

Conjunctival effects of a selective nasal pollen provocation

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Abstract

Background: Several clinical and experimental observations suggest that allergen deposition in the nose may partially be responsible for the induction of conjunctival symptoms in allergic rhinitis. The aims of this study were to evaluate the induction of conjunctival symptoms by selective nasal allergen provocation and to assess the feasibility of the different tools for evaluation of conjunctival allergic inflammation.

Methods: Grass pollen allergic subjects with rhinoconjunctivitis symptoms during the pollen season ($n = 12$) underwent a nasal sham and grass pollen provocation extra-seasonally. Nasal and conjunctival symptoms were scored using the Visual Analogue Scale (VAS) system at baseline, 15 min, 1 h and 24 h after provocation. In addition to Peak Nasal Inspiratory flow (PNIF) measurements, conjunctival inflammation and vascular congestion were evaluated and histamine and substance P levels in tear fluid were measured.

Results: Selective nasal grass pollen provocation induced ocular pruritus, lacrimation and conjunctival vascular congestion. PNIF values correlated inversely with lacrimation ($r = -0.71$, $P < 0.001$) and ocular pruritus ($r = -0.41$, $P < 0.05$). Four out of 11 patients showed a conjunctival eosinophilic inflammation and levels of histamine ($r = 0.73$, $P < 0.05$) and substance P ($r = 0.67$, $P = 0.05$) in tear fluid correlated with conjunctival symptoms.

Conclusion: Selective nasal grass pollen provocation induced conjunctival inflammation, ocular pruritus and lacrimation, which correlated with histamine and substance P levels in tear fluid and inversely with the PNIF values. These data show a naso-ocular interaction in allergic rhinitis and offer objective tools for evaluation of conjunctival inflammation in allergic rhinoconjunctivitis.

Along with the classic triad of nasal obstruction, rhinorrhea, and sneezing, nasal inhalation of allergens in sensitized subjects may lead to symptoms beyond the nose, such as conjunctival, otologic and laryngeal symptoms (1). Conjunctival symptoms are often overlooked, despite the large proportion of patients with allergic rhinitis having these complaints. Indeed, almost half the patients with allergic rhinitis suffer from red, itching or watery eyes, and 15% state that their ocular symptoms are the most bothersome aspect of their condition (2). Furthermore, approximately 70% of the patients with allergic rhinitis report their conjunctival symptoms to be at least as severe as their rhinitis symptoms (3). The diagnosis of allergic conjunctivitis is mostly based on

clinical signs such as a milky or pale pink conjunctiva with vascular congestion that may progress to conjunctival swelling, ocular pruritus and lacrimation. To confirm the diagnosis, skin prick tests are performed as well as a determination of specific and total serum IgE levels. Additional tests such as conjunctival challenges, conjunctival impression cytology and measurement of IgE in tear fluid are performed upon specific indications (4, 5).

Three mechanisms have been proposed to explain how conjunctival symptoms develop in patients with allergic rhinoconjunctivitis. Most evidently, direct exposure of the conjunctiva to airborne allergens leads to an IgE-mediated mast cell activation in sensitized patients, which parallels the

immune reaction in the nose and results in the release of preformed mediators such as histamine, leukotriene C₄, prostaglandin D₂, IL-4 and IL-5 (6, 7). This early phase response peaks within 15 min after allergen exposure and is followed by the late phase response occurring between 6 and 24 h after allergen exposure (6). The late phase conjunctival response is characterized by an up-regulation of adhesion molecules such as eotaxin, VCAM-1 and ICAM-1 and by an influx of inflammatory cells such as eosinophils, neutrophils, macrophages and basophils (8). The systemic immune response after allergen inhalation may also contribute to the conjunctival inflammation after allergen inhalation. We have previously reported that allergens deposited onto the nasal mucosa rapidly enter the systemic circulation and hence activate circulating immune cells with the release of IL-5 by mast cells (9). A systemic release of inflammatory mediators such as IL-5 may contribute to the up-regulation of adhesion molecules such as eotaxin, VCAM-1 and ICAM-1 in the conjunctiva and the development of eosinophilic inflammation on the conjunctival surface (10). Finally, neural mechanisms originating from nasal inflammation may contribute to the generation of conjunctival inflammation in allergic rhinitis, as several clinical and experimental observations point into the direction of a naso-ocular reflex. A unilateral nasal provocation with allergens results in a local histamine release with an increase in nasal vascular permeability, a naso-nasal secretory reflex and rapidly occurring bilateral ocular pruritus and lacrimation (11). The mediators responsible for this naso-ocular reflex are not clear, but substance *P* may be involved (12). Substance *P* has been reported to be released by sensory nerve endings upon activation by mast cell-derived mediators like histamine (13) and may induce the conjunctival vascular congestion, lacrimation and ocular pruritus (12). Nasal provocations have also produced ocular itching in a variable percentage of patients (14–16), suggesting that ocular symptoms may be induced in part by a naso-ocular reflex. The best illustration of the contribution of nasal allergen deposition and inflammation to the induction of conjunctival symptoms comes from a study performed in pollen allergic patients being exposed to pollen in the field with or without the presence of allergen filters in the nose. Prevention of allergen deposition in the nose reduced both nasal as well as ocular symptoms of allergic disease (17).

The aims of our study were to evaluate the induction of conjunctival symptoms via selective nasal allergen deposition and present different research tools for studying conjunctival allergic inflammation, including mediators thought to be involved in this naso-ocular interaction.

Material and methods

Study design

Twelve grass pollen allergic volunteers, aged between 21 and 34, with rhinoconjunctivitis symptoms during the pollen season were recruited via an announcement on the University website. Subjects were asked to participate in an allergen provocation study of the Department of Otorhinolaryngol-

ogy of the University Hospitals Leuven. The following exclusion criteria were used: present or past wearing of contact lenses, ocular pathology or current ocular treatment, and history of asthma. Other exclusion criteria were the use of nasal or oral steroid treatment ≤ 6 weeks prior to the study, the use of nasal or oral antihistamine treatment ≤ 4 weeks prior to the study, and past or ongoing immunotherapy for grass pollen.

On day 0, a screening was completed in order to confirm that patients fulfilled all the inclusion and had none of the exclusion criteria. An informed consent was signed and grass pollen sensitization was confirmed with a skin prick test. The standard skin prick tests were carried out with house dust mite, timothy grass, smooth meadow grass, orchard grass, nettle, plantago, oxeye daisy, mugwort, alder, birch, hazel, horse, cat, dog, rabbit, alternaria, aspergillus and cladosporium (HAL Allergy, Leiden, the Netherlands). All subjects underwent the provocations in February and March 2009, i.e. prior to the grass pollen season of 2009. A selective nasal sham provocation (saline with 0.5% phenol, HAL Allergy) was performed at the baseline visit (day 0). Using a 200–1000- μ l micropipette, 250 μ l of the provocation solution was instilled onto the nasal mucosa of the medial and lateral wall of the nasal cavity, as shown on Fig. 1A. Direct contact of the pipette with the nasal mucosa was avoided. Analyses were carried out prior to as well as at 15 min, 1 h and 24 h after the provocation. The following parameters were investigated: PNIF measurements, evaluation of nasal and conjunctival symptoms, collection of tear fluid for measuring histamine and Substance *P* levels, evaluation of conjunctival vascular hyperemia and conjunctival impression cytology to study conjunctival eosinophilic inflammation (Fig. 1B–C). The early time point of 15 min after the provocation was chosen for analysis as we intended not to interfere with the conjunctival symptoms and inflammation by mechanical stimulation of tissue sampling, nor did we want to miss any marker of neurogenic inflammation. One h after the provocation, analysis was performed to characterize the reaction in the conjunctiva at the end of the early phase allergic reaction. On day 7, a selective nasal provocation with a high dose of grass pollen extract (mixed grass pollen, 10 000 AU/ml, HAL Allergy, phenol 0.5%) was performed to screen the recruited patients for the induction of nasal and conjunctival symptoms, which was determined by a Visual Analogue Scale (VAS) score of 5 for at least 2 of the nasal and conjunctival symptoms. The provocation dose of grass pollen is 10 times higher compared to other studies and was chosen to ensure a potent induction of nasal inflammation allowing conclusions on a naso-ocular interaction. In view of the high concentration grass pollen dose for provocation, patients undergoing the provocations were not blinded to the type of provocation. On day 14, a second nasal grass pollen provocation was performed, followed by the same analyses as described after the sham provocation. Twenty-four h later (day 15), patients were asked to evaluate their nasal and conjunctival symptoms using VAS scores, and tear fluid and conjunctival cells were harvested. The experimental protocol was approved by the local medical ethics committee.

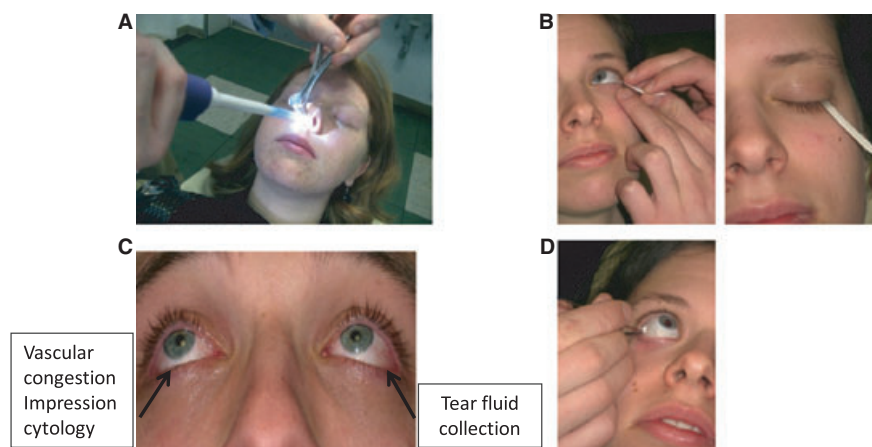


Figure 1 (A) Selective nasal provocation was performed with a pipette and a tip in which 250 μ l of the challenge solution was applied to both nostrils without any mucosal contact. (B) The collection of tear fluid in the left eye with Schirmer strips. (C) Evaluation of conjunctival reaction after selective nasal provocation, with the

evaluation of tear fluid production in the left eye, and the evaluation of vascular congestion and conjunctival impression cytology in the right eye. (D) Conjunctival impression cytology in which a filter paper is gently pressed against the conjunctival surface to collect cells from the conjunctiva.

Evaluation of nasal and conjunctival symptoms

Evaluation of the major nasal and conjunctival symptoms was performed before and at 15 min and 1 h after the sham provocation, and before and at 15 min, 1 h and 24 h after the grass pollen provocation. Nasal and conjunctival symptoms were evaluated using a VAS with 0 indicating a total absence of symptoms and 10 indicating the most severe symptoms. Nasal symptoms were sneezing, runny nose, blocked nose and itchy nose. Conjunctival symptoms comprised ocular pruritus and lacrimation. In addition to the VAS scoring, patients were also asked to score their global nasal and conjunctival symptoms during the previous grass pollen season on a symptom scale from 0 to 3 with 0, none; 1, mild; 2, moderate; and 3 severe and before and at 15 min, 1 h and 24 h after the provocation. Total ocular and nasal symptom score were calculated for the evaluation of nasal and conjunctival symptoms during the previous grass pollen season and at 15 min after the grass pollen provocation.

Peak nasal inspiratory flow (PNIF)

Nasal congestion was measured by the peak nasal inspiratory airflow using the PNIF device (Cement Clarkx International, Essex, UK). Hereby, an anesthesia mask was placed over the mouth and nose of the patients after expiration, and patients were asked to close their mouth and forcefully inspire air through the nose. The best value out of three consecutive measurements with a variability of $\leq 10\%$ in between measurements was recorded (18–21).

Conjunctival vascular congestion

Conjunctival vascular hyperemia was evaluated on the right eye of the patients by an ophthalmologist (S.L.) based on the

Cornea and Contact Lens Research Unit (CCLRU) ocular hyperemia grading scale (zero to four units) interpolated into 0.5 increments (22). This scale has been independently evaluated and verified. The conjunctiva was observed with the naked eye using a headlamp. Digital photographs were taken for subsequent analysis and verification.

Collection of tear fluid secretion and measurement of histamine and substance P

Tear fluid was collected from the left conjunctival surface using paper Schirmer strips (Haag-Streit, Harlow, Essex, UK, Fig. 1B). The subject was asked to close the eyes for the duration of the collection. This technique is painless and atraumatic for the patient, and leads to the rapid collection of approximately 30 μ l of fluid without the need for local anesthesia (Fig. 1C). The Schirmer strip with the containing tear fluid was then removed and placed in an Eppendorf tube and incubated for 24 h with 300 μ l of saline at 4°C for elution of the secretions. After 24 h, the tubes were vortexed, the Schirmer strips were squeezed to the bottom of the tubes, and the supernatants were aliquoted to Eppendorf tubes and frozen at -20°C for subsequent analysis. Histamine levels in the collected tear fluid were measured using a commercially available competitive enzyme-linked immunoassay sensitive to 1 ng/ml (Labor Diagnostike Nord GmbH & Co, Nordhorn, Germany). Substance P levels were measured in tear fluid using a commercially available competitive enzyme-linked immunoassay sensitive to 3.9 pg/ml (Cayman, MI, USA).

Conjunctival impression cytology

Conjunctival impression cytology is a noninvasive technique that allows the sampling of the superficial conjunctival

Table 1 Visual Analogue Scale scoring (0–10 cm) for the nasal symptoms, sneezing, runny, blocked and itchy nose and the conjunctival symptoms, ocular pruritus and lacrimation, as reported by the patients

Symptoms	SHAM provocation (day 0)			GRASS POLLEN provocation (day 14)			
	Baseline	15 min	1 h	Baseline	15 min	1 h	24 h
Sneezing	0.17 ± 0.11	0.05 ± 0.04	0.02 ± 0.02	0.20 ± 0.14	4.00 ± 0.88**	0.30 ± 0.15	0.72 ± 0.46
Runny nose	0.55 ± 0.24	0.39 ± 0.23	0.39 ± 0.14	0.30 ± 0.12	5.84 ± 0.93***	2.33 ± 0.52*	2.10 ± 0.50**
Blocked nose	1.12 ± 0.64	0.64 ± 0.55	0.58 ± 0.47	0.40 ± 0.16	6.64 ± 1.15**	5.28 ± 1.12**	3.10 ± 0.96*
Itchy nose	0.02 ± 0.02	0.11 ± 0.06	0.05 ± 0.03	0.02 ± 0.02	1.82 ± 0.59**	0.82 ± 0.31*	0.58 ± 0.38
Ocular pruritus	0.13 ± 0.06	0.29 ± 0.10	0.24 ± 0.14	0.04 ± 0.04	1.50 ± 0.65	0.55 ± 0.24	0.61 ± 0.21*
Lacrimation	0.10 ± 0.09	0.23 ± 0.16	0.10 ± 0.06	0.21 ± 0.19	2.50 ± 1.00*	0.97 ± 0.68	0.46 ± 0.43

Data are expressed as means ± SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

epithelial layers for morphological and cytological examination of the ocular surface. Impression cytology was performed on the right temporal bulbar conjunctiva and on the inferior fornical conjunctiva. The average results of both samples were calculated for each patient. For this purpose, cellulose acetate filter paper sheets (type HAWP 2932, Millipore Corp., Bedford, MA, USA) were applied to the lower temporal conjunctiva and to the bulbar conjunctiva on the right side (Fig. 1D). Using a flat, round-tipped forceps, the strips were placed onto the ocular surface and gently pressed for a few seconds using a glass rod (Fig. 1D). They were then removed with a peeling motion and placed onto a plain glass slide. Gentle pressure was applied using a clean glass rod, in order to transfer the cytological sample from the strip to the slide. The slides were fixed and colored with a May-Grunwald-Giemsa coloring in order to visualize the different cell types present in the superficial conjunctival layer. Numbers of eosinophils, neutrophils and epithelial cells were counted using a light microscope with a magnification of 1000 $\times$.

Statistical analysis

All statistical analyses were performed with the GRAPHPAD PRISM program (Prism 4, version 4.03, ©1992–2005 GraphPad Software Inc., San Diego, CA, USA). Comparisons between the different time points before and after nasal provocation were calculated using a nonparametric paired Wilcoxon ranked test. Correlations were calculated with the nonparametric Spearman correlation test. A difference was considered to be significant when $P < 0.05$. Data are represented as means ± SEM.

Results

Induction of nasal and conjunctival symptoms after nasal grass pollen provocation

All patients with rhinoconjunctivitis symptoms during the pollen season had positive skin prick tests for grass pollen ($n = 12$). Seven patients were also sensitized to house dust mite and five patients to birch pollen. One patient with an allergy to birch pollen was excluded at day 7 because he had severe nasal

symptoms prior to the grass pollen provocation. The four other birch pollen allergic patients showed no nasal symptoms at the start of the study and were included in the study.

Before, at 15 min and 1 h after the provocation with sham solution, patients reported no change in any of the following symptoms: sneezing, runny nose, blocked nose and itchy nose (Table 1). However, 15 min after the grass pollen provocation, patients reported a significant increase in the four major nasal symptoms. After 1 h, nasal symptoms progressively became less severe, with runny and blocked nose still being present at 24 h after allergen provocation. Fifteen min and 1 h after sham provocation, patients recorded no conjunctival symptoms (Table 1), whereas lacrimation and ocular pruritus were induced, respectively at 15 min and 24 h after grass pollen provocation. Patients were asked to score their nasal and conjunctival symptoms during the previous grass pollen season on a scale from 0 to 3, and these values were compared with the total nasal symptoms score (TNSS) and total ocular symptom score (TOSS) at 15 min after the grass pollen provocation (Fig. 2). Nasal symptoms after the grass pollen provocation were experienced as being as severe as during the previous grass pollen season (1.68 ± 0.21 vs 2.18 ± 0.12 score, $P = 0.07$, $n = 11$). However, patients

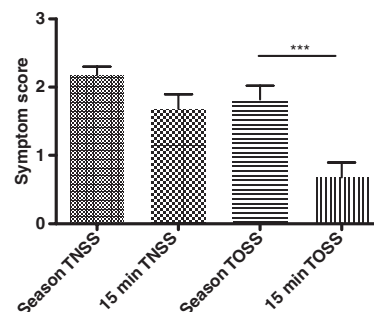


Figure 2 Comparison of the subjective evaluation of total nasal (TNSS) and total conjunctival symptoms (TOSS) 15 min after the grass pollen provocation with the symptoms during the previous grass pollen season. Symptoms were scored on a scale from 0 to 3, with 0, none; 1, mild; 2, moderate; and 3 severe symptoms. The graph represents the means ± SEM *** $P < 0.001$.

Table 2 The Peak Nasal Inspiratory Flow, expressed in l/min, the conjunctival vascular hyperemia score (0–3) and number of eosinophils infiltrated in the conjunctiva, measured at the different time points at sham and grass pollen provocation

Parameters	SHAM provocation (day 0)			GRASS POLLEN provocation (day 14)			
	Baseline	15 min	1 h	Baseline	15 min	1 h	24 h
PNIF (l/min)	144.10 ± 9.02	130.90 ± 8.99	136.80 ± 10.79	157.30 ± 10.10	31.82 ± 10.94***	60.91 ± 16.37**	124.50 ± 12.75**
Conjunctival vascular hyperemia (0–3)	0.09 ± 0.09	0.00 ± 0.00	0.00 ± 0.00	0.05 ± 0.05	0.56 ± 0.20	0.22 ± 0.12	0.50 ± 0.14*
Conjunctival eosinophils; (number)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.16 ± 0.16	1.00 ± 0.48	4.58 ± 3.92

Data are expressed as means ± SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

scored the conjunctival symptoms after grass pollen provocation less severe than those experienced during the grass pollen season (0.68 ± 0.21 vs 1.82 ± 0.20 score, $P < 0.01$, $n = 11$, Fig. 2).

Evaluation of nasal congestion after nasal grass pollen provocation

Peak Nasal Inspiratory flow values were recorded at the different time points in relation to sham and grass pollen provocation. Peak Nasal Inspiratory flow values dropped significantly at 15 min, 1 h and 24 h after the grass pollen

provocation (Table 2). An inverse correlation was found between the PNIF values and the VAS scores for lacrimation ($r = -0.71$, $P < 0.001$) and ocular pruritus ($r = -0.41$, $P < 0.05$) at the different time points after the grass pollen provocation (Fig. 3).

Evaluation of conjunctival inflammation after nasal grass pollen provocation

Twenty-four h after the grass pollen provocation, a significant conjunctival vascular hyperemia was observed (Fig. 4A). This vascular congestion was not induced earlier after provocation or after the sham provocation (Table 2).

Low numbers of eosinophils were found on the conjunctival surface. Four of 11 patients showed eosinophils on the

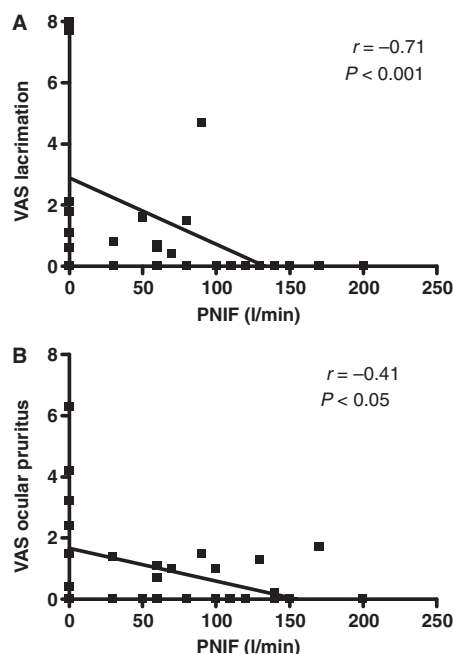


Figure 3 (A) A significant inverse correlation was found between the PNIF values (l/min) and VAS scores for lacrimation (cm) after the grass pollen provocation ($r = -0.71$, $P < 0.001$, $n = 33$). (B) A significant inverse correlation was found between the PNIF values (l/min) and the VAS score for ocular pruritus (cm) after the grass pollen provocation ($r = -0.41$, $P < 0.05$, $n = 33$).

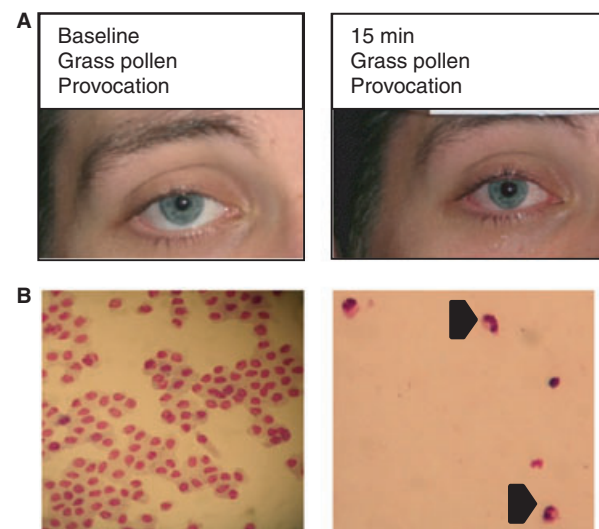


Figure 4 (A) Picture of the conjunctival congestion at baseline (left) and 15 min after the grass pollen provocation (right). (B) Microscopic images of conjunctival superficial cells with on the left side a picture of cells collected before the grass pollen provocation and on the right side an image 24 h after the grass pollen provocation with eosinophils visible (black arrows) surrounded by epithelial cells.

conjunctival surface at 1 h after the grass pollen provocation (Table 2), whereas two of these four patients had eosinophils at 24 h after the pollen provocation (Fig. 4B). The numbers of conjunctival neutrophils and lymphocytes were neglectable at all time points (data not shown).

Substance *P* and histamine could be measured in the tear fluid obtained in 9 of 11 patients, as 2 of 11 patients showed insufficient tear fluid production. No differences in absolute substance *P* levels were found between the different time points after grass pollen provocation (baseline: 51.01 ± 12.93 ; 15 min: 59.63 ± 12.85 ; 1 h: 46.64 ± 8.88 ; 24 h: 42.27 ± 4.21 pg/ml; $n = 9$ for all time-points). Similarly, no differences in histamine levels were found between different time points of analysis after the grass pollen provocation (baseline: 0.75 ± 0.15 ; 15 min: 0.69 ± 0.15 ; 1 h: 0.64 ± 0.09 ; 24 h: 0.78 ± 0.20 ng/ml; $n = 9$ for all time-points). Of note, a correlation was found between substance *P* and VAS scores for ocular pruritus at 15 min after the nasal grass pollen provocation reaching borderline significance ($r = 0.67$, $P = 0.05$, $n = 9$, Fig. 5A). A positive correlation was found between histamine and the VAS scores of lacrimation 24 h after the nasal grass pollen provocation ($r = 0.73$, $P < 0.05$, $n = 9$). Considering the levels of histamine and substance *P* at different time points after allergen provocation, a positive correlation was found between the substance *P* and histamine values in the tear fluid after nasal grass pollen provocation ($r = 0.43$, $P < 0.05$, $n = 26$, Fig. 5B).

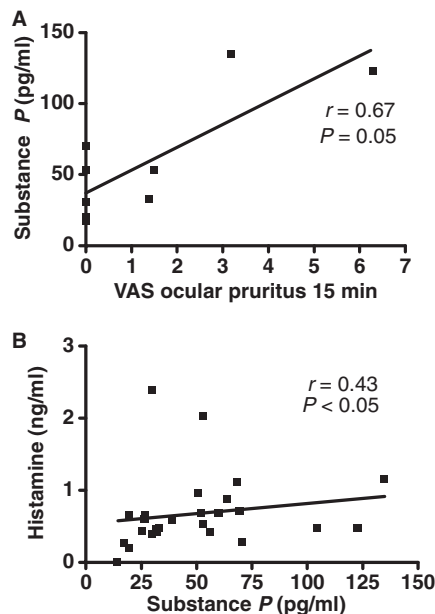


Figure 5 (A) A significant correlation was found between substance *P* levels (pg/ml) and VAS scores for ocular pruritus at 15 min after the nasal grass pollen provocation ($r = 0.67$, $P = 0.05$, $n = 9$). (B) A significant correlation ($r = 0.43$; $P < 0.05$; $n = 26$) was found between the histamine (pg/ml) and substance *P* levels (pg/ml) produced in the tear fluid after the grass pollen provocation.

Discussion

We here present several research tools for the evaluation of conjunctival allergic inflammation in patients with allergic rhinoconjunctivitis, and we demonstrate the induction of conjunctival and nasal symptoms as well as conjunctival vascular congestion after a selective nasal grass pollen provocation. The novelty of this study relates to the selectivity of the nasal pollen provocation on the one hand and the simultaneous evaluation of different subjective and objective nasal and conjunctival parameters on the other hand. Interestingly, the substance *P* and histamine levels in tear fluid correlate with the VAS scores for ocular pruritus and lacrimation after grass pollen provocation and the PNIF values correlate inversely with the VAS scores for ocular pruritus and lacrimation.

A novel technique for pollen provocation is reported here, allowing the evaluation of the contribution of selective nasal allergen deposition to conjunctival inflammation in allergic rhinoconjunctivitis. Patients receive a high-dose provocation with grass pollen on the nasal mucosa in an unblinded way on day 7 and day 14. The pollen allergen extract is applied in solution to the nasal mucosa of both nasal cavities using a micropipette. Using this method, allergens are unlikely to come into direct contact with the conjunctiva and airborne spreading of allergens is less likely to occur than in case of nebulization or application of the allergen with a spray. Traditional methods of nasal provocations using nebulizers or sprays (16, 23) cannot be used here as they do not prevent any contact of the allergen with the conjunctiva. Allergen disks containing the allergen (11, 24–26) are not used here either as any mechanical contact stimulation may trigger a naso-ocular reflex. With the technique of contact-free allergen deposition onto the nasal mucosa, a high dose of grass pollen extract is applied in order to study the conjunctival effects of a potent allergic nasal inflammation, allowing conclusions on the naso-ocular interaction in allergic rhinoconjunctivitis. The fact that patients are evaluated before and after the grass pollen provocation on both the presence of symptoms as well as on cellular level, vascular congestion and mediators in tear fluid allows us to conclude that residual effects of the provocation of day 7 are unlikely to influence the data generated on day 14 significantly. As expected, nasal allergen provocation induces a rapid nasal congestion and severe nasal symptoms, which are maintained for 24 h after the provocation. Beside nasal symptoms, nasal allergen deposition elicits conjunctival symptoms like ocular pruritus, lacrimation and conjunctival vascular hyperemia. As direct contact of the allergen with the conjunctiva is unlikely to take place with this technique of provocation, two possible mechanisms can be proposed for the generation of the conjunctival inflammation and symptoms after nasal allergen deposition. Firstly, systemic absorption of the allergen through the nasal mucosa may take place (9), leading to a systemic activation of the immune response and eventual induction of conjunctival inflammation. This mechanism may be involved in the late phase allergic response with conjunctival symptoms, conjunctival vascular hyperemia and eosinophilic inflammation being present at 24 h after the provocation. However, the rapid time course of the induction of

conjunctival symptoms occurring already at 15 min after nasal allergen contact suggests the existence of a naso-ocular neural reflex mechanism. Allergic inflammation in the nose may trigger the trigeminal nerve endings inside the nasal mucosa, leading to activation of a reflex loop via the trigeminal ganglia (27). The efferent nerve endings in the conjunctival surface may then initiate and contribute to the conjunctival reaction after nasal allergen provocation. Indeed, the ocular surface is highly innervated (13) and mediators like substance *P*, calcitonin gene-related peptide (CGRP), VIP (vaso-active intestinal peptide), neuropeptide tyrosine (NPY) and nerve growth factor (NGF) are widespread. These neural mediators maintain homeostasis in the conjunctiva and may modulate the pathogenesis of allergic conjunctivitis. Increased levels of substance *P*, NGF and VIP are found in plasma and conjunctiva of patients with vernal keratoconjunctivitis, suggesting that an altered expression of neuropeptides and neurotrophins may be involved in this condition (13, 28–30).

Substance *P* is an important mediator of this neurogenic inflammation and is found in the tear fluid of patients with allergic conjunctivitis and vernal conjunctivitis (12, 28). It is known that substance *P* and CGRP cause the recruitment, activation and functional activity of eosinophils, neutrophils, mast cells and T cells (13, 31, 32). Substance *P* may therefore contribute to the influx of eosinophils in the conjunctiva seen in this study in 4 of 11 patients after the nasal grass pollen provocation. Of these four patients with increased numbers of eosinophils, two are also sensitized to house dust mite, one to birch pollen and the 4th to grass pollen only. Based on the sensitization profiles of the studied individuals, we can only speculate on the reason why 4 of 11 patients show conjunctival eosinophils. Poly-sensitization does not interfere with the results as 1 of 4 patients is mono-sensitized to grass pollen. Also, when mono-sensitized patients are compared to poly-sensitized patients, no differences are seen in conjunctival eosinophilic influx, vascular hyperemia, PNIF measurements and nasal and conjunctival symptoms. Neural hypersensitivity as well as inflammatory mechanisms associated with eosinophilic influx beyond allergic inflammation may be responsible for the observed eosinophilia. These four patients display high individual conjunctival hyperemia scores and report high VAS scores for nasal and conjunctival symptoms but these scores are similar to the ones from other patients where no eosinophilic influx was seen. Therefore, it seems that only a portion of patients with allergic rhinoconjunctivitis develops a detectable eosinophilic inflammation via nasal inflammation. This observation resembles the data of bronchial eosinophilic inflammation after nasal allergen provocation. Previous allergen provocation studies have demonstrated the influx of bronchial eosinophils in a variable percentage of patients with allergic rhinitis where in a few studies all patients show bronchial eosinophilia (33, 34), but others cannot show an eosinophilic influx in the bronchi (25). Here, subjects with asthma and lower airway symptoms are excluded to avoid serious asthmatic reactions on the high dose of grass pollen and because the focus is the induction of conjunctival symptoms. However, none of the 12 challenged subjects show lower airway symptoms after a high-dose nasal

grass pollen provocation. Other studies have also demonstrated that allergen provocations with filter disks or nebulizers do not induce nasal symptoms to the same extent in all patients studied (14–16).

Levels of substance *P* are measured in the tear fluid at different time points before and after grass pollen provocation. No difference is found in absolute levels between the time points of analysis. Among other neural mediators, neurokinin A is also measured in tear fluid, but these values are around detection limit (data not shown). However, the importance of substance *P* in tear fluid is illustrated by the fact that its levels correlate with the VAS scores for ocular pruritus at 15 min after grass pollen provocation. Substance *P* contributes to the release of histamine from mast cells via binding to the NK1 receptor on mast cells (13, 31, 32). This confirms the possible role of substance *P* and herewith the involvement of a neural pathway in the naso-ocular interaction in allergy. Furthermore, substance *P* and histamine levels correlate with each other in tear fluid, illustrating a close interplay between both mediators. In allergic rhinoconjunctivitis, histamine is considered the main mediator driving vasodilatation, vascular permeability, cell proliferation, tissue growth and repair (35). In spite of the lack of any induction of histamine levels in tear fluid after pollen provocation, there is a significant correlation between histamine levels and the VAS scores for lacrimation at 24 h after the grass pollen provocation. Moreover, an inverse correlation is found between the measured PNIF values and the VAS scores for lacrimation and ocular pruritus at the different time points after grass pollen provocation. These data illustrate the existence of a close naso-ocular interaction in allergic rhinitis.

When patients are asked to score their nasal and conjunctival symptoms after the grass pollen provocation in comparison with the previous grass pollen season (2008), they report that the severity of total nasal symptoms like sneezing, runny, blocked and itchy nose are similar after the grass pollen provocation and during the previous grass pollen season. However, the conjunctival symptoms after nasal grass pollen provocation are less severe than during the previous grass pollen season. When subjects are asked to compare nasal and conjunctival symptoms after the grass pollen provocation with symptoms experienced during the subsequent grass pollen season, i.e. during May and June 2009, similar data are obtained (data not shown). It is obvious that conjunctival symptoms during natural allergen exposure are elicited by a combination of direct conjunctival allergen contact and indirect mechanisms like systemic immune response after allergen inhalation and/or naso-ocular reflex mechanisms. Therefore, seasonal conjunctival symptoms are more severe than after experimental nasal pollen provocation with high dose of allergen.

Several research tools are currently available for the evaluation of conjunctival inflammation like clinical assessment and grading of conjunctival injection by slit lamp microscopy (22), determination of tear inflammatory cytokines by ELISA (8, 36, 37), flow cytometry to detect inflammation, apoptosis and Th1/Th2 profile in the conjunctiva (38, 39), conjunctival brush cytology, and biopsy of the conjunctiva allowing

the quantification of inflammatory changes (40, 41). Here, we intend to evaluate the feasibility of different relatively non-invasive techniques: (i) tear fluid collection with the measurements of important mediators like histamine and substance P, (ii) conjunctival vascular congestion and (iii) conjunctival impression cytology. The collection of tear fluid with Schirmer paper strips is relatively easy and noninvasive for the patients; however, the low amount of tear fluid collected allows only a limited number of analyses. Evaluation of conjunctival vascular congestion with a validated scoring system is a quick and adequate method to assess vascular injection and chemosis (22). Conjunctival impression cytology is an atraumatic technique to collect superficial conjunctival inflammatory cells. However, this method is only useful in the active phase of the disease when more granulocytes are attracted to the conjunctival surface.

In conclusion, the present study shows an induction of conjunctival symptoms like ocular pruritus and lacrimation

after a selective nasal grass pollen provocation. These symptoms correlate with the production of histamine and substance P in the tear fluid and correlate inversely with the measured PNIF values. Therefore, this study confirms the close naso-ocular interaction in allergic rhinitis.

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