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Original Article

Regression of mature corneal lymphatic vessels by intracorneal ranibizumab injection

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Abstract: Objective: Ranibizumab is a Fab fragment of a recombinant, humanized, monoclonal anti-vascular endothelial growth factor (VEGF) antibody. This study analyzes the possibility of regressing lymphangiogenesis and hemangiogenesis by intracorneal ranibizumab injection. In addition, the effect of ranibizumab on corneal endothelial cells (CECs) of mice is also studied. Methods: Hemangiogenesis and lymphangiogenesis were induced in female BALB/c mice using the murine model of suture-induced inflammatory neovascularisation. The treatment group received an intracorneal injection of ranibizumab (controls: phosphate buffered saline (PBS)). Corneas were excised at different time points (1 day, 5 days, and 10 days) after the injection, and corneal whole mounts were stained with CD31, LYVE-1, and alizarin red S to quantify hemangiogenesis, lymphangiogenesis, and corneal endothelium. The morphology was analyzed by using the image analysing programme Cell^F and Image J image analysis programme, respectively. Results: In accordance with our previous findings, lymphatic vessels and blood vessels could be reduced after an intracorneal ranibizumab injection: One day after the injection, lymphatic vessels were reduced by 18% ($P = 0.4$), blood vessels were reduced by 22% ($P = 0.083$); after 5 days and 10 days, lymphatic vessels were reduced by 50% ($P = 0.002$) and 63% ($P < 0.001$), respectively, and blood vessels were reduced by 52% ($P = 0.0031$) and 68% ($P < 0.001$), respectively. The corneal endothelial morphology showed no significant differences after the intracorneal ranibizumab injection for 10 days (all $P > 0.05$). Conclusions: This study is the first to demonstrate that the intracorneal ranibizumab injection is a novel technique to specifically induce regression of corneal lymphatics and blood vessels without affecting corneal endothelial cells.

Keywords: Cornea, hemangiogenesis, lymphangiogenesis, neovascularization, endothelial cells, ranibizumab

Introduction

Corneal transparency and optimal vision require an avascular cornea. The cornea possesses redundant antiangiogenic mechanisms that actively maintain corneal avascularity, collectively accounting for corneal angiogenic privilege [1]; however, corneal angiogenic privilege is not absolute. Several conditions such as chemical burns, infections, limbal stem cell deficiency, or trauma can interfere with this (lymph) angiogenic privilege and cause ingrowths of pathological blood and lymphatic vessels from the limbus into the corneal centre [2]. This is a major cause for blindness worldwide. Further, these vessels are one of the main risk factors for immune mediated allograft rejection after corneal transplantation (keratoplasty) in patients [3]. Hos et al. used the mouse model of high-risk corneal transplantation, where corneal avascularity was abolished by a severe inflammatory stimulus prior to keratoplasty [4]; they recently found that lymphatic vessels in particular, but not blood vessels, defined the high-risk status of vascularized corneas, and that anti (lymph) angiogenic treatment significantly promoted corneal allograft survival. Removal of draining lymph nodes could also significantly improve graft survival in the murine model [5]. In brief, pre-existing stromal lymph and blood vessels are strong risk factors for immune rejection after corneal transplantation [6, 7].

With regard to corneal neovascularization, both topical and subconjunctival administration of

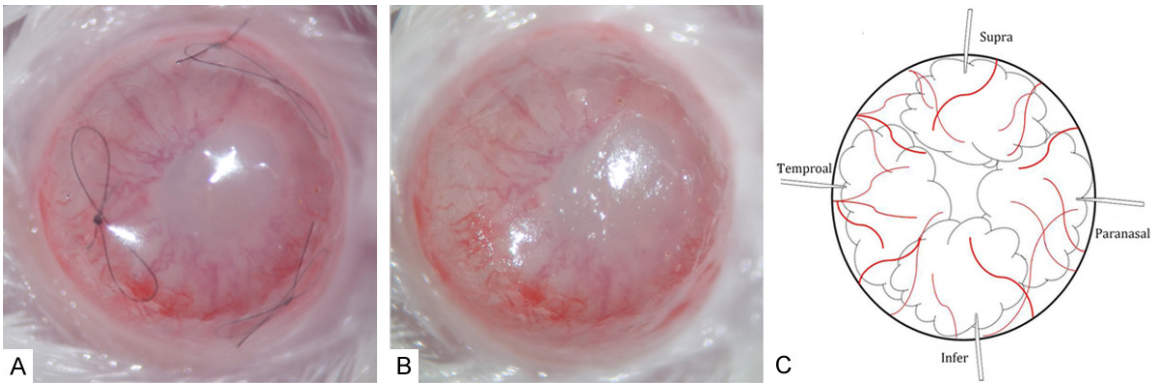

Figure 1. A. Intrastromal sutures inducing neovascularization into the cornea. Three 11-0 nylon sutures were placed in the corneal stroma; after 14 days, blood and clinically invisible lymphatic vessels grew from the limbus into the corneal centre. B. The ranibizumab was injected in the corneal stroma after removal of the corneal sutures. C. The sites of the intracorneal ranibizumab injection; the dotted line indicates the range of the liquid diffusion.

Bevacizumab were shown to be effective in diminishing vascularization [8]. However, penetration of the drug through an intact epithelium is considered an issue since its molecular weight is high, thereby limiting the absorption after topical administration [9]. Subconjunctival injections guarantee better delivery, although local side-effects have been reported [9]. Other disadvantages of these methods are the high cost of topic preparations as well as potential systemic side-effects associated with subconjunctival administration. Because systemic anti-VEGF exposure is associated with severe and potentially life-threatening adverse events, it is prudent to pursue the route of administration that minimizes systemic exposure [10]. Intracorneal injection has been used to treat a case of corneal neovascularization, showing encouraging results [11]. A small case series on subconjunctival and intracorneal injections of Bevacizumab for the treatment of lipid keratopathy has also been published [12]. Therefore, we analyzed whether it is possible to regress existing lymphatic and blood vessels in the cornea by the intracorneal injection of ranibizumab.

Materials and methods

Animals and anaesthesia

All animal protocols were approved by the local animal care committee and in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research; moreover, institutional guidelines regarding animal experimentation were followed. Mice were anaesthetized with an intraperitoneal injection of a combination of 8 mg/kg ketamine (Ketanest; Godecke, Berlin, Germany) and 0.1 ml/kg xylazine (Rompun; Bayer, Leverkusen, Germany). For the suture-induced inflammatory corneal neovascularization assay, 6-8 week-old female BALB/c mice were used.

Mouse model of suture-induced inflammatory corneal neovascularization

The mouse model of suture-induced inflammatory corneal neovascularization was used as previously described. Before corneal neovascularisation, each animal was anaesthetized. Then, three 11-0 nylon sutures (Serag Wiessner, Naila, Germany) were placed averagely in three parts of each corneal stroma (**Figure 1A**). The outer point of entry was chosen near the limbus, and the inner exit point was the corneal centre equidistant from the limbus to obtain standardized angiogenic responses. Sutures were left in place for 14 days [7], and the intracorneal ranibizumab or PBS injection was administered after removal of the corneal sutures (**Figure 1B**).

Ranibizumab injection into the peripheral corneal stroma

The injections were given under a surgical microscope. The treatment group (n = 15) received an intracorneal injection of 2.5 μL ranibizumab (10 mg/mL) for each quadrants [13, 14] using a 33 gauge Hamilton needle (Hamilton Messtechnik GmbH, Höchst, Ger-

many). The control group (n = 15) received an intracorneal injection of 2.5 μL phosphate buffered saline (PBS) for each quadrants. Ranibizumab was injected into the corneal stroma, next to the neovessels and avoiding the blood vessels (**Figure 1C**). The exact site of the injection depended on how far from the limbus or how deep in the cornea the vessels were found. The four quadrants of the cornea were injected in each session. Each cornea was injected with 10 μL of ranibizumab or PBS in four quadrants. Ranibizumab or PBS was injected in four sites in the corneal stroma including supra, infer, paranasal, and temporal (**Figure 1C**). When central involvement was observed, the needle was introduced in the affected quadrant, adjacent to the vessels, but out of the visual axis. After the injection, the mice were prescribed topical antibiotics (moxifloxacin) q.i.d. for three days.

Morphological analysis of corneal hemangiogenesis and lymphangiogenesis

Thirty mice were used for the assessment of hemangiogenesis and lymphangiogenesis (treatment group with ranibizumab (n = 15) and control group with PBS (n = 15)). Five corneas of each group were excised at 1 day, 5 days, and 10 days after the intracorneal ranibizumab injection. Corneal blood and lymphatic vessels were stained in whole mounts with CD31-fluorescein isothiocyanate (FITC) (Acris Antibodies GmbH, Hiddenhausen, Germany) as a pan-endothelial marker and LYVE-1 (AngioBio, DelMar, USA) as a specific marker for lymphatic endothelial cells, as described previously [15-17]. Further, LYVE-1 was detected with a Cy3-conjugated goat anti-rabbit secondary antibody (Dianova GmbH, Hamburg, Germany). Isotype control was assured with a FITC-conjugated normal rat IgG for CD31 and with a normal rabbit IgG for LYVE-1 (both Santa Cruz Biotechnology, Santa Cruz, California, USA).

Whole mounts were analyzed with a fluorescence microscope (BX53, Olympus Optical, Hamburg, Germany), and digital pictures were taken with a digital camera (XM10, Olympus, Hamburg, Germany). The areas covered with blood or lymphatic vessels were detected with an algorithm established in the image analysing programme Cell^F (Olympus, Hamburg, Germany) [15-18]. Briefly, 9 to 12 images at a 100 × magnification were taken of the corneas and automatically assembled to one whole image. Then, an algorithm was used to detect the areas covered with blood or lymphatic vessels in an image-analysis programme (analysis^B; Soft Imaging System). Different filters modified grey-scale images of whole mounts before analysis. Thereafter, the total area of the cornea was defined along the limbus. The area covered by blood and lymphatic vessels was determined by setting a threshold that included the bright vessels and excluded the dark background in the measurements. These areas covered vessels correlated with the total area of the cornea (vessel ratio).

Corneal endothelial morphology

Thirty mice were killed with cervical dislocation (treatment group with the intracorneal ranibizumab injection 15 days, n = 15; control group with the intracorneal PBS injection, n = 15), and both corneas were dissected. After making peripheral radial cuts, the specimens were placed on glass slides and the endothelium was stained with alizarin red S (Sigma-Aldrich, Inc. St. Louis, MO). The central part of the endothelium was photographed in a light microscope at 400 × magnification, and the digital photos were analyzed using the Image J image analysis programme (National Institutes of Health, Bethesda, MD). Corneal endothelial cells density (CD), average size (Save), maximum size (Smax), minimum size (Smin), and size standard deviation (SD) were determined for a central cluster of 55 cells in each specimen by marking the cell corners manually [19-21]. The percentages of hexagonal cells (HEX%) was calculated and cell polymegethism was quantified using the coefficient of variation (CV) of cell size, which was calculated as the standard deviation of the cell sizes for a specimen divided by the mean cell size for the same specimen. These cells were divided into six types: polygon (cell edges ≥ 7), hexagon, pentagon, quadrilateral, triangular, and circular. After adjusting the image in terms of multiple filters and contrast, Image J soft-drew the cell borders automatically [18].

The area variation of corneal endothelial cells was measured using the Freehand Selections of the Image J image analysis programme. The Image J area (Z) of individual cell was obtained

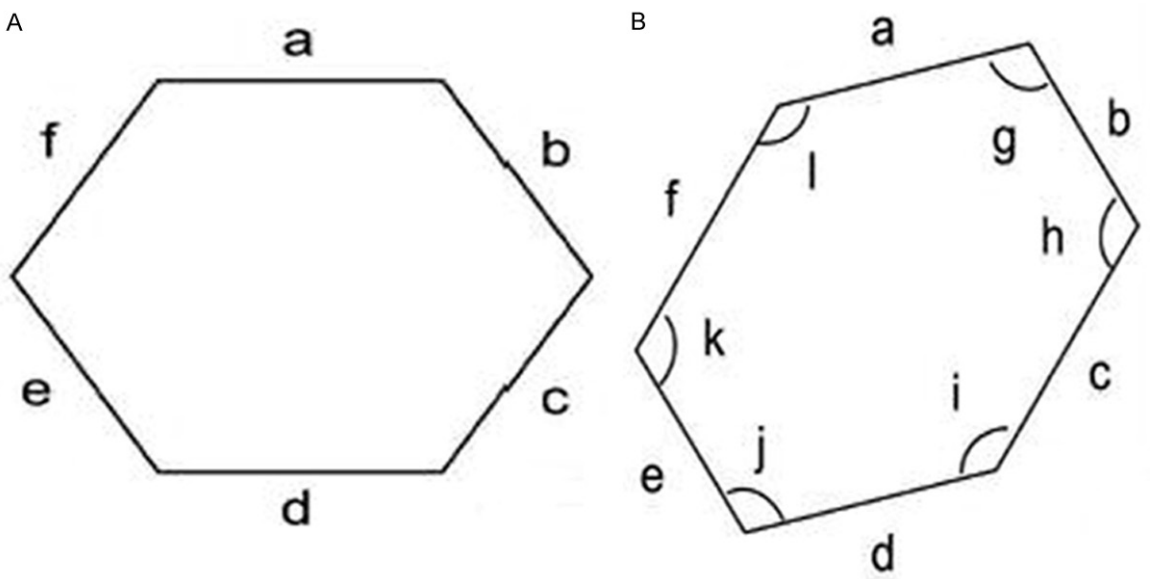

Figure 2. A schematic representation of corneal endothelial cell edges (A) and angles (B).

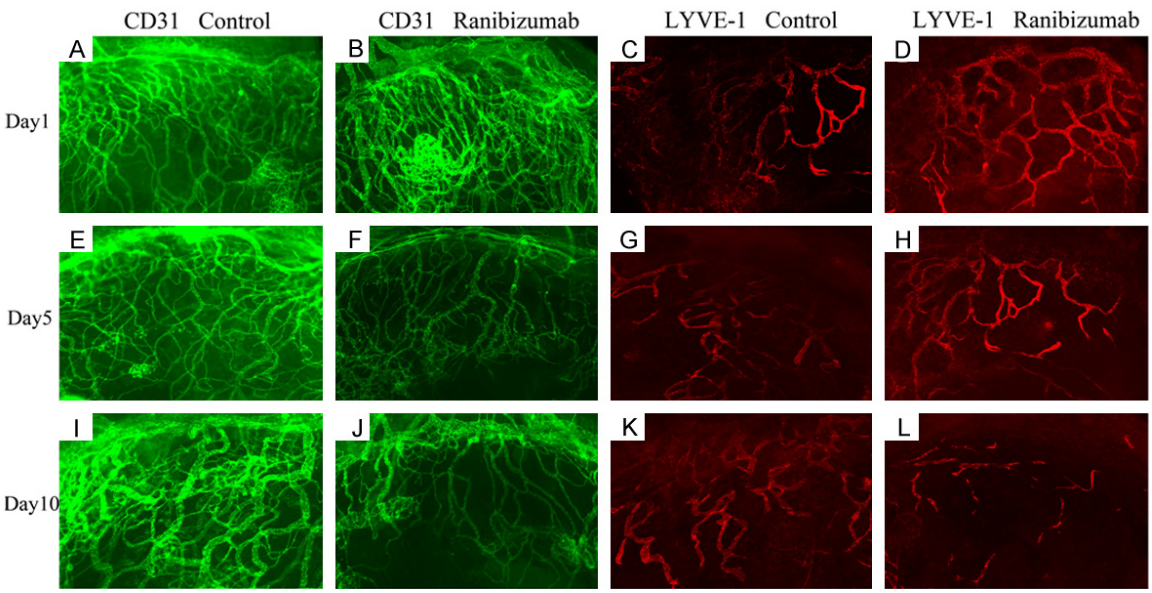

Figure 3. Representative sections of corneal flat mounts after the intracorneal injection of ranibizumab or PBS (control) (magnification × 100). (B, F, J) There is significant difference in the presence of blood vessels after intracorneal injection of ranibizumab when compared with control (A, E, I) in all time points. (D, H, L) After treatment with ranibizumab, corneal lymphatic vessels disappeared, compared with those of control mice (C, G, K).

from a single cell that belonged to the previously selected region. Then, the Image J average cell area (Y) was calculated in the region, which was equivalent to the average area (X) that was calculated automatically by the Image J. When we selected a single cell Image J area (Z), the following is the real area (A) calculation formula of each cell: A = Z*Y/X. The other area parameters were calculated on the following basis: Save, Smax, and Smin. The cells were classified into the following categories on the basis of area (A, μm^2): $1000 < A \leq 800$, $800 < A \leq 600$, $600 < A \leq 400$, $400 < A \leq 200$, and the constituent ratio was calculated. The edge variation (Ev) of corneal endothelial cells were calculated by the following formula: Ev = {(a-d) +

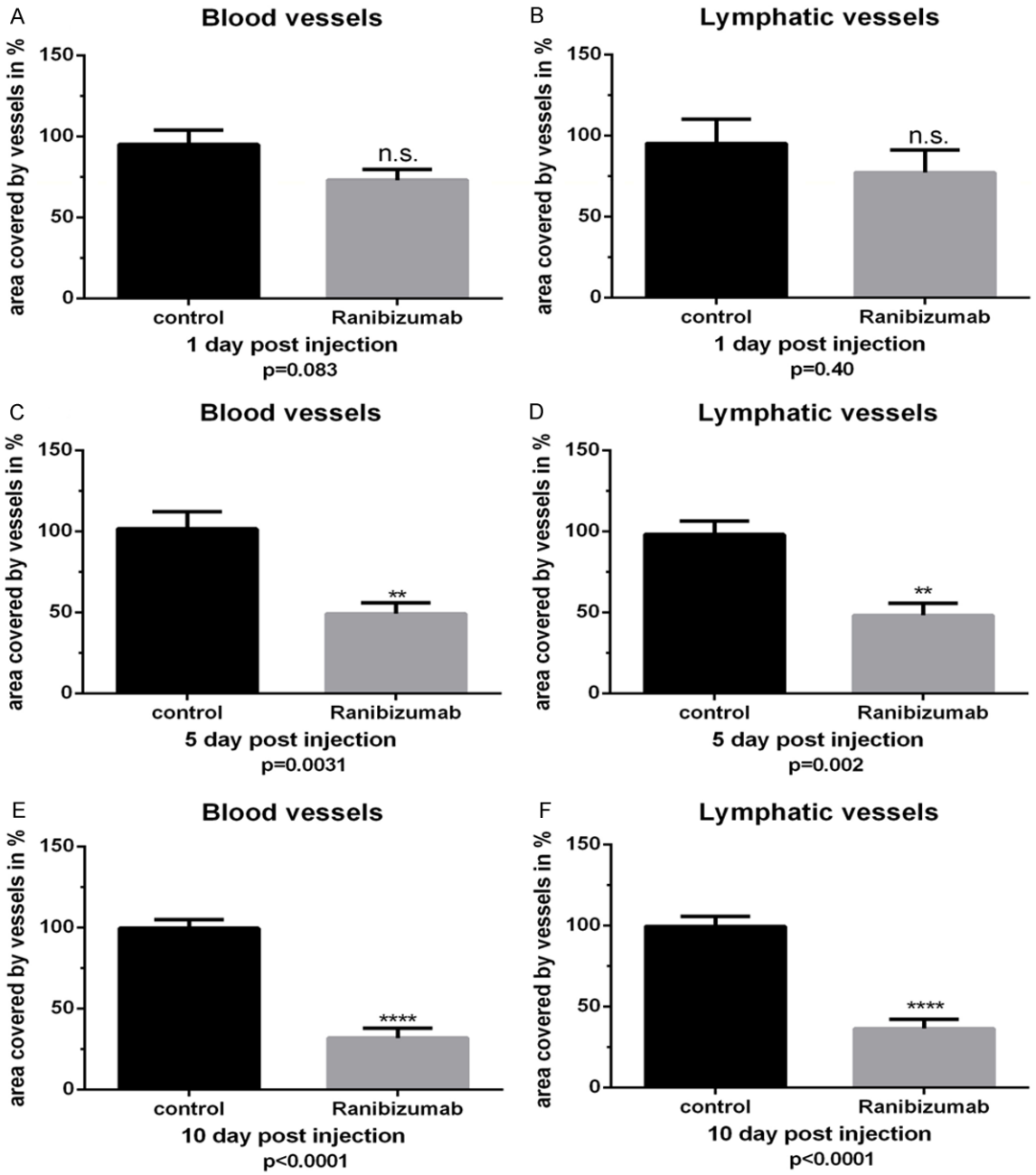

Figure 4. Blood and lymphatic vessels can significantly be regressed by intracorneal injection of ranibizumab. Compared with control group, one day after injection, no obvious regression of blood (A) and lymphatic (B) vessels. Five days after injection, blood vessels showed a reduction of 52% (C), and lymphatic vessels showed a reduction of 50% (D). Ten days after injection, blood vessels showed a reduction of 68% (E), and lymphatic vessels showed a reduction of 63% (F). Data expressed as mean + SEM.

(b-e) + (f-c)}/(a+b+c+d+e+f)*%. Further, a, b, c, d, e, and f were defined as the six sides of the hexagonal corneal endothelial cell. The opposite edges of ideal hexagonal are parallel and equal in length. Therefore, the ideal hexagonal value is zero (**Figure 2A**). The angle variation (Av) of corneal endothelial cells was calculated by the following formula: Av = (g-h) + (i-h) + (i-j) + (j-k) + (i-k) + (i-g). In theory, the internal angle of a regular hexagon is 120°. However, the six

angles were often not equal. The difference between them can help us understand the angle variation (**Figure 2B**).

Statistical analysis

Statistical analysis was done using Microsoft Excel 2003, and graphs were drawn using Prism6, V.6.02 (GraphPad Software, San Diego, California, USA). A two-tailed unpaired t test was used to detect the difference of the effect of the intracorneal ranibizumab injection on corneal lymphangiogenesis, hemangiogenesis, and corneal endothelial morphology among the groups, respectively.

Results

Effect of intracorneal ranibizumab injection on corneal lymphangiogenesis and hemangiogenesis

It was found that existing mature lymphatic vessels and blood vessels in the cornea, induced by the inflammatory neovascularisation assay, can significantly be reduced by intracorneal ranibizumab injection (**Figure 3**). Corneas treated with ranibizumab, excised one day after the intracorneal injection, showed 18% regression of lymphatic vessels ($P = 0.40$; treatment group $n = 5$, control group $n = 5$) (**Figure 4B**). Five days after the intracorneal injection, lymphatic vessels were significantly reduced by 50% ($P = 0.002$; treatment group $n = 5$, control group $n = 5$) (**Figure 4D**), and 10 days after the intracorneal injection, the lymphatic vessel area was significantly reduced by 63% ($P < 0.0001$; treatment group $n = 5$, control group $n = 5$) (**Figure 4F**). The intracorneal ranibizumab injection also had an effect on mature corneal blood vessels. Corneal blood vessels were reduced significantly at different time points: one day after the intracorneal injection, the reduction was 22% ($P = 0.083$; treatment group $n = 5$, control group $n = 5$) (**Figure 4A**); five days after the injection it was 52% ($P = 0.0031$; treatment group $n = 5$, control group $n = 5$) (**Figure 4C**); and ten days after the injection it was 68% ($P < 0.0001$; treatment group $n = 5$, control group $n = 5$) (**Figure 4E**). Our results show the regression of mature lymphatic vessels and blood vessels in the mice cornea after the administration of the intracorneal ranibizumab injection (**Figure 3**).

Effect of intracorneal ranibizumab injection on corneal endothelial cells

Both groups showed the morphology of corneal endothelial cells between the PBS group and ranibizumab group. All parameters of corneal endothelial cell morphology measured in both groups showed no significant difference (**Table 1**).

Discussion

The medical treatment of corneal neovascularization includes steroids, nonsteroidal anti-inflammatory drugs, vascular endothelial growth factor inhibitor, cyclosporine, vitamin C, apoptosis inducing factor, plasminogen, peroxiredoxin-6, and gene therapy [22-27]. The surgical treatment of corneal neovascularization includes laser therapy, photodynamic therapy, superficial keratectomy, and fine-needle diathermy [28, 29]. However, topical eye drops for corneal neovascularization as the common treatment in ophthalmology, cause the following problems: (1) the drug action does not usually last long; (2) the drug can be affected by limited penetration through an intact epithelium [9]. Meanwhile, there is the rebound phenomenon of corneal neovascularization in the short term after treatment for some common surgical intervention [30]. Garcia-Valenzuela et al reported a complicated case of recurrent fungal keratitis with endophthalmitis, following a contaminated penetrating keratoplasty that ultimately was controlled with a new treatment modality: intrastromal injections combined with intravitreal injection of amphotericin B, which led to the eradication of the corneal fungal plaques and the intraocular infection. They believed that intrastromal injections of amphotericin B may offer a less invasive, in-office alternative to recurrent fungal keratitis [31]. In this study, we first introduced the therapeutic approach of administering intracorneal ranibizumab injection for corneal neovascularization and found that it has a regression effect on pre-existing mature pathological corneal lymph vessels and blood vessels in the cornea. The 33-gauge Hamilton needle was injected along the limbus into the corneal stroma to avoid the optical centre of the cornea; thus, each cornea was injected at the four sites in the corneal stroma to expand the therapeutic surrounding range to the maximum extent pos-

Table 1. Parameters of the corneal endothelium morphology after intracorneal ranibizumab injection

Group	CD (cells/mm^2)	CV (%)	Area parameter (μm^2)			Cell proportion							Dark zone	
						HEX			Polygon	pentagon	quadrilat-eral	Other shapes	Number (cells/mm^2)	Area (μm^2)
			Save	Smax	Smin	Hexagonal (%)	Ev (%)	Av (°)						
1	2757 ± 91	33.2 ± 10.6	352.4 ± 14.1	563.4 ± 64.3	143.6 ± 22.2	66.1 ± 2.3	14.7 ± 2.2	147.4 ± 16.8	16.1 ± 2.3	3.9 ± 2.0	2.7 ± 1.4	11.4 ± 5.2	0.16 ± 0.12	75.0 ± 26.8
2	2785 ± 127	29.0 ± 11..2	351.2 ± 15.6	551.6 ± 73.7	154.2 ± 29.3	65.7 ± 3.6	14.5 ± 2.5	148.2 ± 15.1	15.7 ± 3.6	3.1 ± 1.7	2.5 ± 1.5	12.9 ± 8.0	0.24 ± 0.11	81.1 ± 24.8
T value	-0.387	0.605	0.128	0.270	-0.644	0.198	0.149	-0.079	0.198	0.670	0.278	-0.337	-1.130	-0.373
P value	0.709	0.562	0.902	0.794	0.537	0.848	0.885	0.939	0.848	0.522	0.788	0.745	0.291	0.719

Group 1 included mice with the intracorneal PBS injection and group 2 included mice with the intracorneal ranibizumab injection. (n_1 = 15, n_2 = 15); CD = cell density; CV = coefficient of variation, expressing the degree of cell polymegethism; Save = average cell size; Smax = maximum cell size; Smin = minimum cell size; HEX = percentages of hexagonal cell; Ev = edge variation; Av = angle variation.

sible. One day after the administration of the intracorneal injection, although there was a little dropsy in the cornea, conspicuous corneal oedema was not found and corneal transparency also wasn't affected. Five days after the intracorneal injection, corneal neovascularization gradually disappeared and the central area of the cornea remained transparent. There could be a few possible reasons for this: It is possible that there was no drug precipitation in the cornea and the stroma collagen fibres did not break when we completed the intracorneal injection. Further, we did not observe corneal scar healing in the study. As we know, after cataract phacoemulsification or small incision cataract surgery, the corneal incisions are usually closed by intracorneal stroma injections of water; even if there was a white appearance immediately after the injection, the oedema could quickly recover to become transparent within one day. However, it is necessary to pay attention to the fact that the thickness of the mouse cornea is only about 71.5 μm [32], which can easily be pierced during the injection. Considering that the human cornea is much thicker, particularly almost 0.66 + 0.076 mm in the peripheral cornea [33], this provides a more safe and accurate surgical manipulation site.

There were several mediators in the corneal angiogenesis as well, including basic fibroblast growth factor, transforming growth factor, platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) [9, 34, 35]. The role of VEGF has been extensively proven, and it is often considered the most prominent angiogenic factor [9]. VEGF neutralizing agents have proven invaluable in the treatment of pathologic conditions, such as neovascular age-related macular degeneration and diabetic retinopathy; furthermore, recent findings suggest that VEGF inhibition may be an effective therapeutic modality for corneal neovascularization [36, 37]. Ranibizumab is a humanized monoclonal anti-VEGF antibody that has been licensed for the treatment of pathological choroidal neovascularization due to various aetiologies; it has demonstrated efficacy both in regressing new vessels and improving visual acuity [38-42]. Compared to other forms of administration, such as subconjunctival injection and topical administration, the intrastromal injection possibly allows greater exposure of the corneal vessels to the drug and also guarantees lower likelihood of treatment failure due to a patient's lack of compliance. Bucher et al. reported that the topical application of ranibizumab in mice with epithelium debridement significantly reduces the density of blood and lymphatic vessels in the corneas of treated mice; however, the magnitude of change was less than that revealed in the results of our study [43].

In a recent study, researchers found the deterioration in rabbits' endothelial cell morphology after intracameral injection of 1 and 0.5 mg ranibizumab [44]. However, intravitreal injections of 0.5 mg ranibizumab do not appear to cause substantial changes in the corneal endothelium [45]. Therefore, it is necessary to observe the morphology of corneal endothelial cells after the intracorneal ranibizumab injection. In our study, there were no significant differences between the PBS and ranibizumab groups in terms of cell density, coefficient of variation, area parameter, and cell proportion of corneal endothelial cells. In this study, we also first introduced the area variation, edge variation, and angle variation parameters for the corneal endothelial cells morphology evaluation. Our study did not involve the function of corneal endothelial cells after the intracorneal ranibizumab injection, but at least we proved that it did not cause statistically significant changes on the morphology of corneal endothelial cells after we injected 10 μL of ranibizumab in the four corneal quadrants. In conclusion, our experiments show that the intracorneal ranibizumab injection can induce regression of corneal lymphatic vessels and blood vessels without affecting the morphology of corneal endothelial cells.

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Disclosure of conflict of interest

None.

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