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Changes in tear protein pattern after photorefractive keratectomy

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PURPOSE. *Changes in tear protein composition of patients who underwent photorefractive keratectomy (PRK) were analyzed.*
METHODS. *Tear samples were obtained from 23 eyes of 23 patients immediately before PRK and on the fourth postoperative day with glass capillaries. Tear proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Digital image analysis and evaluation of the densitometric data of the electrophoretic separations were done with BioDoc-Analyze.*
RESULTS. *Analysis of discriminance found a significant difference in the protein patterns ($p<0.001$). This type of analysis of the electrophoretic densitographs uses all peak information simultaneously. A significant decrease ($p<0.005$) in three of the main protein peaks – lactoferrin, immunoglobulin A heavy chain, and lysozyme – was also found after PRK.*
CONCLUSIONS. *Excimer laser ablation of the cornea has an acute effect on lacrimal gland protein secretion. Changes in tear composition may lead to feelings of dryness and to a decrease in tear film stability postoperatively. (Eur J Ophthalmol 2003; 13: 525-31)*

KEY WORDS. *Tear proteins, Electrophoresis, Photorefractive keratectomy*

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INTRODUCTION

Photorefractive keratectomy (PRK) corrects refractive errors by changing the curvature of the anterior surface of the cornea directly. After removal of the epithelium – mechanically, chemically, or by laser – photoablation removes the Bowman membrane and the anterior layers of the stroma in the central cornea. As a consequence, the surface of the cornea is changed; the new surface is composed of injured marginal epithelial cells and proliferating epithelial cells, collagen fibers, extracellular matrix, and keratocytes. It stays in touch with the tear film in the first 3 to 4 days, until the re-epithelization is completed Furthermore, photoablation damages the sensory neural network of the superficial cornea, and regeneration requires a few months (1, 2).

The composition of the tear fluid changes after PRK. The concentration of several substances, which are secreted to the tear fluid by the lacrimal gland or locally in the eye, changes in the first postoperative days. An increase in the excretion of plasmin was shown

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(3), although the activity of plasmin (3) and the plasminogen activator (4) decreased. The examined cytokines, such as hepatocyte growth factor, transforming growth factor beta 1, vasoendothelial growth factor, platelet deriving growth factor, and tumor necrosis factor alpha, were found to be increased following PRK (5). The release of neuropeptide calcitonin gene-related peptide (6) and some extracellular matrix proteins, such as fibronectin (7) and tenascin (5), also increased. On the contrary, levels of ascorbic acid, which is the major scavenger of superoxide radicals in tears, decreased significantly (8).

To our knowledge, there is no information on changes in overall protein composition in tears after PRK. It is well known that the protein composition of tears changes in some other conditions. Lysozyme and lactoferrin, two of the main protein components of tears, were shown to be decreased in keratoconjunctivitis sicca (9-11). Postoperative decrease in the concentration of lactoferrin (12) and increase in the level of serum albumin (13) were found during tear analysis in eyes operated for senile cataract. In previous studies, a multivariate analysis of discriminance of the electrophoretic pattern of tear proteins was performed (14-16). This procedure includes not only single peaks or spots in the calculation, but all peaks detected in the densitographs of the electrophoretic lanes. Significant changes in protein pattern were found in patients with dry eye (15, 16), patients with diabetes (17), and contact lens wearers (14).

In the present study, we analyzed changes in the tear protein composition of patients who underwent PRK treatment. The tear samples were collected before surgery and on the fourth postoperative day.

METHODS

Patients and surgery

Effect of PRK on tear fluid proteins: Tear samples were obtained from 23 patients (23 eyes) who underwent PRK. The mean age of patients was 32.9 ± 11.0 years (range 19 to 60 years). The left or right eye was selected randomly when both eyes were treated in one section. Each patient signed informed consent before the laser treatment and volunteered to give tear fluid samples. The study protocol followed the tenets of the Declaration of Helsinki.

Preoperative investigations included determination of refraction, uncorrected (UCVA) and best-corrected visual acuity (BCVA), corneal topography, ultrasonic pachymetry, and routine slit-lamp and fundus examination. The eyes were treated with an Aesculap-Meditec MEL70G-scan 193 nm flying spot ArF excimer laser (flux density: 200 mJ/cm^2, repetition rate: 50 Hz). After topical anaesthesia with Oxibucain HCl drops (40 mg/10 ml), epithelial abrasion was performed mechanically with a hockey-knife. During the laser treatment, movement of the eye was prevented by means of a suction-ring. The spherical equivalent was 3.2 ± 4.9 D (-10.0 D to +7.0 D) preoperatively; emmetropia was attempted in every case. Postoperatively, the eyes were patched for one night; antibiotic eyedrops were prescribed, to be used five times daily until complete re-epithelization of the cornea; after that, steroid eyedrops were given in decreasing dosages for 3 to 6 months. The postoperative examinations included UCVA, BCVA, refraction, pachymetry, slit-lamp biomicroscopy, and evaluation of the haze. The haze was evaluated according to the Hanna scale (18).

Effect of nasal stimulation on tear fluid proteins: The question arises whether the changes in tear composition after excimer laser treatment are influenced by the increased postoperative tear flow, as the epithelial defect (caused by the PRK) induces reflex tearing. To examine this, we carried out a control study on nine randomly selected eyes of nine healthy volunteers. The mean age of patients was 30.7 ± 4.0 years (range 26 to 35 years). Tears were collected without stimulation (normal tears). After this, reflex tears were obtained from each eye. Tear flow of each eye was calculated from the collected volume and the time needed for the collection. The mean tear flow (volume/time) was 1.06 ± 0.55 μL/min for the normal tears and 11.75 ± 9.00 μL/min for the reflex tears.

Tear sample collection: Tears were collected with 50-mL glass capillaries from the tear meniscus at the lateral canthus.

Unstimulated tear samples were obtained immediately before PRK, on the fourth postoperative day, and from volunteers. Care was taken not to touch the conjunctiva, in order to avoid the stimulation of tear production. The collected quantity was 5 to 10 ml.

Reflex tear collection from volunteers: For obtaining reflex tears, nasal stimulation with a small cotton-tipped applicator was applied on the ipsilateral side (19). The tears were stored at –70 ˚C until use.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Tear samples were centrifugated at 12,000 g for 3 to 5 minutes and 2 μL of each sample was diluted with 10 μL sample buffer (1 M Tris-HCl, pH = 7.5, 2% SDS, 2% bromphenol-blue, 2% dithiothreitol), 8 μL bidistillated water, 2 μL iodoacetamide, and 1 μL glycerin. The tear proteins were separated by SDS-PAGE on discontinuous slab gels (20), with stacking gel: 0.5 M Tris, pH 6.8, 0.04% SDS; 13.5% separating gel: 1.5 M Tris, pH 8.8, 0.04% SDS; electrode buffer: 192 mM glycine, 25 mM Tris, pH 8.3, 1% SDS (20). Molecular weights were established using marker proteins (Bio-Rad, Munich, Germany, molecular weight standards broad range). The electrophoretic separations were stained using the Sypro Orange procedure (Sigma, St. Louis, MO).

Digital image analysis of electrophoretic separations

Digital image analysis and evaluation of the densitometric data of the electrophoretic separations were done using BioDocAnalyze (Biometra, Göttingen, Germany), as described elsewhere (21-23). BioDocAnalyze created densitometric data files for each electrophoretic lane (separation of one tear sample), which show the grey-intensity values (eight-bit grey values) against the relative mobility in x-axis (Rf values). ScanPack evaluates height, area, molecular weight, and Rf value for all peaks in this densitometric data file and also includes a photographic-quality half-tone bitmap. From each densitographic data file, a vector containing 70 variables was built, each variable corresponding to a Rf region. (The Rf axis was broken into 70 classes and each variable of the vector represents 1/70 of the Rf region between 0 and 1.) Each variable of the data vector represents the percentage area of the densitometric data file at this particular Rf region. These data vectors were compiled in a database for subsequent calculations.

Statistical procedure

Discriminant analysis of the data vectors: From the densitometric data vectors a multivariate discriminant analysis was performed, which tests the null hypothesis that mean data vectors of the different groups derive from the same multivariate normally distributed population. The statistical calculations were performed by Statistica 6.0 (Statsoft, Tulsa, OK).

The area under densitometric curve (AUC), which characterizes the total protein concentration of the samples, and the number of peaks were compared by paired t-test.

Analysis of the mean percentage areas of the main peaks: Five selected main peaks represent molecular weights corresponding to the most frequent or biologically important proteins of the human tear: lactoferrin, albumin, immunoglobulin A (IgA) heavy chain, lipocalin (tear-specific prealbumin), and lysozyme. The mean percentage areas of these five peaks were calculated in the two groups and compared by paired *t*-test.

RESULTS

Effect of PRK on tear fluid proteins

The number of protein peaks was compared in tear samples collected from the 23 eyes before and 4 days after PRK. No significant change occurred (Tab. I). The multivariate analysis of discriminance found a significant difference in the protein patterns ($F=3.7769$, $p<0.0014$) between samples collected before and after PRK. The AUC also changed significantly after the excimer laser treatment (Tab. I).

Figure 1 shows a plot of the canonical root of the analysis, giving discrimination of all lanes in the discriminant space.

Changes in the five main protein peaks – lactoferrin, albumin, IgA heavy chain, lipocalin (tear-specific prealbumin), and lysozyme – were detected and evaluated. The results are shown in Figure 2. A decrease could be detected in all of the main protein peaks. The decrease was highly significant ($p<0.005$) in lactoferrin, IgA heavy chain, and lysozyme peaks, and in the case of lipocalin it was borderline significant. The albumin density did not change significantly.

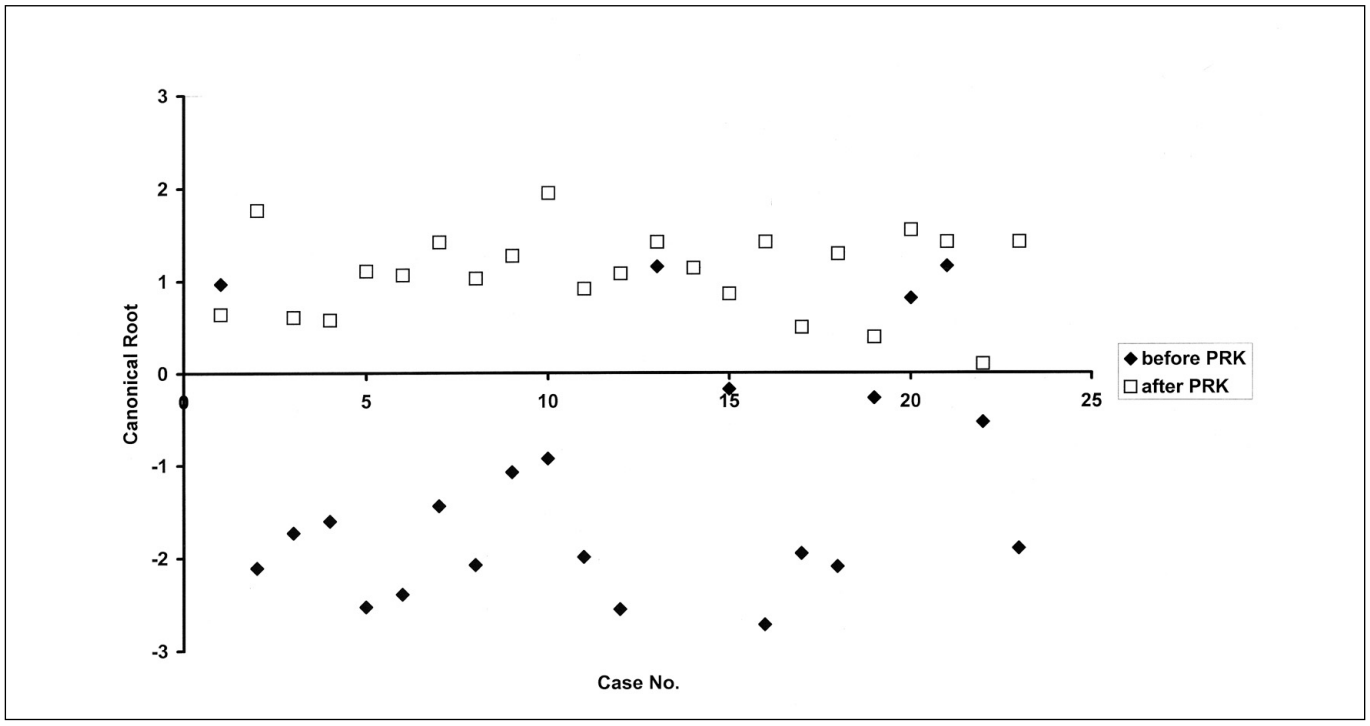

Fig. 1 - *The canonical root of the analysis of discriminance of tear samples obtained before and 4 days after photorefractive keratectomy was plotted versus the case number.*

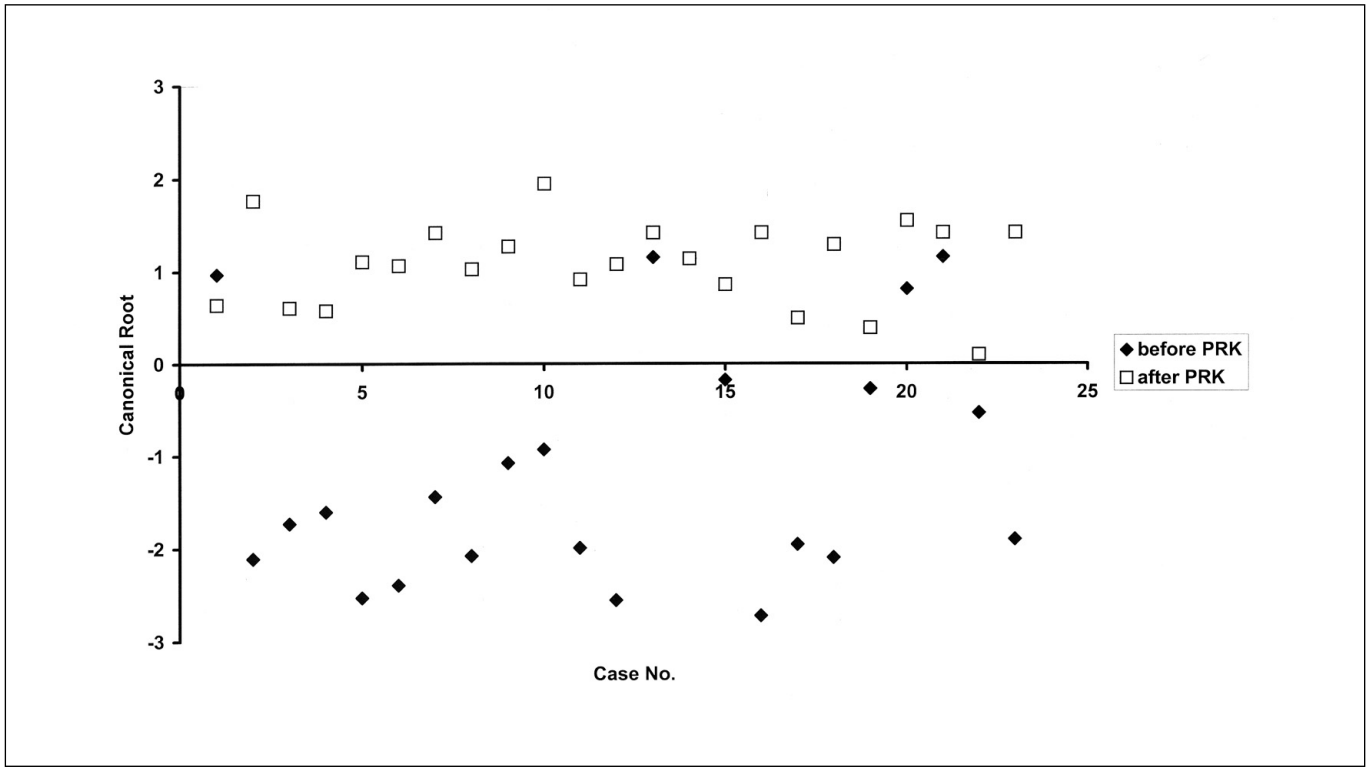

Fig. 2 - *Areas under the mean peaks (lactoferrin, albumin, immunoglobulin A heavy chain, lipocalin, lysozyme) before and after photorefractive keratectomy (n=23). *Highly significant (p<0.005) differences.*

Effect of nasal stimulation on tear fluid proteins

The summary of the comparison of the unstimulated and reflex tear samples of the healthy volunteers can be found in Table II. The only significant change was found in the density of albumin, which decreased considerably. In the other main proteins (lactoferrin, IgA heavy chain, lipocalin, and lysozyme), the AUC and the number of the peaks did not change significantly.

DISCUSSION

We performed electrophoretic analysis of tear proteins before PRK and on the fourth postoperative day in 23 eyes of 23 patients. Comparing the number of peaks pre- and postoperatively, there was no significant increase. Therefore, it is likely that new proteins did not appear in the tear fluid. When we compared the protein patterns, the analysis of discriminance found a significant difference between the two groups of samples. This type of analysis of the electrophoretic densitographs uses all peak information simultaneously. In earlier studies using this method, significant changes in protein patterns were found in patients with dry eye (15, 16), patients with diabetes (17), and contact lens wearers (14), even when there was no significant difference in the main protein peaks. These results document the high sensitivity of this complex image analysis.

In the present study, a significant decrease in three of the main protein peaks – lactoferrin, IgA heavy chain, and lysozyme – was found after PRK. Some borderline significant decrease was observed in the concentration of lipocalin (tear-specific prealbumin). These proteins are known to originate from the lacrimal gland (24). On the other hand, the level of serum albumin, which originates from the conjunctival vessels (24), decreased slightly, but not significantly.

The question arises whether these changes in tear composition were influenced by differences between preoperative and postoperative tear flow. Although we tried to collect the tear samples atraumatically and the postoperative samples were collected only on the fourth day, when the epithelial defect has just healed, reflex tearing occurred in most of the eyes. For testing this hypothesis, we performed a control study on healthy volunteers. We found that the difference be-

TABLE I - CHANGES IN THE NUMBER OF PROTEIN PEAKS AND THE AREA UNDER DENSITOMETRIC CURVE (AUC) IN TEAR SAMPLES AFTER PHOTOREFRACTIVE KERATECTOMY (PRK) IN 23 PATIENTS

Peak	Before PRK, mean ± SEM	4 days after PRK, mean ± SEM	p value (paired *t*-test)
Number of peaks	8.65 ± 0.41	7.65 ± 0.48	0.091
AUC	185155 ± 22460	119725 ± 13713	0.004

TABLE II - DIFFERENCES BETWEEN UNSTIMULATED AND REFLEX TEAR SAMPLES IN NUMBER OF PROTEIN PEAKS, AREA UNDER DENSITOMETRIC CURVE (AUC), AND DENSITY OF MAJOR PROTEIN PEAKS

Variable	Unstimulated tear samples, mean ± SEM		Reflex tear samples, mean ± SEM		p value (paired *t*-test)
Number of peaks	8.55 ± 0.38		7.66 ± 0.47		0.154
AUC	176,957 ± 13,169		144,427 ± 14,776		0.134
Lactoferrin	40,651 ± 6721		38,692 ± 4374		0.732
Albumin	18,616 ± 4654	density	6048 ± 2286	density	0.005
Immunoglobulin A heavy chain	7878 ± 1151		4841 ± 1073		0.062
Lipocalin (prealbumin)	16,246 ± 1939		17,032 ± 1647		0.737
Lysozyme	21,379 ± 3721		16,817 ± 2194		0.300

tween the unstimulated tear and the reflex tear is different from that caused by the laser treatment. This result is congruent with the opinion of others (25-28), who note that there are different types of tear proteins, according to the change in their levels with progressively increasing tear flow rate (25-27). Their behavior depends on their origin and the regulation of their production (28). Lactoferrin, lipocalin, and lysozyme are regulated lacrimal gland proteins; that is, their rate of secretion is controlled by the rate of stimulation, so their level does not decrease with increasing tear flow. Therefore, our finding that their concentration decreased after PRK cannot be explained by reflex tearing. Secretory IgA, however, belongs to the group of constitutively secreted lacrimal gland proteins; the rate of its secretion may fall behind the fluid secretion rate with increasing flow. Because of this, the decrease in IgA concentration after excimer laser treatment may partly be a consequence of the dilution of the tear fluid.

The mechanism that gives rise to changes in the levels of tear proteins after PRK treatment is unknown. We suspect that wounding of the corneal epithelium and surface stroma and injury of the corneal sensory nerves (1, 2) may interfere with the regulatory mechanisms of the protein synthesis of the lacrimal gland.

Symptoms and signs of dry eye are common in the first months after PRK (29, 30). The change in tear composition after excimer laser treatment may also lead to feelings of dryness and a decrease in tear film stability. This hypothesis is supported by the finding that the tear composition is altered in sicca syndrome (9, 15, 16, 31). However, our results differ largely from those of previous studies of patients with dry eye. In previous studies, an increase in the number of peaks could be found, and we concluded that the dry eye syndrome is closely related to this increase in protein peaks. However, in this study following PRK, we observed dry eye clinically, but could not find an increase in the number of peaks. Thus the pathogenesis of dry eye following PRK may differ from the idiopathic pathogenesis.

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