Bilateral Limbal Stem Cell Alterations in Patients With Unilateral Herpes Simplex Keratitis and Herpes Zoster Ophthalmicus as Shown by In Vivo Confocal Microscopy

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Purpose. The purpose of this study was to investigate the limbal changes in the palisades of Vogt (POV) in patients with herpes simplex keratitis (HSK) and herpes zoster ophthalmicus (HZO) with the application of in vivo confocal microscopy (IVCM).

Methods. We enrolled 35 eyes of 35 consecutive patients with HSK and 4 patients with HZO in this observational study. Thirty-five participants were also recruited from a healthy population as the control group. All subjects were examined by IVCM in addition to routine slit-lamp biomicroscopy. The IVCM images of the corneal basal epithelial cells, corneal nerve, and the corneoscleral limbus were acquired and then were analyzed semiquantitatively.

Results. The rate of absent and atypical POV was significantly higher in the affected eyes of patients with HSK than in the contralateral eyes and eyes of controls (88.57% vs. 65.71% vs. 17.14%, $P < 0.01$). In the HZO group, the rate of absent and atypical POV was 100% in the affected eyes and 50% in the contralateral eyes. When compared to the contralateral unaffected eyes and control eyes, the average density of the central basal epithelial cells and the sub-basal nerve plexus density and the total number of nerves in the central area of the affected eyes were significantly lower in the HSK group (1541 ± 704.4 vs. 2510 ± 746.8 vs. 3650 ± 746.1 cells/mm^2, $P < 0.0001$). Spearman's rank correlation showed that the presence of absent and atypical POV had a significant negative correlation with central corneal basal epithelial cells ($r_s = -0.44979$, $P < 0.0001$), the density of total nerves ($r_s = -0.49742$, $P < 0.0001$), and the total nerve numbers ($r_s = -0.48437$, $P < 0.0001$). A significant positive correlation was established between the presence of absent and atypical POV and HSK severity in affected eyes in the superior, inferior, nasal, and temporal quadrants ($r_s = 0.68940$, $r_s = 0.78715$, $r_s = 0.65591$, and $r_s = 0.75481$, respectively, $P < 0.0001$) and the contralateral eyes ($r_s = 0.51636$, $r_s = 0.36207$, $r_s = 0.36990$, $r_s = 0.51241$, correspondingly, $P < 0.0001$).

Conclusions. Both eyes of patients with unilateral HSK and HZO demonstrated a profound and significant loss of limbal stem cells, which may explain the fact that HSK and HZO are risk factors for limbal stem cell deficiency (LSCD) in both eyes. The loss of LSCs was strongly correlated with the sub-basal nerve plexus and central basal epithelial cell alterations as shown by IVCM.

Keywords: herpes simplex keratitis (HSK), limbal stem cell (LSC), herpes zoster ophthalmicus (HZO), basal epithelial cell, corneal nerve

The most common forms of viral keratitis are caused by herpes simplex virus, varicella-zoster virus (VZV), and adenovirus, whereas less common forms include cytomegalovirus (CMV) and rubella virus.[1] Furthermore, 80% of Europeans are positive for herpes simplex virus (HSV)-1 antibodies,[2–4] and up to 30% of the population is affected by an episode of herpes zoster virus infection during their lifetime.[5–7] Furthermore, viral infection can induce immune-mediated damage throughout all layers of the cornea, resulting in corneal scarring, thinning, lymphangiogenesis, and neovascularization, which may lead to visual impairment and eventual blindness.[8,9]

Corneal neovascularization in viral keratitis can occur subsequent to the destruction of the limbus.[10,11] The limbus is located between the cornea and the sclera tissue and is approximately 1.5 mm wide in adult human eyes. The limbal stroma is organized into a series of radial elevations with fibrovascular centers known as palisades of Vogt (POV).[12,13] POV harbor a high density of limbal stem cells (LSCs) and maintain corneal epithelial homeostasis and clarity.[14–16] The barrier function of the limbus makes the normal cornea stay avascular, and the corneal epithelial cells may play a significant role in maintaining the angiogenic balance in favor of avascularity. Direct damage to LSCs and/or the destruction

Table 1. Baseline Characteristics of Controls and Patients With Viral Keratitis

Characteristics	Controls	Viral Keratitis	χ^2/F Value	*P* Value
Patients, *n*	35	39		
Sex, male/female	19/16	24/15	0.5299	0.4667*
Age, mean ± standard deviation	43.54 ± 15.54	43.94 ± 16.10	1.21	0.5783*

Data presented as values are expressed as mean ± standard deviation.
* $P > 0.05$ (χ^2 test) compared to controls.

of their niche microenvironment leads to limbal stem cell deficiency (LSCD).[17,18] There was also a recent retrospective study that found the prevalence of LSCD increased in the setting of viral keratitis.[19]

In vivo confocal microscopy (IVCM) can be used to observe the subtle changes in the limbus. IVCM is a novel tool that allows for quasi-histological in vivo optical sections of the cornea and to investigate quantifiable parameters, such as cell density and size.[20] Based on previous work, the corneal basal cell density, sub-basal nerve density, and the structure of the POV were identified as potential parameters to evaluate the function of the LSCs.[21–23] However, the correlation between viral keratitis and the damage to LSCs has barely been studied.[19] To obtain objective evidence of the changes in LSCs in patients with viral keratitis, we investigated the correlations among the morphological changes in POV and the degree of corneal nerve and corneal basal cell density loss in both eyes of unilateral herpes simplex keratitis (HSK) and herpes zoster ophthalmicus (HZO) cases with IVCM, and we also identified the differences in the POV between the HSK and HZO groups.

Methods

This was a prospective, cross-sectional and single-center study. We enrolled 35 patients with a diagnosis of unilateral HSK and HZO who were recruited from the Eye Hospital, the First Affiliated Hospital of Harbin Medical University, Harbin, China, between August 20, 2019 and October 5, 2020. The corneal lesions in these patients were central to the corneal. Both eyes, affected and contralateral clinically unaffected, were included as separate groups. No significant difference was observed in age and gender between the viral keratitis group and the control group (Table 1).

Thirty-five age-matched and sex-matched healthy subjects were also included. The study excluded subjects with any other comorbidities, such as a history of previous infectious keratitis, ocular inflammatory disease, ocular trauma, ocular surgery, or systemic disease, known to affect corneal epithelial healing, or immunosuppression, other than diabetes or chronic corneal pathologies (cystic corneal disorders and epithelial basement membrane dystrophies).

Patients with HSK and HZO were divided into three groups based on the severity: the epithelial keratitis group, the stromal keratitis without ulceration group, and the stromal keratitis with ulceration group. In the epithelial keratitis group, the corneal dendrites were linear and branching with terminal bulbs and punctate epithelial keratitis in HSK or punctate epithelial keratitis and pseudodendrites in patients with HZO (Fig. 1A2, 4–1 A1). The clinical manifestations in the stromal keratitis without ulceration group of HSK and HZO included unifocal, multifocal, or diffuse stromal edema, an immune ring, cornea thinning, and sectoral or diffuse stromal neovascularization (Fig. 1A3, 4–2 A1). Stromal keratitis with ulceration and necrotizing stromal keratitis and ulcerating interstitial keratitis was present, with significant keratolysis and even corneal perforation (Fig. 1A4, 4–3 A1, 4–4 A1).

This study received the approval of the Institutional Ethics Committee of the First Affiliated Hospital of Harbin Medical University (Approval No. IRB-AF/SC-04/02.0) and followed the tenets of the Declaration of Helsinki. Written informed consent was obtained from all of the subjects.

In Vivo Confocal Microscopy Images

Laser scanning IVCM (Heidelberg Retina Tomograph 3 with Rostock Cornea Module, Heidelberg Engineering GmbH, Heidelberg, Germany) images of the central cornea were obtained from all subjects. This microscope uses a 670-nm wavelength diode laser source, and it is equipped with a 63 × objective immersion lens with a numerical aperture of 0.9, allowing a scanning area of 400 × 400 mm with a magnification of up to 800 times and a resolution of approximately 1 mm. Before the examination, one drop of 0.4% oxybuprocaine hydrochloride (Benoxil; Santen Pharmaceutical, Japan) was applied to the lower conjunctival sac.

A particular focus on the sub-basal nerve plexus and the central basal epithelial cells was adopted. Images of the central cornea of the enrolled eye were obtained first. We collected the superior, nasal, inferior, and temporal limbus.

Subjects were then instructed to look downward, upward, and toward the left and right to allow examination of the limbus, and images of the limbal area were examined by one experienced masked ophthalmologist. Images of the POV were classified as typical, atypical, or absent. The typical POV was characterized as a wavy epithelium–stromal boundary or alternating epithelium–stromal cords, with a bright fringe of hyper-reflective basal epithelial cells and a slender blood vessel along each stromal papilla (Fig. 1B1, Fig. 2B1). An atypical POV was defined as hyper-reflective stromal cords without a bright fringe of hyper-reflective basal epithelial cells, which appeared to be double contoured or parallel lines (Fig. 1B2, B3). If neither alternating epithelium–stromal cords nor hyper-reflective stromal cords were found in a quadrant, the region was classified as having no POV (Fig. 1B4).[24]

Image Analysis

Selected confocal images from each eye were independently reviewed by two experienced confocal microscopists (authors L.X.T. and W.Y.B.). The rate of POV was calculated by the percentage of patients with typical, atypical, or absent POV. The specific parameter measured per frame was the average nerve fiber width, which was expressed as μm/mm^2 by ACC Metrics (A Dabbah; Imaging Science and Biomedical Engineering, University of Manchester, Manchester, UK). The following corneal nerve parameters were determined for each image: total nerve density by tracing all visible nerve fibers in the image and calculating the length of the nerve fibers, which was expressed as μm/mm^2, and the total number of nerves were counted manually per frame and were reported as number/frame. For measuring the sub-basal nerves, we used ImageJ (National Institutes of Health, USA) and Neuron J

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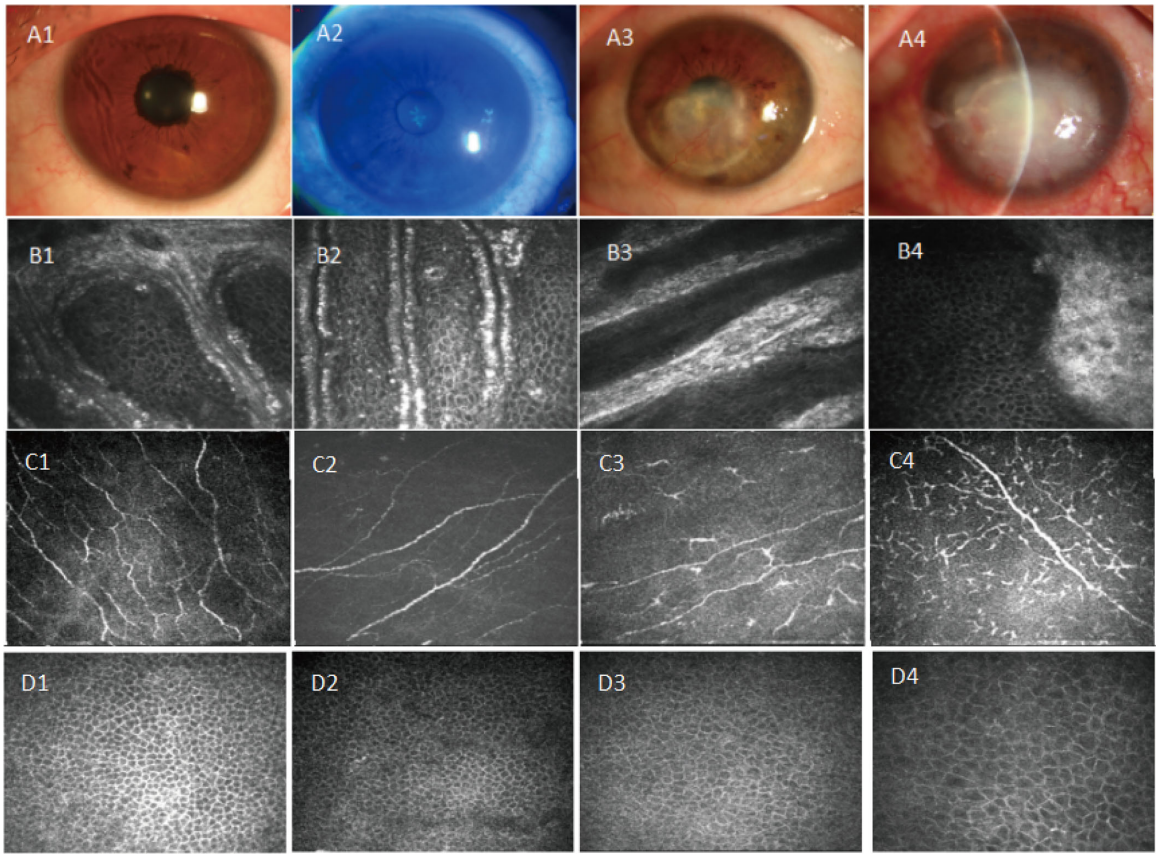

Figure 1. Slit-lamp photographs and in vivo confocal microscopy images (IVCM) of affected eyes with HSK. (**A1-D4**) Slit lamp photograph of the normal controls group, the epithelial keratitis group, the stromal keratitis without ulceration group, and the stromal keratitis with ulceration group (**A1–A4**). A wavy epithelium–stromal boundary with a bright fringe of hyper-reflective basal epithelial cells and a slender blood vessel along each stromal papilla were defined as typical POV in the control group (**B1**). The atypical POV was classified with hyper-reflective stromal cords without a bright fringe of hyper-reflective basal epithelial cells, which appeared to be double contoured or parallel lines in affected eyes of the epithelial keratitis group and the stromal keratitis without ulceration group (**B2, B3**). The absence of POV was identified to the absence of hyper-reflective basal epithelial cells around stromal cords in affected eyes of the stromal keratitis with ulceration group (**B4**). IVCM images of corneal nerves in the control group, the affected eyes of epithelial keratitis group, the affected eyes of stromal keratitis without ulceration group, and affected eyes of stromal keratitis with ulceration group (**C1–C4**). IVCM images of corneal basal epithelial cells in the control group, the affected eyes of stromal keratitis without ulceration group, and the affected eyes of stromal keratitis with ulceration group (**D1–D4**).

(https://www.imagescience.org/meijering/software/neuronj/), a semiautomated nerve analysis plug-in program for ImageJ. The number of cells within the frame was counted in manual mode (Cell Count Software; Heidelberg Engineering GmbH) and the cellular densities were output by the software automatically. Then, the average cellular densities were calculated. Two masked observers evaluated the images, and the averaged values were subjected to analysis. The mean of three images was used for each calculation. For the morphologic analysis, the three to four most representative structures in three images for each eye were chosen. If the difference between the two observers was higher than 10%, a third observer assessed the images, and the average of these 3 values was used in the analysis.

Statistical Analysis

Statistical analyses were performed using SAS software (version 9.4; SAS Institute, Cary, NC, USA). ANOVA and χ^2 tests were performed to compare the rate of the presence of POV, cellular density, and corneal nerve parameters among the affected eyes, contralateral unaffected eyes, and healthy controls. Chi-square tests and multiple tests were used for pairwise comparisons, and Bonferroni was used to adjust for the level of multiple tests. Covariance analysis and an ordered logistic regression model were used to investigate the changes in the differences between the above factors after age adjustment. The rank-sum test was performed to compare the relationship between POV presence per quadrant of the bilateral eyes of patients with HSK with the progression of disease. Spearman's rank correlation tests were performed to determine the correlations between the corneal nerve parameters and the presence of absent and atypical POV, as well as the densities of corneal basal epithelial cells. Spearman's correlation coefficient analysis was applied to assess the correlation between the presence of absent and atypical POV per quadrant and the severity of HSK. Any $P < 0.05$ was considered to indicate statistical significance.

Results

Thirty-five eyes of 35 patients with HSK and 4 eyes of 4 patients with HZO with corneal involvement were studied,

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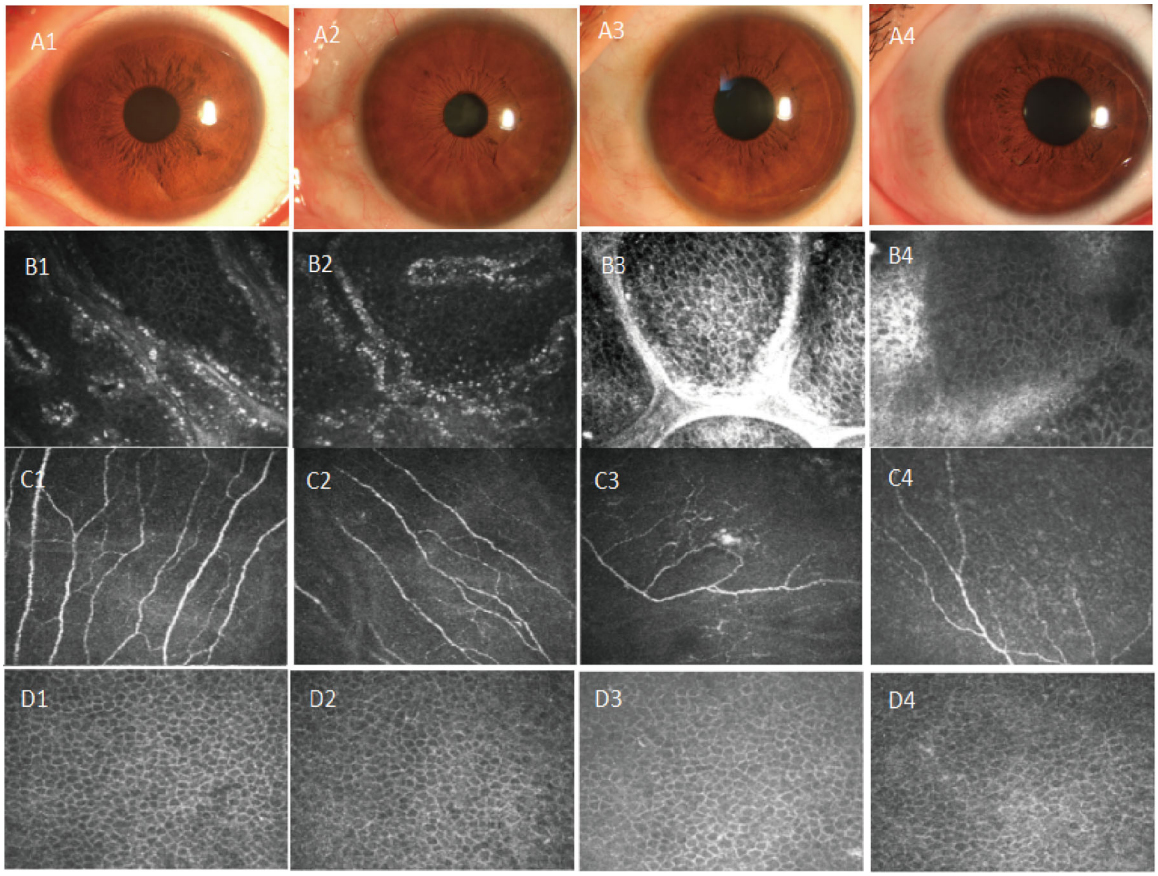

Figure 2. Slit-lamp photographs and in vivo confocal microscopy images (IVCM) of the contralateral unaffected eyes with HSK (**A1–D4**). Slit lamp photograph of normal controls, the epithelial keratitis group, the stromal keratitis without ulceration group, and the stromal keratitis with ulceration group (**A1–A4**). IVCM images of typical POV in the control group (**B1**), the atypical POV in the contralateral unaffected eyes of epithelial keratitis group, and the stromal keratitis without ulceration group (**B2, B3**), and the absence of POV in the contralateral unaffected eyes of the stromal keratitis with ulceration group (**B4**). IVCM images of corneal nerves in the control group, the contralateral unaffected eyes of epithelial keratitis group, the contralateral unaffected eyes of the stromal keratitis without ulceration group, and the contralateral unaffected eyes of stromal keratitis with ulceration group (**C1-C4**). IVCM images of corneal basal epithelial cells in the control group, the contralateral unaffected eyes of stromal keratitis without ulceration group, and the contralateral unaffected eyes of stromal keratitis with ulceration group (**D1–D4**).

as well as their contralateral clinically unaffected eyes. The control group included 35 healthy eyes of 35 volunteers. The demographic data of the patients and controls are presented in Table 1.

The Rate of POV Presence and Alterations by In Vivo Confocal Microscopy

The quantitative analysis of the rate of the presence of POV and their morphology for patients with HSK and the healthy control group is shown in Table 2, Figure 1, Figure 2, Figure 3, Figure 4, Figure 5 and Figure 6. The rates of absent and atypical POV were significantly higher in the affected eyes of patients with HSK than in the control group (88.57% vs. 17.14%, $P < 0.0001$; Fig. 1B1–B4, Fig. 6A, B, C, Table 2). To our surprise, absent and atypical POV in the contralateral unaffected eyes of patients with HSK were also higher than in the controls (65.71% vs. 17.14%, $P < 0.01$; Fig. 2B1–B4, Fig. 6A, B, C, Table 2). Further, eyes affected with HSK

Table 2. In Vivo Confocal Microscopy Parameters of Controls and Patients With Herpes Simplex Keratitis

		Patients With HSK	
	Control Group	**HSK Eyes**	**Contralateral Unaffected Eyes**
With POV (%)	29 (82.86)	4 (11.43)	12 (34.29)
Atypical POV (%)	4 (11.43)	15 (42.86)	14 (40.00)
Without POV (%)	2 (5.71)	16 (45.71)	9 (25.71)
Basal epithelial cell density, cells/mm^2	3650 ± 746.1	1541 ± 704.4	2510 ± 746.8
Number of total nerves (*n*)	13.71 ± 4.68	5.14 ± 2.03	9.74 ± 2.78
Total nerve length, μm/mm^2	2547 ± 673.8	1069 ± 407.4	1894 ± 609.8
Nerve fiber width, μm/mm^2	22.18 ± 2.32	23.20 ± 2.64	20.97 ± 1.65

POV, palisades of Vogt.
Values are expressed as mean ± standard deviation.

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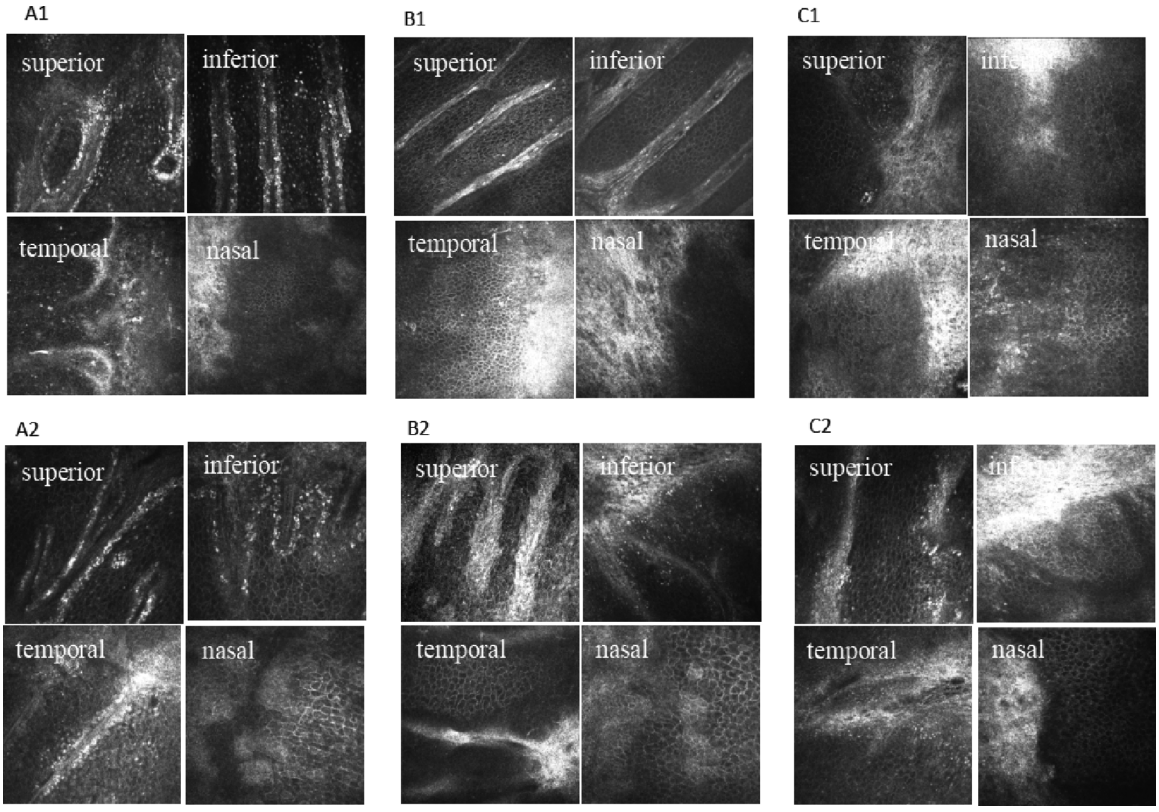

Figure 3. IVCM images of the presence of POV in each quadrant in affected eyes (**A1**) and contralateral unaffected eyes (**A2**) of epithelial keratitis group with HSK. IVCM images of the presence of POV in each quadrant in affected eyes (**B1**) and contralateral unaffected eyes (**B2**) of the stromal without ulceration group with HSK. IVCM images of the presence of POV in each quadrant in affected eyes (**C1**) and contralateral unaffected eyes (**C2**) of the stromal with ulceration group with HSK.

Table 3. The Presence Rate of POV in Bilateral Eyes of Patients With HSK and HZO

		With POV (%)	Atypical and Without POV (%)
HSK	**HSK eye**	4 (11.43)	31 (88.57)
	Contralateral unaffected eye	12 (34.29)	23 (65.71)
HZO	**HZO eye**	0 (0.00)	4 (100.00)
	Contralateral unaffected eye	2 (50.00)	2 (50.00)

compared to the contralateral clinically unaffected eyes had an increased rate of absent and atypical POV parameters (88.57% vs. 65.71%, $P < 0.05$; Fig. 1B2–B4, Fig. 2B2–B4, Fig. 6A, B, C, Table 2). Interestingly, we observed that 100% of affected eyes with HZO had absent and atypical POV, and even in the clinically unaffected eyes of patients with HZO, this rate was also increased by up to 50% (Figs. 4-1, 4-2, 4-3 and 4-4, Table 3).

After subdividing the patients with HSK by the severity of disease into epithelial keratitis (11/35), stromal keratitis without ulceration group (18/35), and stromal keratitis with ulceration group (6/35), a significant statistical difference was found among groups and the presence of POV ($P < 0.0001$). In addition, the presence of absent and atypical POV of the affected eyes with HSK (superior = $P < 0.0001$, inferior = $P < 0.0001$, nasal = $P < 0.0001$, and temporal = $P < 0.0001$; Table 4, Table 5 and Table 6) and the contralateral unaffected eyes (superior = $P < 0.05$, inferior = $P < 0.05$, nasal = $P < 0.05$, and temporal = $P < 0.05$; Table 6) per quadrant were statistically significant with the severity of the disease. In short, a more serious degree of HSK resulted in lower expression of POV in each quadrant with both eyes. Logistic regression analysis showed that the rate of the presence of absent and atypical POV in patients with HSK had no significant correlation with ages in each group (Table 4).

Table 4. *P* Value for Pairwise Comparison of the Presence of POV, Basal Epithelial Cell Density, and Nerve Parameters After Age Adjustment

			Age Adjustment	
	χ^2/F	*P* Value	χ^2/F	*P* Value
The presence of POV	39.3495	<0.0001	18.9334	<0.0001[a]
			34.0343	<0.0001[b]
Basal epithelial cell density	72.71	<0.0001	71.98	<0.0001
Number of total nerves	57.31	<0.0001	57.12	<0.0001
Total nerve length	58.11	<0.0001	58.29	<0.0001
Nerve fiber width	8.70	0.0003	8.65	0.0003

Covariance analysis and an ordered logistic regression model were used to investigate the changes in the differences between the above factors after age adjustment (parallel line test $\chi^2 = 1.719$, $P = 0.633$).

[a] Compared between the contralateral unaffected eye and controls.

[b] Compared between affected eye and controls.

Table 5. *P* Value and LS Mean (95% CI) for Pairwise Comparison of the Presence of POV, Basal Epithelial Cell Density, and Nerve Parameters

	HSK Eye vs. Contralateral Unaffected Eye	HSK Eye vs. Control Group	Contralateral Unaffected Eye vs. Control Group
The presence of POV[#]	5.9945[*]	36.1967[***]	17.0589[**]
Basal epithelial cell density[^]	−969.4 (−1395.74, −543.06)[***]	−2109.8 (−2536.14, −1683.46)[***]	−1140.4 (−1566.74, −714.06)[***]
Number of total nerves[^]	−4.6 (−6.55, −2.65)[***]	−8.57 (−10.52, −6.62)[***]	−3.97 (−5.92, −2.02)[***]
Total nerve length[^]	−824.99 (−1159.53, −490.44)[***]	−1478.32 (−1812.86, −1143.78)[***]	−653.34 (−987.88, −318.8)[***]
Nerve fiber width[^]	2.23 (0.93, 3.54)[**]	1.03 (−0.28, 2.33)	−1.21 (−2.51, 0.1)

[*] $P < 0.05$.
[**] $P < 0.01$.
[***] $P < 0.0001$.
[#] Chi-square tests was used for pairwise comparisons.
[^] Multiple tests was used for pairwise comparisons, and Bonferroni was used to adjust for the level of multiple tests.

Table 6. *P* Value for Pairwise Comparison of the Presence of POV in Each Quadrant of the Bilateral Eyes With HSK

Quadrant	Control	Epithelial Keratitis	Stromal Keratitis Without Ulceration	Stromal Keratitis With Ulceration	χ^2/F	*P* Value
Affected eyes						
Superior	1 (1-1)	2 (1-3)	3 (2-3)	2 (2-3)	37.5188	<0.0001
Inferior	1 (1-2)	2 (2-3)	3 (2-3)	3 (3-3)	42.8624	<0.0001
Temporal	1 (1-2)	2 (2-3)	2 (2-3)	2.5 (2-3)	32.1473	<0.0001
Nasal	1 (1-1)	2 (2-3)	2 (2-3)	3 (2-3)	40.9433	<0.0001
Contralateral unaffected eyes						
Superior	1 (1-1)	2 (1-2)	2 (1-3)	2 (2-2)	19.4099	0.0002
Inferior	1 (1-2)	2 (1-2)	2 (1-2)	2 (2-3)	9.8647	0.0198
Temporal	1 (1-2)	2 (1-3)	1 (1-2)	3 (2-3)	17.0303	0.0007
Nasal	1 (1-1)	1 (1-2)	2 (1-2)	2 (2-2)	18.5636	0.0003

The rank sum test was performed to compare the relationship between the presence of POV in per quadrant of the bilateral eyes of patients with HSK with the progression of disease.
1: Typical POV.
2: Atypical POV.
3: Without POV.

Central Basal Epithelial Cells and Corneal Sub-Basal Nerve Plexus by IVCM

The average density of the central basal epithelial cells in the affected eyes with HSK was significantly lower than in clinically unaffected eyes and the control group (1541 ± 704.4 vs. 2510 ± 746.8 vs. 3650 ± 746.1 cells/mm^2, $P < 0.0001$; Fig. 1D1–D4, Fig. 2D1–D4, Fig. 6D, Table 2). A summary of the corneal nerve parameters for the eyes with HSK, their contralateral clinically unaffected eyes, and the healthy control group is reported in Table 2. In particular, the total nerve density was reduced to 1069 ± 407.4 µm/mm^2 in affected HSK eyes and to 1894 ± 609.8 µm/mm^2 in contralateral eyes, compared to 2547 ± 673.8 µm/mm^2 in controls (respectively, $P < 0.0001$; Fig. 1C1–C4, Fig. 2C1–C4, Fig. 6F, Table 2). The total number of nerve fibers demonstrated a significant reduction in the affected eyes of HSK compared to the contralateral unaffected eye and the control group (5.14 ± 2.03 vs. 9.74 ± 2.78 vs. 13.71 ± 4.68 n/mm^2, respectively; $P < 0.0001$, Fig. 1C1–C4, Fig. 2C1–C4, Fig. 6E, Table 2). Interestingly, in the affected eyes of patients with HSK, the increase in the average nerve fiber width was significantly higher than that in the contralateral unaffected eyes (23.20 ± 2.64 vs. 20.97 ± 1.65, $P < 0.01$; Table 2).

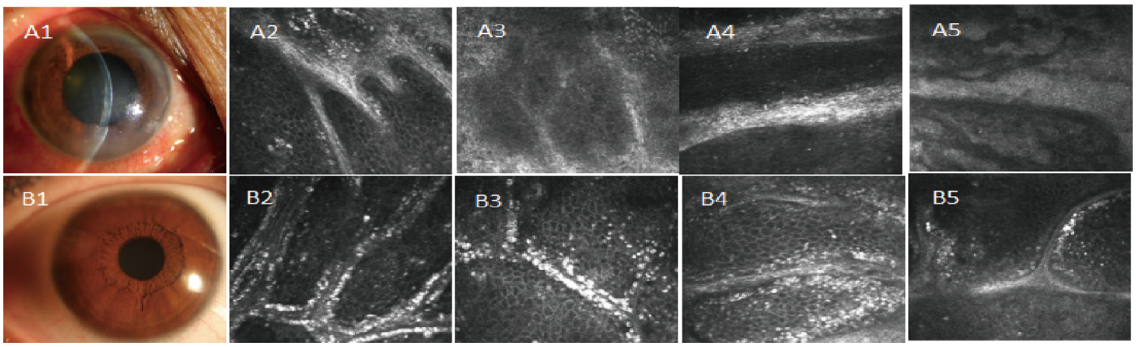

Figure 4-1. Slit-lamp photographs of the bilateral eyes with HZO in the epithelial keratitis group (**A1**, **B1**). IVCM images of superior, inferior, temporal, and nasal quadrant of the presence of POV in affected eyes (**A2–A5**) and the contralateral unaffected eyes (**B2–B5**) with HZO of epithelial keratitis group.

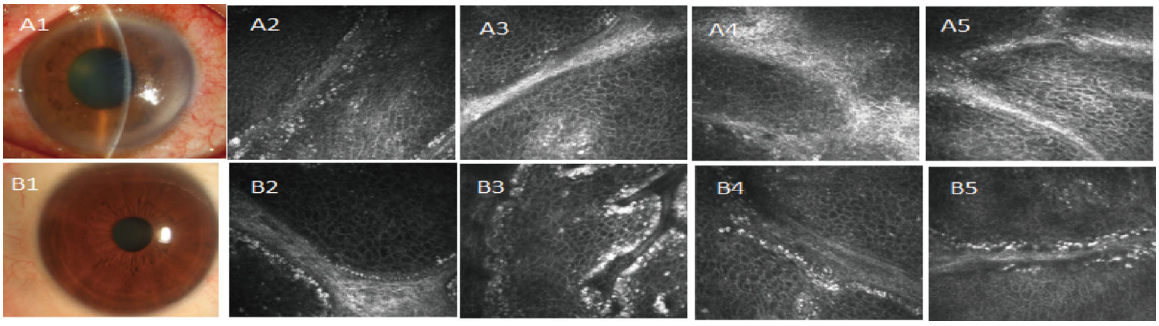

Figure 4-2. Slit-lamp photographs of the bilateral eyes with HZO in the stromal keratitis without ulceration group (**A1, B1**). IVCM images of superior, inferior, temporal, and nasal quadrant of the presence of POV in affected eyes (**A2–A5**) and the contralateral unaffected eyes (**B2–B5**) with HZO of stromal keratitis without ulceration group.

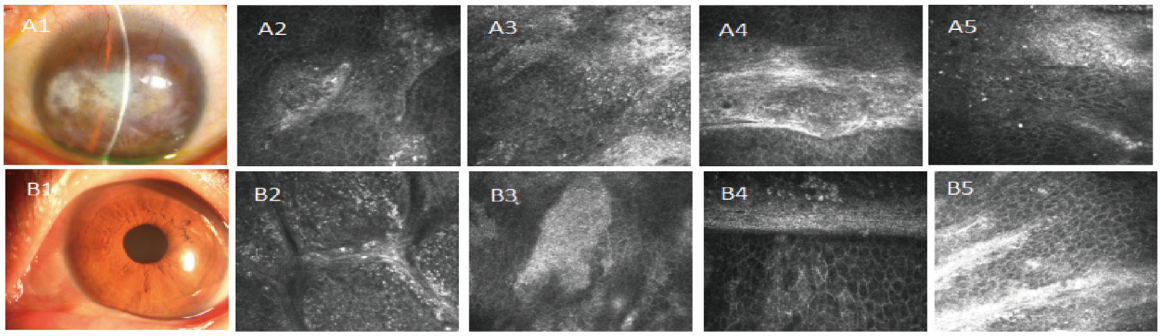

Figure 4-3. Slit-lamp photographs of the bilateral eyes with HZO in the stromal keratitis with ulceration group (**A1, B1**). IVCM images of superior, inferior, temporal, and nasal quadrant of the presence of POV in affected eyes (**A2–A5**) and contralateral unaffected eyes (**B2–B5**) with HZO of the stromal keratitis with ulceration group.

We did not find significant differences in the corneal nerve, central basal epithelial cells, or ages by logistic regression analysis.

Correlation of the Rate of the Presence of Absent and Atypical POV With Central Basal Epithelial Cells and Corneal Nerve Parameters by IVCM

Spearman's rank correlation showed that the presence of absent and atypical POV had a significant negative correlation with central corneal basal epithelial cells in each group by IVCM ($r_s = -0.44979$, $P < 0.0001$; Fig. 5A). A similar correlation was found for the presence of absent and atypical POV and the density of total nerves and total nerve numbers ($r_s = -0.49742$, $r_s = -0.48437$, respectively; $P < 0.0001$, Figs. 5B, C). However, we did not observe a significant correlation between the presence of absent and atypical POV and corneal nerve fiber width (CNFW; $r_s = -0.04093$, $P = 0.6785$, Fig. 5D).

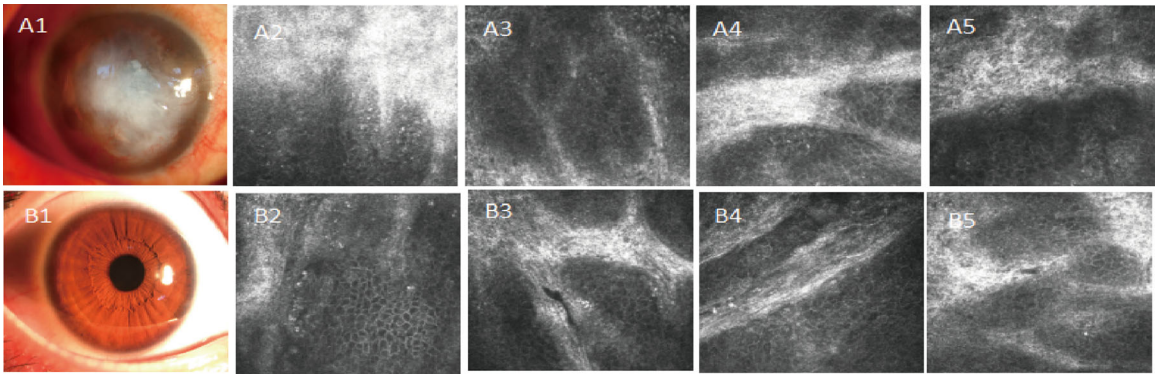

Figure 4-4. Slit-lamp photographs of the bilateral eyes with HZO in the stromal keratitis with ulceration group (**A1, B1**). IVCM images of superior, inferior, temporal, and nasal quadrant of the presence of POV in affected eyes (**A2–A5**) and the contralateral unaffected eyes (**B2–B5**) with HZO of stromal keratitis with ulceration group.

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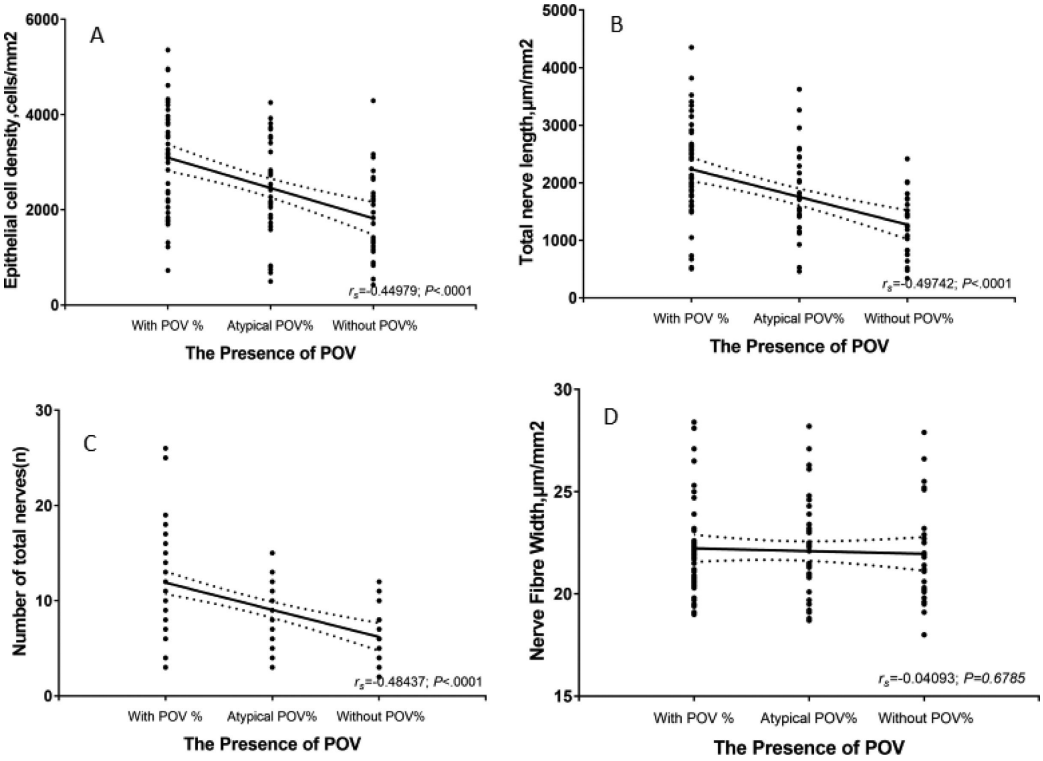

Figure 5. Statistically significant correlations among corneal the presence of POV and/or corneal parameters per group. The presence of POV was significantly inversely correlated with basal epithelial cell density (**A**), total nerve density (**B**), number of total nerves (**C**), and no significant correlation with neve fiber width (**D**). Spearman's rank correlation coefficient (r) and *P* values are shown. *P* value of less than 0.05 was considered statistically significant.

Correlation of the Rate of the Presence of Absent and Atypical POV in Per Quadrant With the Severity of HSK

Spearman's rank correlation was employed to evaluate the correlation between the presence of absent and atypical POV per quadrant and the severity of HSK. A significant positive correlation was found between the presence of absent and atypical POV and the severity of HSK in affected eyes in superior, inferior, nasal, and temporal quadrant (r_s = 0.68940, r_s = 0.78715, r_s = 0.65591, r_s = 0.75481, respectively; $P < 0.0001$, Figs. 7A, B, C, D). Similar correlation was observed in the contralateral eyes of HSK patients (r_s = 0.51636, r_s = 0.36207, r_s = 0.36990, r_s = 0.51241, respectively; $P < 0.0001$, Figs. 8A, B, C, D).

Changes of the Presence of POV in Each Quadrant of HZO With the Severity of HZO

Four patients with HZO were investigated in this study, including one patient with mild HZO (Fig. 4-1), one patient with moderate HZO (Fig. 4-2), and two patients with severe HZO (Fig. 4-3, 4-4). We found that the presence of POV in patients with HZO in both eyes gradually disappeared with the severity of the HZO by IVCM. In addition, we also established that the presence of POV in four of the patients with HZO was more obvious in the upper and lower quadrants than in the temporal and nasal quadrants.

Discussion

Previous studies by IVCM found that there was a significant decrease in corneal innervation and an increase in dendritic cells in subjects with HSK and HZO.[25–27] However, there has been no prospective study about the changes in the presence of POV in patients with HSK and HZO. To our knowledge, this is the first prospective study to systematically analyze the POV, central basal epithelial cells and sub-basal nerve changes and their potential correlations in patients with HSK and HZO by IVCM. We observed damage to the LSCs in most patients with HSK and in all patients with HZO. Furthermore, our results also showed that POV damage and loss of the corneal nerve and central basal epithelial cells were commonly seen in the contralateral eyes in clinically unilateral patients with HSK and HZO.

Carreno-Galeano et al. reported that 30% of patients with HZO and 7% of patients with HSK had LSCD.[19] Our current results supported their findings. LSCD is characterized by absent and atypical POV, neovascularization, and conjunctivalization of the corneal surface.[28,29] As noted above, we found that the rates of the presence of absent and atypical POV were significantly higher in the affected eyes of patients with unilateral HSK than the controls (88.57% vs. 17.14%, $P < 0.0001$), and all of the four affected eyes of the four patients with HZO had lost the normal structure of POV. Moreover, we found that the average density of the central corneal basal cells, corneal nerve, and the number of nerves in the HSK eyes were

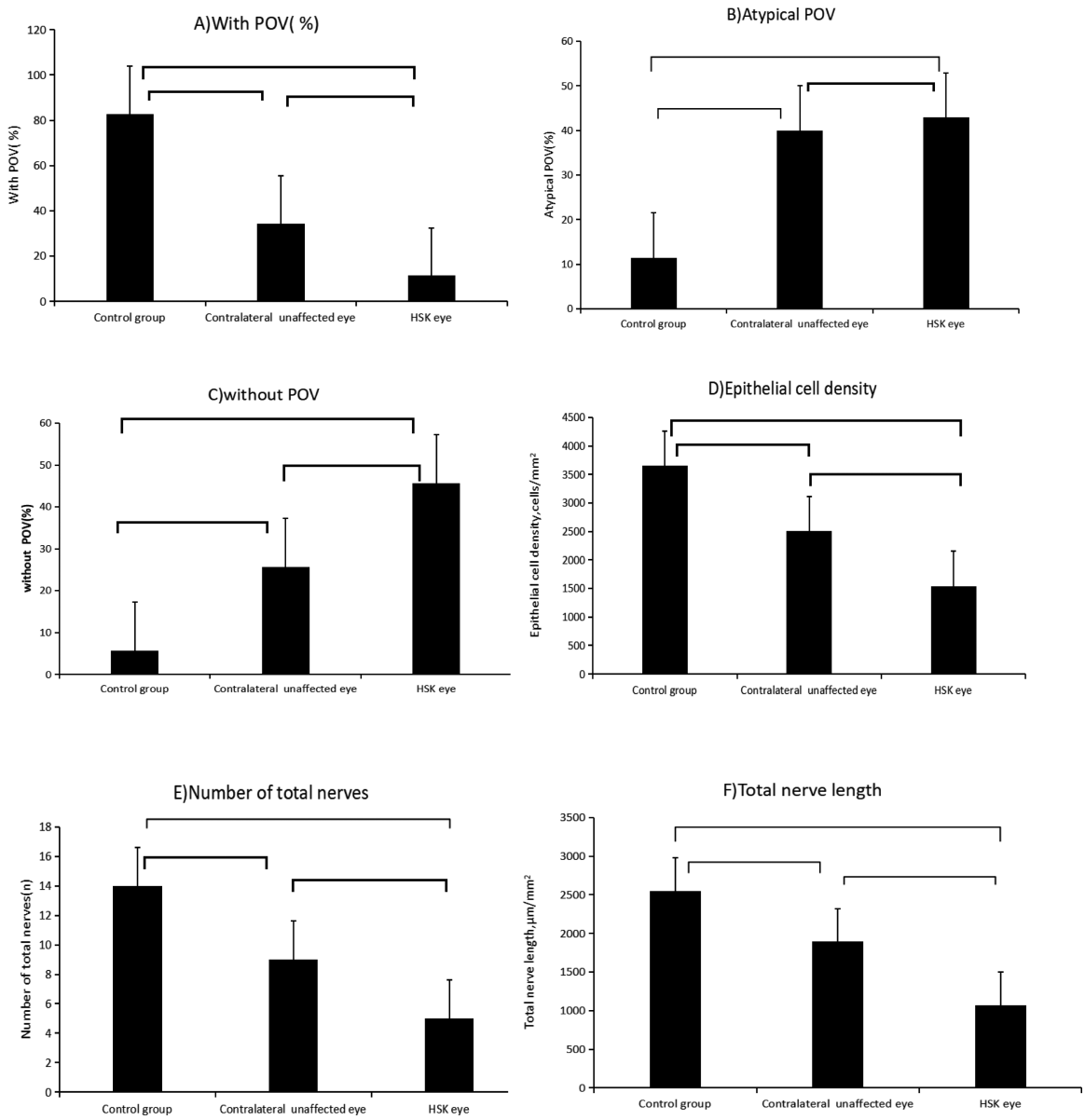

Figure 6. Comparison of the POV alterations, corneal nerve alterations, and basal epithelial cell density in the affected eyes in patients with HSK group, the contralateral unaffected eyes group, and the normal control group. Affected eyes with HSK showed significantly diminishment of the presence of POV compared to the contralateral clinically unaffected eyes and control group. (**A**) With POV. (**B**) Atypical POV. (**C**) Without POV. Error bars represents standard error from the mean. * $P < 0.05$ compared to contralateral unaffected eye. ** $P < 0.01$between contralateral unaffected eye and controls. *** $P < 0.0001$ compared to controls. The density of basal epithelial cells and corneal nerve parameters in patients with HSK showed significant diminishment in affected eyes, contralateral eyes and controls. *** $P < 0.0001$ among affected eyes, contralateral unaffected eyes, and the control group. Statistical analysis performed by χ^2 test with Bonferroni correction for multiple comparisons.

significantly lower than in the control group (1541 ± 704.4 vs. 3650 ± 746.1 cells/mm^2; 1069 ± 407.4 vs. 2547 ± 673.8 μm/mm^2; 5.14 ± 2.0 vs. 13.71 ± 4.68 n/mm^2, respectively; $P < 0.0001$). These observations may suggest that the alterations in patients with HSK and HZO were similar to the early changes in LSCD, which may lead to significant damage to the POV and a reduction in basal cell density and corneal nerves.[30] A previous report has described the corneal basal cell density and sub-basal nerve density changes as potential parameters in the different stages of LSCD,[30,31] and the reduction of corneal nerves is more severe in patients with HZO than in patients with HSK,[32] which confirms our previous findings. In addition, one of the sequelae of HSK and HZO is neurotrophic keratitis (NK),[33] which can result in persistent corneal epithelial defects and corneal sensory nerve damage and can cause dysfunction of the LSCs.[34]

Another reason for the loss of LSCs may be damage to their microenvironment (niche). This niche is characterized by the POV and a distinct blood supply, which is a specific local microenvironment that houses stem cells and controls their self-renewal and reproduction. The POV is one facet of the limbal niche architecture.[35] Common causes of damage to this niche include chemical or thermal injuries, chronic

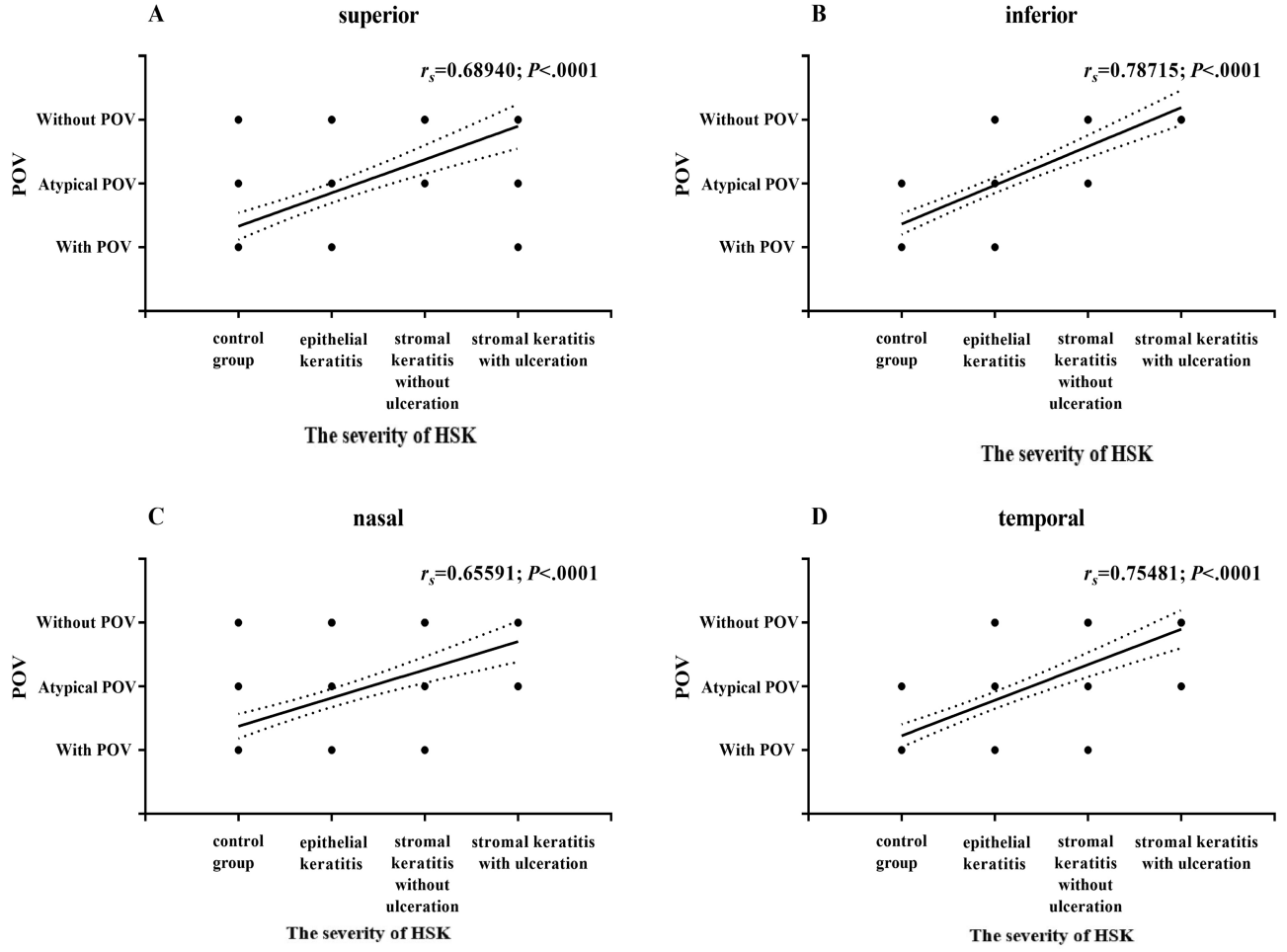

Figure 7. Statistically significant correlations among corneal the presence of POV and the severity of HSK in affected eyes in per quadrant. The presence of POV shows significantly positive correlation with the severity of HSK in affected eyes in the superior (**A**), inferior (**B**), nasal (**C**), and temporal quadrant (**D**). Spearman's rank correlation coefficient (r) and *P* values are shown. *P* value of less than 0.05 was considered statistically significant.

inflammation, and iatrogenic injury caused by ocular surgeries, and so on.[31] Huang et al.[36] demonstrated that the limbal microenvironment plays an important role in maintaining limbal epithelial stem cell survival. The present study found that the absence of POV in the affected eyes of patients with HSK and HZO is likely due to inflammation, which may cause significant injury to the limbal microenvironment.[37]

The differences in the loss of POV between HSK and HZO may be because viral replication during VZV re-activation seems to spread to the entire ganglion, culminating in peripheral disease within the distribution of the entire dorsal root ganglia, whereas in HSV, its re-activation only takes place within one neuron.[32] This may lead to the reduction of the corneal nerves in patients with HZO being more severe than in patients with HSK, which is consistent with the study of Hamrah et al.[38] and will eventually lead to the loss of POV, associated with corneal nerve loss. Moreover, another reason that may lead to this difference is age. In our study, the age of patients with HZO was older than in the patients with HSK group. A previous study showed age-related changes in the microstructure of the limbus in healthy subjects, with a decreasing tendency in the rate of the presence of POV with age.[39] Kennedy et al.[40] proved that the incidence of HZO increases with age but in patients with HSV infection, the role of age is less prominent. We hypothesize that the reason why 11.43% of patients with HSK have a normal POV structure may be that they have a shorter process of HSK than those who have absent and atypical POV. The corneal nerve changes caused by the short-term course of HSK cannot affect the POV, and the nerve factors released before the infection may still be effective to support the POV. With an extension of the course of the disease, the nerve damage is aggravated, and the nerve factors released before are gradually consumed, leading to damage to the POV.

Interestingly, although changes in the rate of the presence of absent and atypical POV was noted in affected eyes of patients with HSK and HZO, they were also higher in contralateral clinically unaffected eyes (65.71% vs. 17.14%, in the HZO group 50%, $P < 0.01$). Furthermore, our study also found a significant negative correlation among the density of the total nerves, total nerve numbers, the density of the corneal basal cells, and the presence of absent and atypical POV after HSV infection had the same trend in the contralateral eyes as in the affected eyes ($r_s = -0.49742$, $r_s = -0.48437$, $r_s = -0.44979$, respectively; $P < 0.0001$). Although the mechanism of the loss of POV in the contralateral eyes in patients with HSK and HZO is still unknown, we

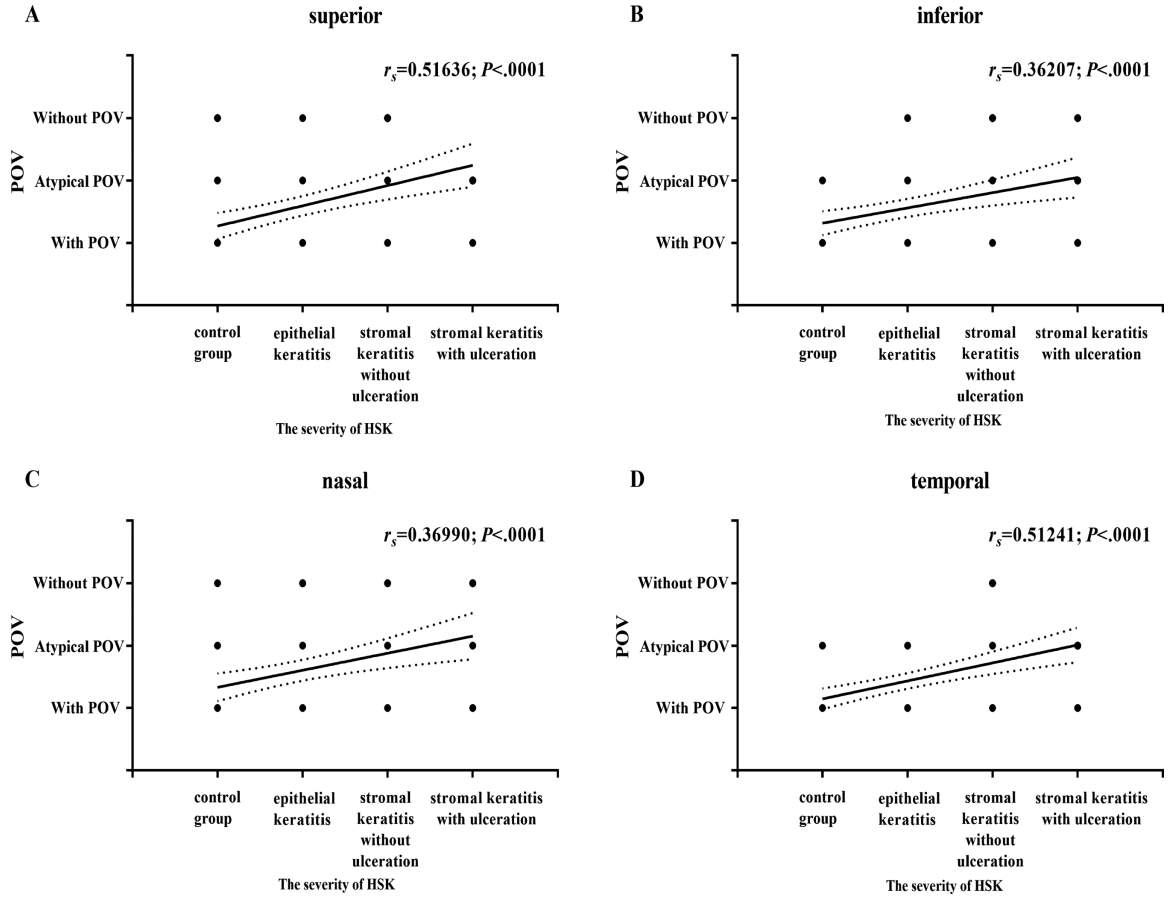

Figure 8. Statistically significant correlations among corneal the presence of POV and the severity of HSK in the contralateral unaffected eyes in per quadrant. The presence of POV shows significantly positive correlation with the severity of HSK in the contralateral unaffected eyes in the superior (**A**), inferior (**B**), nasal (**C**), and temporal quadrant (**D**). Spearman's rank correlation coefficient (r) and *P* values are shown. *P* value of less than 0.05 was considered statistically significant.

do know that the contralateral clinically unaffected eyes of these patients have a reduction in the sub-basal nerve plexus compared to healthy subjects, and a previous study revealed bilateral nerve alterations in unilateral disease, and sub-basal nerve density changes as potential parameters in the different stages of LSCD were mentioned.[41] Thus, a reduction in corneal nerves may lead to damage of LSCs in the contralateral unaffected eye of patients with viral keratitis. Moreover, in animal models of corneal nerve injury caused by experimental eye surgery, changes in nerve growth factor (NGF) protein and mRNA in the early postoperative period were found to be correlated with the reduction of the corneal nerve plexus.[42,43] NGF is constitutively expressed in the basal limbus with lower expression in the basal corneal epithelium and NGF signaling is a key promoter of LSC proliferation, colony-forming efficiency, and a maintainer of the LSC phenotype. It can be inferred that the reduction of NGF in patients with viral keratitis may result in a direct destructive loss of LSCs.[44–46] Another possible reason may be that the contralateral unaffected eyes of patients with HSK and HZO are probably experiencing a particularly proinflammatory period during the infection, with a likelihood of disturbing the LSC niche.

In this study, we established that the presence of POV gradually disappeared with the aggravation of HSK and HZO. The possible reason is that corneal neovascularization occurs in stromal keratitis.[47] The neovascularization may damage the limbal barrier function and damage the LSCs. Furthermore, the immune-mediated inflammation during the aggravation of HSK and HZO may also be one of the causes of LSC injury. Stromal keratitis is characterized by recurrent or chronic stromal inflammation due to retained viral antigens that trigger an antigen–antibody complement cascade.[47] The recruitment of inflammatory cells causes LSC injury and can lead to corneal opacity.[48] In addition, our results are consistent with those of Ghouali et al.,[49] who found that POVs were more predominantly located in the superior and inferior quadrants than in the nasal and temporal quadrants. These authors suggested that the possible reason for this phenomenon was the predominance of the stem cell niche in regions covered by the lids. The lids protect the limbus from slight external and mechanical damage.

Several limitations encountered in this study need to be highlighted. First, additional studies on larger populations of patients with HSK and HZO are required to assess the correlation between the presence of POV and the severity or duration of these diseases. Although we only recruited 39 patients with viral keratitis, the results were still significant and remarkable. Second, animal studies are needed

to confirm any other associated factors, which may help increase our understanding of the mechanisms involved in the LSC's homeostasis and the pathophysiology of these patients.

Conclusion

In conclusion, we found that both eyes of patients with unilateral HSK and HZO have damage to the POV, and there was a significant correlation among the presence of POV, the corneal basal cells, and corneal nerves in these patients. These findings might provide new insights into the protection of LSCs in viral keratitis, which may avoid the development of LSCD in these patients in the future.

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