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## Plasminogen activator activity and inhibition in rabbit tears after photorefractive keratectomy

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### Abstract

Plasminogen activator is a normal component of tear fluid that plays a role in corneal wound healing processes. This work examines whether inhibitor-induced low levels of plasminogen activator activity (PAA) during corneal re-epithelialization after excimer laser photorefractive keratectomy (PRK) correlates with the eventual occurrence of haze in rabbit eyes. Tear samples were collected with glass capillaries from 16 eyes of eight New Zealand rabbits, using i.m. injection of pilocarpine hydrochloride for stimulation. Tears were collected before and after PRK surgery, and then daily for 5 days, and every fourth day thereafter for 3 months. Both eyes underwent PRK treatment. One eye of each rabbit was treated as a control while the contralateral eye was treated with aprotinin, a serine protease inhibitor, over the first 7 days. PAA in the tear samples was measured by a spectrophotometric method using human plasminogen and chromogenic peptide substrate S-2251. For the eight control eyes after PRK, the PAA values were significantly lower (day 1) and higher (days 2 and 3) than the equilibrium PAA ( $p < 0.001$ ). The corneas remained clear in each of these control eyes. For the eight contralateral aprotinin-treated eyes after PRK, the PAA values on days 1–7 were significantly lower than the equilibrium PAA ( $p < 0.001$ ). All eight of these aprotinin-treated eyes developed corneal haze after 2 months. There was no significant difference ( $p = 0.06$ ) between control and aprotinin-treated eyes for the equilibrium PAA after 19 days. We conclude that a corneal wound healing abnormality (haze) develops in rabbit eyes after PRK when PAA levels are reduced using aprotinin for a week following PRK.

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**Keywords:** plasminogen activator; photorefractive keratectomy; refractive surgery; corneal wound healing; rabbit tears

### 1. Introduction

The cornea is responsible for two-thirds of the refracting power of the eye, making it a candidate for surgical procedures aimed at refractive correction. The qualities of the wounds made by the ArF excimer laser suggested the concept of using such a laser to directly photoablate the cornea after removal of the epithelial layer (Marshall et al., 1986). The effect is to reprofile the surface of the cornea, defining a new anterior radius of curvature, thereby altering the optical power of the cornea. Marshall et al. (1986) termed this technique photorefractive keratectomy (PRK). The procedure is a reasonably safe, effective and predictable

technique for correcting low to moderate myopia. Nevertheless, the most frequently reported complications include glare, haloes, difficulty with night vision, decreased contrast sensitivity, transient increases in intraocular pressure, mild subepithelial haze and myopic regression (Chan et al., 1997; Hadden et al., 1999).

Numerous laboratory investigations, principally utilizing rabbit and monkey models, have generally demonstrated rapid and stable re-epithelialization over the area of excimer laser ablation. After injury, reformation of the basement membrane and anchoring fibril network is necessary for tight adhesion of the epithelium to the stroma, and the healing epithelium must synthesize and assemble these structures (Gipson et al., 1987, 1989). After mechanical keratectomy wounding, full re-assembly of the adhesion complex generally requires 1–2 months in rabbits (Gipson et al., 1989). In both rabbit and monkey eyes, the ablation

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zone is initially clear, but a diffuse reticular haze, sometimes accompanied by a localized or diffusely dense scar, has been commonly observed (Marshall et al., 1988; Gaster et al., 1989; Hanna et al., 1989). Loss of transparency peaks at approximately 1 month and then slowly diminishes over 6 or more months. Opacification may completely disappear, but some degree of light scattering may persist throughout a follow-up period in most animal studies.

The human cornea exhibits less dense opacification than animal corneas, but has a similar time course of onset and resolution. Most clinical series report maximal, although generally mild, haze developing within 1–2 months after treatment, marked improvement in haze by 6 months, and even further improvement with little or no haze by 12 months (Gartry et al., 1991; Seiler and Wollensak, 1991; Dutt et al., 1994). In addition to the results with animal studies, early experience suggested that haze in humans was correlated with ablation depth (Moller-Pedersen et al., 1998a) and smaller ablation zones. In particular, there were patient complaints about haloes and glare under dim illumination. Surprisingly, the human cornea tolerates an increased ablation depth associated with a larger diameter ablation zone as evidenced by an overall reduction in haze as well as less refractive regression and improved predictability (Sher et al., 1992; O'Brart et al., 1995).

Additional risk factors for haze have been discussed (Azar et al., 1997; Chan et al., 1997; Carones et al., 1999; Hadden et al., 1999; Siganos et al., 1999). These include higher levels of myopic correction, noncompliance with postoperative steroid medication, steroid-induced intraocular pressure response and collagen vascular disease and other autoimmune diseases.

Although the exact mechanisms underlying post-PRK complications are unknown, it is generally suspected that individual variations in corneal wound healing play a significant role in post-PRK refractive regression and haze formation (Moller-Pedersen et al., 1998b; Csutak et al., 2000). In a previous human study (Csutak et al., 2000), low plasminogen activator (urokinase-type) activity sustained over a period of 3 days, evidenced in tear fluid, was an accompanying sign of the six PRK cases that developed haze. The normal plasminogen activator activity (PAA) pattern seen in 71 eyes included a higher than normal PAA level on the third postoperative day and no subsequent haze. In that study, it was not possible to distinguish whether the low PAA was a primary phenomenon or was a result of some other primary event. However, based on its known importance in the wound healing process, the low PAA could have been a possible cause of the defective corneal healing resulting in haze.

The aim of this study is to determine whether low levels of PAA could induce haze formation after PRK surgery. This is accomplished by using aprotinin, a serine protease inhibitor, to artificially suppress PAA during the period of re-epithelialization after PRK surgery in rabbit eyes.

Analysis of frequently sampled tears provides a quantification of the PAA.

## 2. Materials and methods

Sixteen eyes of eight normal healthy New Zealand rabbits (3.0–3.5 kg) underwent PRK surgery for 3D, 6D or 9D spherical refractive correction using the Schwind Keratom II ArF excimer laser (193 nm), performed by the same surgeon at Vital-Laser LLC, Department of Ophthalmology, Faculty of Medicine, Medical and Health Science Center, University of Debrecen. Animals were handled and treated in adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. De-epithelialization was performed with a blunt Keratome Blade knife after epithelial marking with a 6.0–6.5 mm Hoffer trephine. The epithelium was scraped gently from periphery to centre. Residual epithelial debris was removed with a sterile microsponge. Both eyes of a rabbit received the same corrective surgery: 3D, 6D or 9D. The laser surgeries were characterized by a 6.0 mm ablation zone and an ablation depth of 34  $\mu\text{m}$  for 3D, 68  $\mu\text{m}$  for 6D or 102  $\mu\text{m}$  for 9D. For each rabbit, one eye served as a control while the contralateral eye received inhibitor as described below.

Topical anesthetic (0.4% oxybuprocaine hydrochloride) eyedrops were administered to both eyes twice before the surgery. General anaesthesia was accomplished by intravenous injection of ketamine–xylazine in the ratio 60–5 mg kg<sup>-1</sup>. The PRK surgeries were performed on the morning of day 1. The postoperative treatment of both eyes included antibiotic eyedrops, Ciloxan (Alcon), administered from morning to early evening, 12 times (hourly) on day 1 and five times (every 2 hr) on five additional days (postoperative days 2–5 and 7). Flucon (Alcon) and Tears Naturale (Alcon) were also given from morning to early evening, five times daily (every 2 hr) during the first postoperative month, reduced to four times daily (every 3 hr) for the second month and three times daily (every 4 hr) for the third month.

In addition, the left eye of each rabbit received 1 drop of 10 000 KIU ml<sup>-1</sup> aprotinin (Gordox, Richter Gedeon Rt., Budapest, Hungary). The aprotinin was administered during the morning through early evening, 12 times (hourly) on day 1 and five times (every 2 hr) on five additional days (postoperative days 2–5 and 7). This was designated the aprotinin group. The right eyes were not treated with aprotinin and were designated the control group. After the seventh day, identical treatment (Flucon and Tears Naturale) was used on both eyes. No other treatment was used during the 6-month follow-up period.

Tear samples for PAA analyses were obtained on the day before the PRK surgery (day 0). Tear samples were collected within minutes after PRK (day 1), before treatment with any eyedrops. Daily on postoperative days 2–5, on postoperative day 7 and every fourth day thereafter

for 3 months, tears were sampled in the morning prior to the administration of any eyedrops on the given day.

Samples consisted of tears collected with glass capillaries (Tózsér et al., 1989; van Haeringen and Glasius, 1976) using i.m. injection of pilocarpine hydrochloride ( $5 \text{ mg kg}^{-1}$ ) for stimulation. Tears were taken from the lower tear meniscus and care was taken not to touch the conjunctiva. The same collection method was used throughout the study. The duration of the sampling time was recorded and the secretion rate was calculated in  $\mu\text{l min}^{-1}$ , dividing the obtained tear volume by the time of sample collection. Samples used in this investigation had secretion rates of  $10\text{--}50 \mu\text{l min}^{-1}$ . Samples were centrifuged (1800 rpm) for 8–10 min right after sample collection and supernatants were deep-frozen at  $-80^\circ\text{C}$  and were thawed only once for measurements.

PAA was measured in the tear samples by a spectrophotometric method using human plasminogen and a plasmin-specific chromogenic peptide substrate, D-valyl-L-leucyl-L-lysyl-*p*-nitroanilide (S-2251) (Shimada et al., 1981). This assay is sensitive predominantly to urokinase-like plasminogen activator (Tózsér et al., 1989). Plasminogen and the S-2251 were purchased from Chromogenix (Milan, Italy). Urokinase standard was purchased from Choay (Paris, France). This assay is suitable to measure plasmin activity but can also be used for determining PAA by adding plasminogen to the reagents. PAA was measured as described by Shimada et al. (1981) with the modifications of Tózsér and coworkers (Tózsér et al., 1989; Tózsér and Berta, 1990; Csutak et al., 2000).

All rabbits received follow-up examinations daily on postoperative days 2–5, on postoperative day 7 and every fourth day thereafter for 3 months and then weekly from the third to sixth month following the PRK procedure. Determination of haze was made during each examination without any knowledge of the plasminogen activator levels for any of the rabbits. The haze grading system of Hanna was adopted (Hanna et al., 1990).

Standard statistical procedures were used to compare plasminogen activator activities within and between different groups using *t*-tests for means with unequal variances and paired *t*-tests to compare results on contralateral eyes. Differences having probability, *p*, less than 0.05 were considered significant, and  $p < 0.001$  was considered highly significant.

### 3. Results

Fig. 1 shows the time course of the PAA over the first 21 days after PRK for each of the control rabbit eyes. Table 1 gives mean values of the PAA for the early measurement days and the average PAA for days 19–91. In Table 1 and the Figures, ‘day 0’ represents the day before PRK surgery, ‘day 1’ is the day of the surgery, and the succeeding days follow. The mean PAA value before surgery (day 0) was not significantly different ( $p = 0.16$ ) than the 19–91-day equilibrium PAA level. The PAA values were significantly ( $p < 0.001$ ) lower (day 1) and higher (days 2 and 3) than the equilibrium PAA level. The PAA on day 4 was significantly ( $p = 0.02$ ) higher than the equilibrium level, but from day 5 and beyond, there were no significant differences with the equilibrium value. The corneas remained clear in these control eyes over the 6-month follow-up period.

Fig. 2 shows the first 21 days of PAA for each of the contralateral aprotinin eyes. Mean PAA levels for various measurement days are given in Table 1. The aprotinin suppressed the PAA to a mean value averaged over days 1–7 of  $0.13$  ( $0.15$ )  $\text{IU ml}^{-1}$ . The mean PAA prior to surgery (day 0) was not significantly different ( $p = 0.18$ ) than the equilibrium PAA level of  $2.88$  ( $0.22$ )  $\text{IU ml}^{-1}$ , averaged over days 19–91. PAA on days 1–7 were significantly ( $p < 0.001$ ) lower than the equilibrium PAA level. The mean PAA on day 11 remained significantly lower ( $p = 0.002$ ) than the equilibrium PAA level, but from day 15 onwards, there were no significant differences with the equilibrium

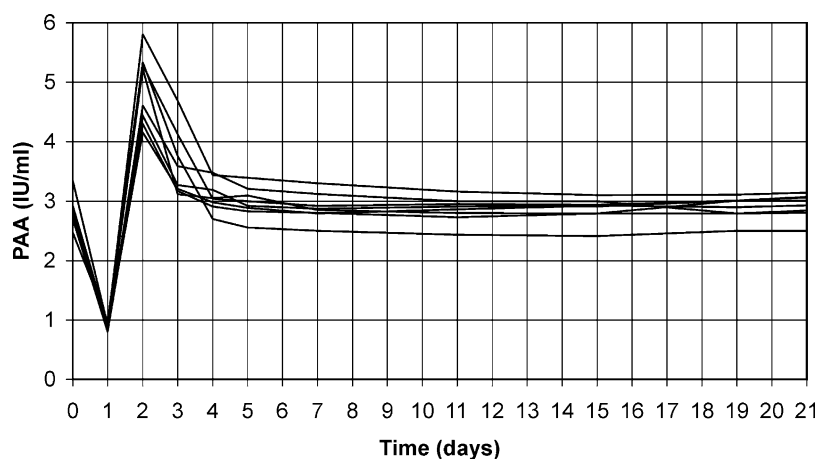


Fig. 1. Plasminogen activator activities in rabbit tears following PRK, for the eight eyes in the control group that were not treated with aprotinin.

Table 1

Mean values (S.D.) and comparison of PAA in rabbit tears after PRK for the eight eyes in the control and the eight eyes in the aprotinin groups

Day	PAA: control (IU ml <sup>-1</sup> )	PAA: aprotinin (IU ml <sup>-1</sup> )	PAA comparison control vs. aprotinin ( <i>p</i> )
0	2.82 (0.26)	2.77 (0.26)	0.46
1	0.87 (0.06)	0.42 (0.47)	0.03
2	4.90 (0.60)	0.18 (0.21)	<0.001
3	3.62 (0.56)	0.04 (0.03)	<0.001
4	3.10 (0.26)	0.03 (0.04)	<0.001
5	2.99 (0.25)	0.05 (0.05)	<0.001
7	2.89 (0.24)	0.05 (0.04)	<0.001
11	2.86 (0.21)	2.63 (0.18)	0.009
15	2.86 (0.21)	2.76 (0.18)	0.06
19–91	2.93 (0.20)	2.88 (0.22)	0.06

Significant difference when  $p < 0.05$ .

PAA value. All eight of these aprotinin eyes developed corneal haze of at least grade 1 between the first and third month. Through the 6-month follow-up period, one of these eyes remained at grade 1, one progressed to grade 3, and the remaining six eyes progressed to grade 2.

The difference between the presurgical (day 0) mean PAA for the two groups was not significant ( $p = 0.46$ ) nor was the equilibrium (days 19–91) mean PAA significantly different ( $p = 0.06$ ) for the two groups. The post-surgical PAA levels were not correlated with the ablation depth nor with the tear flow values calculated for each eye.

#### 4. Discussion

Plasminogen activator is a normal component of tear fluid whose concentration is influenced by biochemical transformations in the cornea. Normal values of PAA in rabbit tears are seen in Figs. 1 and 2 on day 0, prior to PRK and in the later months following the surgery. The two groups, control and aprotinin, consist of contralateral eyes and therefore the plasminogen activator activities before surgery (day 0) and the equilibrium levels (days 19–91) are expected (and observed) to be not significantly different.

The tear samples were collected from the rabbits in the early morning 8–15 hr after the previous administration of any eyedrops from the previous day and prior to the instillation of eyedrops on the given day. The lack of PAA in the tear samples from the aprotinin-treated eyes reflects the fact that the residual aprotinin was sufficient to inhibit the plasminogen activator present in the tears regardless of the source of the plasminogen activator.

The influence of corneal wounding (after PRK) is seen in Fig. 1 for the control group of rabbit eyes. First, there is a drop in PAA after surgery (day 1). This is followed by an overshoot to a level above the initial PAA on day 2 and lasting for a few days. Finally, there is a return to the initial PAA level. This pattern of PAA seen in Fig. 1 for the control group resembles the pattern seen for humans who experience normal wound healing without the occurrence of haze (Csutak et al., 2000). In the case of humans, the PAA fell to a level near zero after PRK, then rose above initial levels on postoperative day 3 and returned to the initial level by postoperative day 5. The PAA pattern in Fig. 1 is also consistent with that reported for rabbits with normal wound healing following anterior keratectomy (van Setten et al., 1989).

In contrast, the PAA pattern found in Fig. 2 for the aprotinin group was engineered to resemble the pattern

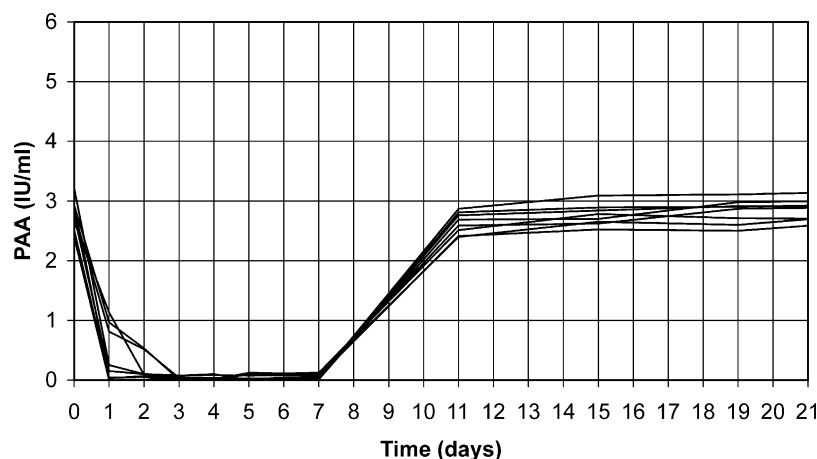


Fig. 2. Plasminogen activator activities in rabbit tears, for the eight eyes in the aprotinin group that were treated with aprotinin for seven days following PRK.

found in humans who presented abnormal wound healing and experienced haze (Csutak et al., 2000). In the human abnormal healing case, the PAA fell to near zero after PRK and remained low through the third postoperative day. By the fifth postoperative day, the PAA was below but returning to the initial level. Here, the PAA level was deliberately suppressed using aprotinin in the aprotinin group of rabbit eyes over a period of 7 days, as shown in Fig. 2.

Naturally occurring low levels of PAA for several days after PRK in human tears correlated with the development of corneal wound healing abnormality (haze) (Csutak et al., 2000). The inhibitor-induced low levels of PAA for a week following PRK in rabbit tears also correlate with the development of corneal haze.

Aprotinin is a broad spectrum serine protease inhibitor having the ability to inhibit a variety of proteinases, including urokinase-type plasminogen activator, trypsin, chymotrypsin, plasmin and kallikrein (Fritz and Wunderer, 1983; Lottenberg et al., 1988). The measurements of PAA in the tear samples demonstrate the specific effectiveness of the aprotinin in inhibiting PAA.

Given the scope of aprotinin inhibition, there exists the possibility that the induction of haze in the present experiments occurs through a mechanism independent of the plasminogen activator system. In particular, plasmin activity has been detected after PRK (van Setten et al., 1989; Tervo et al., 1994; Lembach et al., 2001) and the mechanism of haze formation may depend on plasmin levels. The urokinase-type plasminogen activator is an enzyme that converts plasminogen to plasmin. The present work shows that aprotinin inhibits plasminogen activator and by inhibiting the cascade at the plasminogen activator stage, plasmin is expected to be significantly diminished. The aprotinin may additionally inhibit any residual plasmin present, but this is secondary to the plasminogen activator inhibiting effect.

Another concern is the possibility that preservative in the aprotinin might contribute to the formation of corneal haze. The aprotinin used in the present work contains preservative and buffer, benzyl alcohol and sodium benzoate, at a concentration of  $0.1 \text{ mg ml}^{-1}$ . We are not aware of any published work linking benzyl alcohol to haze. Although not an examination of aprotinin, a study of the effects of preservatives in non-steroidal anti-inflammatory drug (NSAID) eye drops on normal rabbit corneal epithelium structure did not report haze as one of the consequences (Stroobants et al., 2000). One of the materials included in the study was Indoptol (Merck Sharp & Dohme), which contains benzyl alcohol at a concentration of  $2.5 \text{ mg ml}^{-1}$  (25 times higher benzyl alcohol concentration than in the aprotinin). In a different study, a formulation containing 0.5% (v/v) benzyl alcohol with ketorolac (Alergan) and ketorolac tromethamine was found to be suitable for ophthalmic application without reporting haze formation as a deleterious side effect (Malhotra and Majumdar, 1997).

In previous work (Lohmann and Marshall, 1993), aprotinin had been suggested as a possibility for preventing haze after PRK; however, subsequent work showed a different result (O'Brart et al., 1994). Experiments were performed on human eyes (O'Brart et al., 1994) using 1 drop of  $40 \text{ KIU ml}^{-1}$  aprotinin immediately after PRK surgery, 1 day later and then five times daily for 3 weeks. Compared to controls, disturbances in corneal transparency for the 6D correction group were greater postoperatively and reached statistically significant levels at nine and 12 months in aprotinin treated eyes. That outcome is consistent with our hypothesis. In our experiments, we used a significantly higher concentration of aprotinin but over a shorter period (1 week), identified as the critical time in our earlier work (Csutak et al., 2000). The objective here was to ensure the inhibition of the urokinase-type PAA without concern for dose-response information. Moreover, the objective was to measure the PAA in order to establish that the plasminogen–plasmin cascade was interrupted at the plasminogen activator level.

Using plasminogen deficient mice, early stromal haze (appearance during 1–21 days) occurred in 74% of post-PRK corneas (Drew et al., 2000). Histochemical examination suggested significant fibrin deposition to be associated with this haze. The plasminogen deficiency in these mice would be expected to lead to a corresponding lack of plasmin and a consequent absence of fibrinolysis (Kao et al., 1998). This mechanism is consistent with the present work in that the inhibition of plasminogen activator with aprotinin would be expected to significantly reduce the conversion of plasminogen to plasmin, with consequent effects on fibrinolysis. Histochemistry alone does not explain why the plasminogen deficiency did not cause a similar effect on the remaining 24% of the mice (Drew et al., 2000). Biochemical analysis similar to the present work would provide additional insight regarding activities and levels of deficiency of key enzyme systems.

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