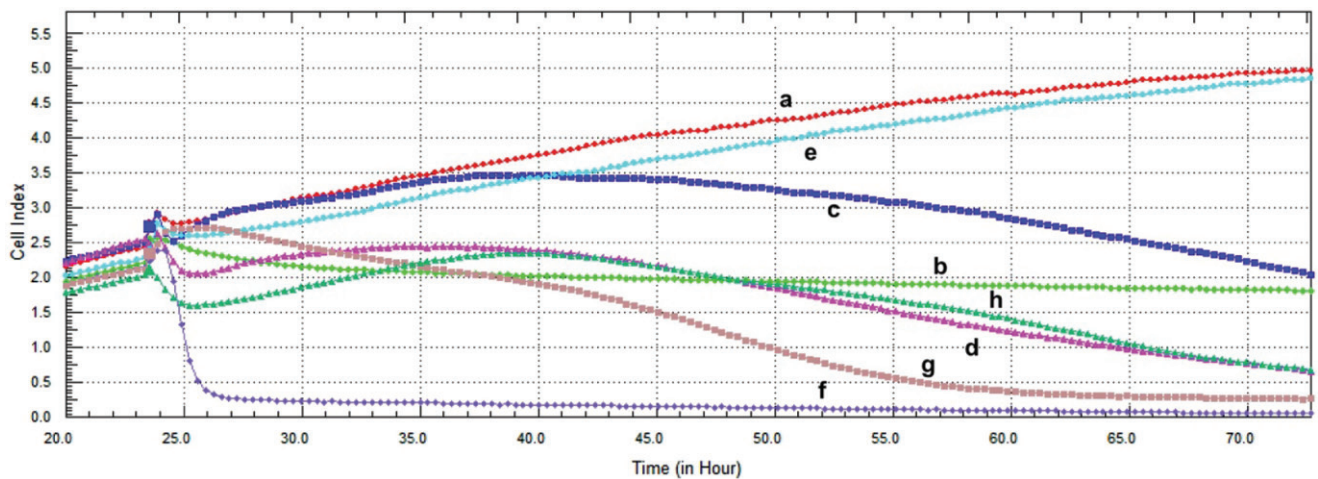


Figure 9. xCELLigence growth curves of control (a) and cells incubated with different pollen extracts: birch 25 mg/mL (b), birch 5 mg/mL (c), common hazel 25 mg/mL (d), common hazel 5 mg/mL (e), European alder 25 mg/mL (f), European alder 5 mg/mL (g), European hornbeam 25 mg/mL (h). A flattening of the growth curve compared to the control suggests cytostatic effects, whereas a decline of the growth curve indicates cytotoxicity.

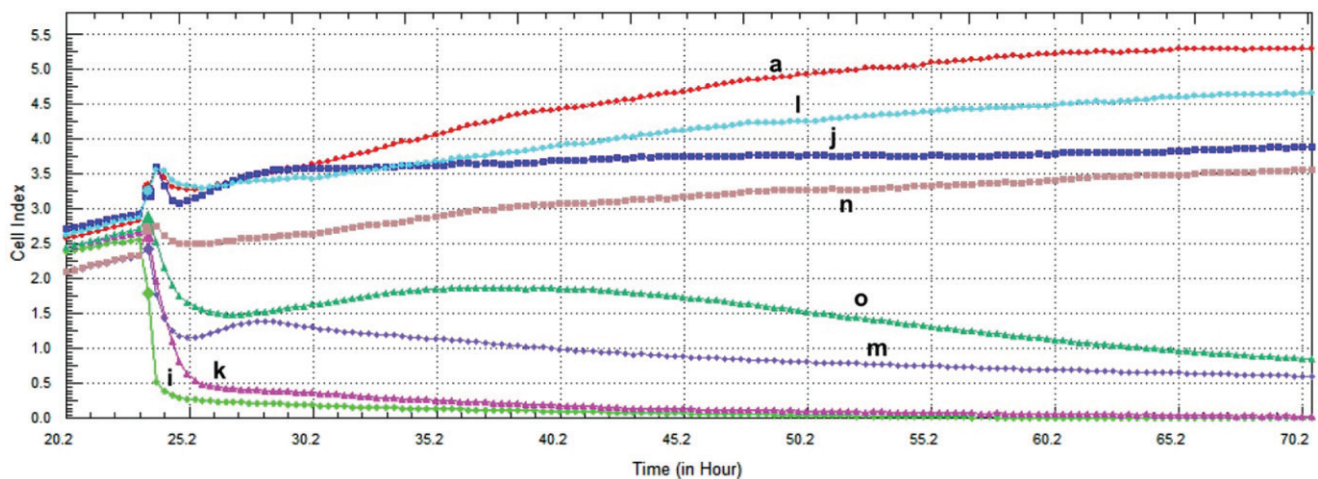


Figure 10. xCELLigence growth curves of control (a) and cells incubated with different pollen extracts: Horse chestnut 25 mg/mL (i), Horse chestnut 5 mg/mL (j), European ash 25 mg/mL (k), European ash 5 mg/mL (l), European chestnut 25 mg/mL (m), European chestnut 5 mg/mL (n), Elder 25 mg/mL (o). A flattening of the growth curve compared to the control suggests cytostatic effects, whereas a decline of the growth curve indicates cytotoxicity.

Table 2. Effects of different pollen species on CI according to RTCA.

Species	Clade	Order	Family	5 mg/mL Pollen extract	25 mg/mL Pollen extract
Birch	Rosids	Fagales	Betulaceae	Cytotoxic	Cytotoxic
Common hazel	Rosids	Fagales	Betulaceae	None	Cytotoxic
Common timothy	Commelinids	Poales	Poaceae	Cytotoxic	Cytotoxic ^a
Corkscrew hazel	Rosids	Fagales	Betulaceae	Cytostatic	Cytostatic ^a
Elder	Asterids	Dipsacales	Adoxaceae	Cytostatic	Cytotoxic
European alder	Rosids	Fagales	Betulaceae	Cytotoxic	Cytotoxic
European ash	Asterids	Lamiales	Oleaceae	Cytostatic	Cytotoxic
European chestnut	Rosids	Fagales	Fagaceae	Cytostatic ^a	Cytotoxic
European hornbeam	Rosids	Fagales	Betulaceae	Cytostatic	Cytotoxic
European silver fir	Pinopsida	Pinales	Pinaceae	None	Cytostatic ^a
Golden foxtail grass	Commelinids	Poales	Poaceae	Cytostatic ^a	Cytotoxic
Greater plantain	Asterids	Lamiales	Plantaginaceae	Cytostatic	Cytotoxic
Horse chestnut	Rosids	Sapindales	Sapindaceae	Cytostatic ^a	Cytotoxic ^a
Korean fir	Pinopsida	Pinales	Pinaceae	None	Cytostatic ^a
Lime tree	Rosids	Malvales	Malvaceae	Cytotoxic ^a	Cytotoxic
Maple	Rosids	Sapindales	Sapindaceae	Cytotoxic	Cytostatic ^a
Orchard grass	Commelinids	Poales	Poaceae	Cytostatic ^a	Cytotoxic ^a
Scots pine	Pinopsida	Pinales	Pinaceae	Cytostatic ^a	Cytotoxic
Walnut	Rosids	Fagales	Juglandaceae	Cytostatic	Cytotoxic ^a
White willow	Rosids	Malpighiales	Salicaceae	Cytostatic ^a	Cytotoxic

^apronounced effect.

The incubation of human epithelial cells with extracts of different pollen species caused changes of cytomorphology, consistent with cell damage. After the incubation with pollen extracts the normally adherently growing cells detached from each other and floated freely in the supernatant. We speculate that this might be due to the destruction of intercellular contact proteins by proteolytic actions³². Cunha could show that the incubation of human alveolar epithelial cells with pollen extracts and isolated serin-endopeptidases of *P. judaica* lead to a loss of cell coherency. This is thought to occur due to the cleavage of a cytoplasmatic tight junction protein, zonula occludens 1³³. Unfortunately, it has yet to be determined whether pollen proteases degrade tight junctions directly or via an indirect mechanism³³. Proteolytic damage of the epithelial barrier promotes the development of further inflammatory and also allergic reactions on mucosal surfaces³². Allergens crossing epithelial barriers are starting the sensitization process at subepithelial dendritic cells. These are presenting allergenic peptides to naive T-lymphocytes, causing the production of specific IgE by plasma cells^{5,34}.

Furthermore, we observed a significant reduction of cell viability as assessed by MTS, WST-1 assay, and RTCA. Our *in vitro* data suggest that eyes exposed in pollen-polluted surroundings *in-vivo* might not only suffer from destruction of tear fluid proteins but also cell damage as well. Data concerning the residence time of pollen on the ocular surface *in-vivo* revealed that pollen can last up to 5 h on the ocular surface after the initial exposure. This should be enough time to perform their enzymatic reactions³⁵. Interestingly, the incubation of human epithelial cells with pollen extracts from Pinaceae, Betulaceae and Juglandaceae at low concentrations statistically significantly increased cell viability for a short period of time. However, this positive effect did not remain significant with an increased pollen concentration or time of incubation. Further studies on the pollen species of these plant families are warranted.

Apparently, there are many personal differences in the reaction following pollen exposure. A possible explanation, why not all humans are affected by non-allergic pollen reactions the same way, might be differing levels of tear fluid and ocular surface antiproteases. Usually, these are present in body fluids and provide protection against enzymatic tissue damage. Examples are α_1 -antitrypsin, α_1 -antichymotrypsin and α_2 -macroglobulin, which have been identified in human tear fluid and nasal secrete as well⁴. A sufficient amount of these antiproteases protects against trypsin and other serine proteases, which are present in pollen^{36,37}. An accumulation of environmental influences, such as exposure to wind, ultraviolet light (UV) and ozone, for example, may increase the sensibility to pollen proteases even more^{38–41}. Recently, it could be shown that UV light irradiation even increased the histamine level of alder and hazel pollen in a dose-dependent manner⁴². The interaction of UV has been well studied, but also other confounding environmental factors like dust and pollution might play an important role. Besides the air pollutants, like nitrogen dioxide (NO₂) and inhalable particulate matter (PM_{2.5} and PM₁₀), often high concentrations of ozone and ultraviolet irradiation are involved. The formation of O₃ depends on the supply of enough substrate, usually

vehicle emissions of NO₂ and on the weather in the respective area. The rate of conversion of NO₂ into O₃ is especially high on days with a lot of sunshine¹⁵. Exposure to O₃ also triggers the appearance of inflammatory cells and mediators, like granulocyte-macrophage-colony-stimulating factor (GM-CSF) as well as interleukin (IL)-6 and IL-8 and fibronectin, in asthmatic subjects^{15,43,44}. Particulate matter might also cause alterations of human tear proteins and conjunctival cells itself⁴¹.

Air pollutants affect pollen in the atmosphere itself, as well as people's airways directly. By interaction with pollen grains, they might play a key role in atopic sensitization and possibly exacerbate allergic diseases^{15,45,46}.

Air pollution leads to mucosal damage and impaired mucociliary clearance in the airways and thereby may pave the way for pollen allergens to immune cells^{15,45}.

Additionally, the allergenic potential of plants is influenced by the reaction of the vegetation with air pollutants and climate changes. There are various factors influencing this reaction, like the type of air pollution, climatic factors, as well as the species of the plant and its nutrition¹⁵. It should be considered that the prevalence of enzymatic pollen reactions affecting mucosal surfaces will be region-dependent, because of the local and seasonal variations in airborne pollen species.

It seems reasonable, that allergic, as well as non-allergic subjects might suffer from enzymatic pollen reactions since the pathomechanism is independent of a possible allergic sensitization. Common anti-allergic treatments, like cromoglycates, H₁-antagonists, and desensitization might be ineffective in protecting from proteolytic tissue damage. Theoretically, the development of antiprotease eye drops or enzyme inhibitors sounds promising⁴⁷. Unfortunately, different pollen species containing a variety of enzymes, some not even specified yet, might complicate this⁴⁸.

Meanwhile, common prevention measures to reduce pollen exposure can be recommended⁴⁹. During pollen seasons, patients should stay outside only in the morning, the night hours and after rain. Outdoor work and exercise should be avoided during high airborne pollen concentrations. It is suggested to often wash face and hair, and to keep the windows closed and pillows free of pollen, especially in the bedroom. Following pollen exposure eye flushes with different rinsing-solutions lead to a significant elimination of pollen grains from the ocular surface¹. By these methods, the burdens of pollen-elicited allergic, as well as non-allergic reactions may be minimized.

Our study, investigating a variety of pollen species from different plant families revealed similar effects on human tear fluid and epithelial cells as observed with hazelnut and birch pollen¹. The present data provide a broad basis for further research on pollen reactions affecting the tear film and ocular surface and underlines the importance of enzymatic pollen reactions affecting secretions lining mucosal membranes and mucosal epithelia of the human body. Thus, further studies on the pollen composition and their reactions' exact pathomechanisms, e.g. the identification of the lysed proteins in tear fluid, diagnosis and therapy are warranted.

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