Nerve Growth Factor Changes and Corneal Nerve Repair after Keratoplasty

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SIGNIFICANCE: Measured tear concentration of nerve growth factor is correlated with postoperative corneal reinnervation among patients who undergo keratoplasty. This may be a future therapeutic target for post-keratoplasty corneal nerve regeneration.

PURPOSE: To determine the relationship between changes in the content of nerve growth factor (NGF) in tear fluid and corneal subepithelial nerve regeneration in patients after keratoplasty.

METHODS: In this retrospective study, 30 eyes of 28 patients (15 males, 13 females; mean age 42.8 [range 16–73] years) who underwent primary keratoplasty for the first time were recruited through the clinics of the Department of Ophthalmology, Jilin University affiliated First Hospital, between May and December 2015. All patients underwent a complete ophthalmic examination preoperatively. Tear fluid samples were collected to detect the content of NFG at different time points in the follow-up period (day 1 preoperatively and days 1, 7, 30, and 90 postoperatively) and analyzed correlations between NFG content and age, infective factors, and variables of the surgical procedure as well as with subepithelial nerve repair at 30 and 90 days postoperatively.

RESULTS: The NFG content in tear fluid on day 1 postoperatively was lower than that on the day preceding surgery; however, it was higher than the preoperative value on postoperative days 7, 30, and 90 ($F = 5.046$, $P < 0.05$). Further, the NFG content of tear fluid at 30 days postoperatively correlated with the surgical procedure (coefficient = –2.775, $P = 0.010$); however, no significant correlation was found on postoperative day 1 (coefficient = –1.315, $P > 0.05$). At all study time points, the NFG content of tear fluid had no correlation with infective factors or age ($P > 0.05$). Postoperatively, at day 30, small nerve buds were observed in the periphery of the corneal graft in 13 eyes (43.3% of cases) but not in 17 eyes (56.7% of cases), which showed a significant correlation with the NFG content of tear fluid (coefficient = –3.370, $P = 0.010$). By postoperative day 90, small nerve buds were observed in the periphery of the corneal graft in 24 eyes (80.0% of cases) and showed a significant correlation with the NFG content of tear fluid (coefficient = –2.750, $P = 0.006$).

CONCLUSIONS: The NFG content in tear fluid increases with the increasing ratio of small nerve buds indicating corneal nerve regeneration. NFG promotes subepithelial nerve regeneration in patients after keratoplasty.

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Penetrating keratoplasty and deep anterior lamellar keratoplasty are, in the present day, important therapies for the treatment of severe keratopathy. However, during keratoplasty, corneal sensory innervation is disrupted and the normal tear film distribution is altered by changes of the ocular surface.[1] Furthermore, dry eye disease and neurotrophic epitheliopathy are significant complications, especially after a penetrating keratoplasty as it disrupts the full-thickness innervation of the cornea,[2–4] with consequent poor efficacy or treatment failure. Therefore, it is crucial to identify the factors essential for maintaining tear film stability and promoting corneal nerve reinnervation after keratoplasty.

Earlier research on corneal grafting has mostly focused on tear film stability,[3,5,6] altered corneal sensation, rate of corneal reinnervation, the number of endothelial cells in the cornea, the size of the corneal reinnervation area,[3,7–10] and so on. Recently, however, there has been increasing attention on nerve regeneration after keratoplasty.[3,8] Nevertheless, there are no research reports on the association of changes in the composition of nerve growth factor in tear fluid and corneal subepithelial neural regeneration. Therefore, this study was conducted to detect tear nerve growth factor content by enzyme-linked immunosorbent assay, combined with evaluation of postoperative subepithelial nerve regeneration by corneal confocal microscopy, after keratoplasty with an aim to explore correlations if any.

METHODS

Patients and Controls

In this retrospective study, 30 eyes of 28 patients (15 males, 13 females; mean age 42.8 [range 16–73] years) who underwent a primary keratoplasty for the first time were recruited through the clinics of the Department of Ophthalmology, Jilin University affiliated First Hospital, between May 2015 and December 2015. The sample size justification for this study referenced other clinical studies on the follow-up of patients who underwent keratoplasty.[10–12]

These included eight cases (eight eyes) of keratoconus, two cases (four eyes) of corneal dystrophy (one of the two corneal dystrophies is granular corneal dystrophy and the other is lattice corneal dystrophy), eight cases (eight eyes) of corneal leukoma, six cases (six eyes) of corneal perforations, and four cases (four eyes) of corneal ulceration. All patients preoperatively underwent a complete ophthalmic examination, and none of the subjects had a history of ocular surgery, other ocular diseases, or any systemic comorbidities. Penetrating keratoplasty was done in 18 cases (19 eyes) that had full-thickness corneal lesions. Similarly, deep anterior lamellar keratoplasty was undertaken in 10 cases (11 eyes) with partial-thickness corneal lesions with intact corneal endothelial function. Patients who had received two or more corneal transplantations were excluded. Patients with noninfective etiology included 10 cases (11 eyes) of corneal dystrophy and keratoconus, whereas patients with infective etiology included 18 cases (19 eyes) of corneal leukoplakia, corneal perforation, and corneal ulcer. Written informed consent was obtained from all subjects. The experimental protocol was established according to the guidelines of the Declaration of Helsinki and was approved by the Human Ethics Committee of Jilin University.

Subjects underwent routine follow-up examinations (preoperatively on the day before penetrating keratoplasty, and on postoperative days 1, 7, 30, and 90), including enzyme-linked immunosorbent assay to detect the nerve growth factor content in tear fluid, slit-lamp examination (Haag-Streit BQ900 + IM900, Switzerland) to detect intra- and postoperative complications, corneal graft transparency, intraocular pressure (TOPCON CT-80, Japan), and corneal confocal microscopy (Confoscan-4; Nidek, Japan). At each follow-up time point, a sample comprising 100 μL tears was collected and the patients were followed up for more than 3 months postoperatively. All surgeries were conducted by the same surgeon (JH), and tear fluid samples were collected by the same physician, with care to avoid stimulation of tear secretion. The method of tear collection was adopted from that of the Sun Yat-sen University research methods,[13] using a Drummond Microcaps tear collection tube (20 μL). Corneal confocal microscopy was performed by the same physician at least twice or more number of times at each follow-up.

Corneal Graft Preparation

Corneal transplants were harvested from cadaveric donors. Eyelid and surrounding skin were administered through routine disinfection; conjunctival sac was disinfected by povidone-iodine, then flushed with physiological saline. Corneal donors were removed by ophthalmic scissors 2–3 mm distances from sclera to corneal limbus, then preserved in corneal preservation solution at 4°C, and transplanted within 24 hours of harvesting.

Surgical Procedures and Postoperative Care

All patients were treated according to the institutional standard protocol for penetrating keratoplasty and deep anterior lamellar keratoplasty. Patients were administered local anesthesia and the eyeball was fixed by traction of the superior and inferior recti with sutures. Diseased corneal tissue was extracted by vacuum trephination. A corneal graft measuring 0.25 mm more than the recipient trephination size was prepared in patients with corneal ulcer or keratoleukoma close to corneal limbus, whereas the same-size corneal grafts were used in patients with keratoconus. For penetrating keratoplasty, the corneal graft was placed on the recipient bed after removal of diseased corneal tissue and sutured to the recipient's cornea by 16 interrupted 10/0 nylon sutures. Then, equilibrium liquid (together with a little viscoelastic agent, if necessary) was injected into the anterior chamber to form a deep anterior chamber. In deep anterior lamellar keratoplasty, the anterior diseased cornea was resected after separation from the deep lamellar endothelium using Anwar's big bubble and the moon-shaped knife technique to expose Descemet's membrane. Thereafter, the corneal graft was placed on the recipient bed (same matching size as with penetrating keratoplasty) and sutured to the recipient cornea by 16 interrupted 10/0 nylon sutures.

Postoperative antibiotic prophylaxis with both local and systemic antibiotics was used against infection. All systemic antibiotics were discontinued 7 days postoperatively, and local antibiotics were stopped 30 days postoperatively. Tacrolimus eye drops, tobramycin and dexamethasone ophthalmic ointment, and dexamethasone injection were used to prevent graft rejection. Tobramycin and Dexamethasone Ophthalmic Ointment (S.A. Alcon-Couvreur N.V., USA) three times a day within 1 month postoperatively, Fluorometholore Eye Drops (concentration 0.1%; Santen Pharmaceutical, Japan) three times a day from 2 months to 6 months postoperatively, and Fluorometholore Eye Drops two times a day from 7 months to suture removal postoperatively were used. The dexamethasone injection was replaced postoperatively by an oral corticosteroid after 7 days. Standard supportive care with vitamin C, vitamin B1, and methylcobalamin oral supplements was administered.

Enzyme-Linked Immunosorbent Assay

The nerve growth factor (human) enzyme-linked immunosorbent assay kit is based on standard sandwich enzyme-linked immunosorbent assay technology. We added an aliquot of 50 μL of standard human nerve growth factor solution into each well of the pre-coated 96-well plates. Then, we added 50 μL of sample diluent buffer into the control well and added 50 μL of each human tear sample into separate empty wells (undiluted; each sample was added to two wells). The plates were covered and incubated at 37°C for 90 min and the contents were discarded. Thereafter, 50 μL of biotinylated anti-human nerve growth factor antibody working solution was added to each well and the plate was incubated at 37°C for 60 min. Then, the plate was washed thrice with 0.01 M phosphate-buffered saline. Fifty microliters of an avidin–biotin–peroxidase complex working solution was prepared and added into each well and the plate was incubated at 37°C for 30 min. The plate was then subjected to five washing cycles with 0.01 M phosphate-buffered saline before the addition of 45 μL 3,3′,5,5′-tetramethylbenzidine color-developing agent into each well and further incubation at 37°C for 30 min. Then, 50 μL of a constituted 3,3′,5,5′-tetramethylbenzidine stop solution was added into each well and the absorbance was read at 450 nm in a microplate reader within 30 min of adding the stop solution.

Statistical Analysis

Data were analyzed using SPSS 23.0 (IBM-SPSS Inc., Chicago, IL). Correlations between nerve growth factor content and age, infective factors, and surgical procedural variables were analyzed using Pearson's correlation coefficient. Nonparametric tests were performed to ascertain whether there were correlations between nerve growth factor content and the rate of subepithelial nerve repair at 30 and 90 days postoperatively. Descriptive statistics

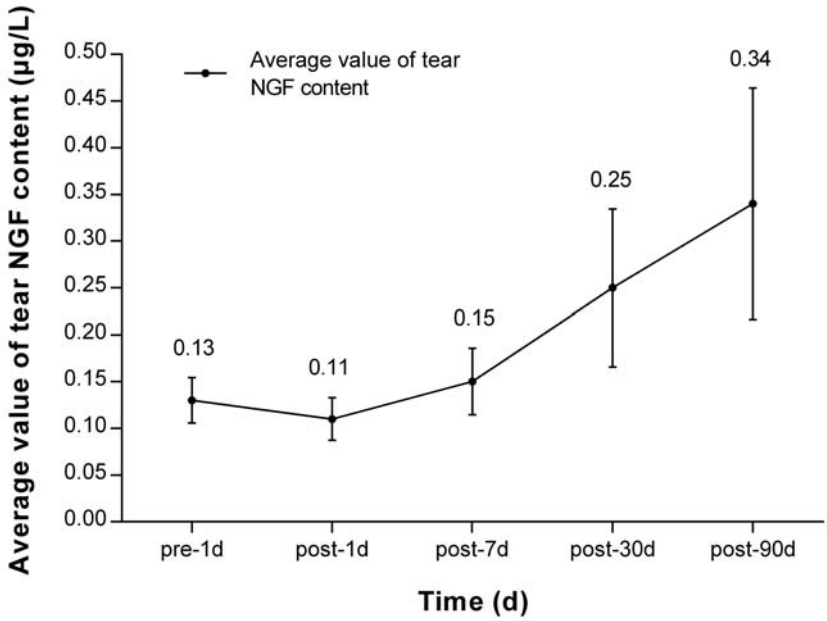

FIGURE 1. Detected tear nerve growth factor content in tear fluid (30 eyes of 28 patients) by enzyme-linked immunosorbent assay. The mean value of tear nerve growth factor content in tear fluid on day 1 postoperatively was lower than that on the day preceding surgery; however, it was higher than the preoperative value on postoperative days 7, 30, and 90 and shows an upward trend ($F = 5.046$, $P < 0.05$). Each data point represents the mean value of tear nerve growth factor content; error bars represent SD; polyline represents variation trend.

are presented as the mean ± SD. *P* values less than 0.05 are considered indicative of statistical significance.

RESULTS

Correlation Between Nerve Growth Factor Content in Tear Fluid and Age, Infective Factors, and Surgical Procedure

The nerve growth factor content in tear fluid on day 1 postoperatively was lower than that on the day preceding surgery; however, it was higher than the preoperative value on postoperative days 7, 30, and 90 ($F = 5.046$, $P < 0.05$; Fig. 1). Further, the nerve growth factor content of tear fluid at 30 days postoperatively correlated with the surgical procedure (coefficient = –2.775, $P = 0.010$); however, no significant correlation was found on postoperative day 1 (coefficient = –1.315, $P > 0.05$). Moreover, there was no correlation between the nerve growth factor content of tear fluid with surgical procedure either at 7 (coefficient = –0.331, $P > 0.05$) or 90 days postoperatively (coefficient = 2.075, $P > 0.05$). The nerve growth factor content of tear fluid had no correlation with infective factors on postoperative days 1 (coefficient = 0.169, $P > 0.05$), 7 (coefficient = 0.020, $P > 0.05$), 30 (coefficient = 1.831, $P > 0.05$), and 90 (coefficient = –0.771, $P > 0.05$). In addition, the nerve growth factor content of tear fluid showed no correlation with age on days 1 (coefficient = 0.390, $P > 0.05$), 7 (coefficient = –1.059, $P > 0.05$), 30 (coefficient = 2.054, $P > 0.05$), or 90 (coefficient = –1.404, $P > 0.05$) postoperatively. There were no significant differences between the patients with noninfective etiology and infective etiology with regard to age ($t = 1.085$, $P > 0.05$) (Table 1).

Association of Nerve Growth Factor Content in Tear Fluid With Rate of Subepithelial Nerve Repair

Postoperatively, at day 30, small nerve buds were observed in the periphery of the corneal graft in 13 eyes (43.3% of cases) but not in 17 eyes (56.7% of cases), which showed a significant correlation with the nerve growth factor content of tear fluid (coefficient = –3.370, $P = 0.010$).

By postoperative day 90, small nerve buds were observed in the periphery of the corneal graft in 24 eyes (80.0% of cases) and

TABLE 1. Correlation between nerve growth factor content and age, infective factors, and surgical procedure postoperatively

	Day 1		Day 7		Day 30		Day 90	
	Coefficient	*P*	Coefficient	*P*	Coefficient	*P*	Coefficient	*P*
Age	0.390	>0.05	–1.059	>0.05	2.054	>0.05	–1.404	>0.05
Infective factors	0.169	>0.05	0.020	>0.05	1.831	>0.05	–0.771	>0.05
Surgical procedure	–1.315	>0.05	–0.331	>0.05	–2.775	0.010	2.075	>0.05

showed a significant correlation with the nerve growth factor content of tear fluid (coefficient = –2.750, $P = 0.006$). However, in six eyes (20.0% of cases), there were no observable small nerve buds at the periphery of the corneal graft. The difference in nerve regeneration between the two follow-up time points was significant ($t = 0.637$, $P = 0.000$) and indicated that small nerve buds were gradually increasing over time (Fig. 2).

Postoperative Complications

Postoperatively, the intraocular pressure remained normal at all follow-up time points. There were no intraoperative or postoperative complications. None of the subjects experienced graft rejection. All patients expressed satisfaction with the surgical outcome.

DISCUSSION

In recent years, nerve growth factor has been widely implicated in several disciplines and fields, with neurotrophic factor being the earliest discovered and most thoroughly researched of all nerve cell growth-regulating factors. The nerve growth factor is primarily expressed by epithelial and stromal cells, which populate the ocular surface in both conjunctiva and cornea.[13] The nerve growth factor accelerates epithelial healing and neural tract repair.[14] The nerve growth factor is a nutrient for neurons and promotes neurite growth; moreover, it induces keratocyte migration and facilitates corneal nerve regeneration.[15,16] Furthermore, it plays an important regulatory role in the development, differentiation, growth, and regeneration of central and peripheral neurons. Ocular nerve growth factor is synthesized and secreted by corneal epithelial cells, endothelial cells, stromal cells, and corneal limbal stem cells that regulate corneal healing by combining with the functional receptor tropomyosin receptor kinase A (TrkA). Several proinflammatory cytokines excreted by injured corneal epithelial cells and stromal keratocytes can upregulate the expression of both nerve growth factor and its receptors.[17]

In our study, the nerve growth factor content in tear fluid proportionally increased with the ratio of small nerve buds. We found small nerve buds at the edge of the corneal graft by postoperative day 30. However, a previous study found that sub-basal nerves were not visible in the central cornea even 1 year after penetrating keratoplasty.[3] Richter et al. detected sub-basal nerves 2 years after keratoplasty.[18] We selected small nerve buds as an observation index because the corneal nerve is too severely injured after a keratoplasty, but small nerve buds may regenerate slowly from the periphery toward the central cornea to develop into mature nerves.

This method could effectively reduce stimulation of tear secretion, with minimal damage or discomfort. The nerve growth factor content in tear fluid on day 1 postoperatively was lower than that recorded preoperatively, which differs from reports of a previous study by Lee on the nerve growth factor content in the tear fluid of patients who underwent laser-assisted in situ keratomileusis. This could be attributed to ocular suture irritation after penetrating keratoplasty and deep anterior lamellar keratoplasty, resulting in reflex lacrimal secretion. At 7, 30, and 90 days postoperatively, the nerve growth factor content significantly increased as compared with preoperative values, and the increase appeared to demonstrate a linear growth trend. It is our inference that stimulation of lacrimal secretion decreased the ocular irritation symptoms significantly and progressively in the postoperative period. In this study, 30 days postoperatively, the nerve growth factor content in the penetrating keratoplasty group was higher than in the deep anterior lamellar keratoplasty group, which may be related to the degree of corneal subepithelial nerve injury. Deep anterior lamellar keratoplasty may have injured the corneal subepithelial neural network more severely within the initial 30 days postoperatively, but the effects of the damage decreased gradually over time in the postoperative period. Our study suggests, therefore, that penetrating keratoplasty has an advantage over deep anterior lamellar keratoplasty within the initial 30 days postoperatively, but the difference between the two procedures in this regard is gradually eliminated after 30 days. Changes in nerve growth factor content did not correlate significantly with age and infection at different time points, but may be related to stimulation of tear secretion, use of corticosteroids and antibiotics, ocular balance, and so on.

Our study has certain limitations. The follow-up time was relatively short because of the difficultly in collecting tear fluid because of severe drying of eyes after day 90 postoperatively. Moreover, we did not collect data for the initial 2 weeks postoperatively. And the tendency

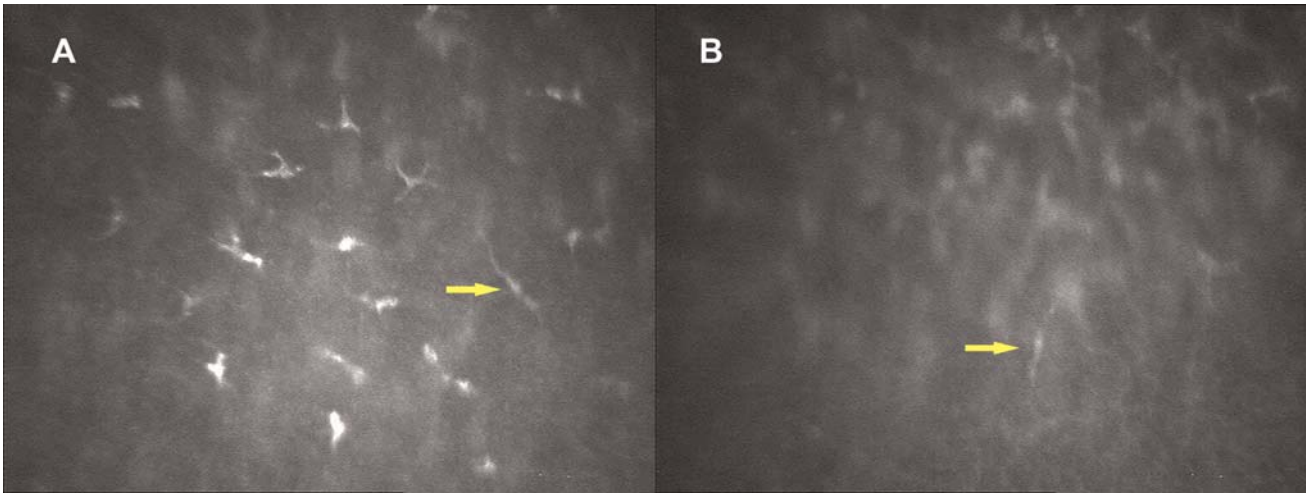

FIGURE 2. Sample of corneal subepithelial small nerve buds (arrow) of one patient's corneal at 30 days postoperatively (A) and 90 days postoperatively (B) by confocal microscopy, scanning around the incision; yellow arrow indicates the corneal subepithelial small nerve buds.

for change in nerve growth factor content was not observed comprehensively. Furthermore, we observe that there is a need to compare improvement in visual acuity in patients after penetrating keratoplasty and deep anterior lamellar keratoplasty in future research.

In conclusion, nerve growth factor promotes subepithelial nerve regeneration in patients after keratoplasty. Our findings have great significance in therapy for postoperative corneal reinnervation among patients who undergo keratoplasty.

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