



In Vivo Confocal Microscopic Evaluation of Corneal Nerve Fibers and Dendritic Cells in Patients With Behçet's Disease

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Central and peripheral nervous system involvement may occur during the course of Behçet's disease (BD). *In vivo* corneal confocal microscopy (CCM) can detect corneal small fiber damage and immune cell density. The aim of this study was to assess central corneal sensitivity, corneal subepithelial nerve plexus morphology and dendritic cell (DC) density in patients with BD. Forty-nine consecutive patients with BD and 30 healthy control subjects were included in this cross-sectional study conducted at a tertiary referral university hospital. Central corneal sensitivity was measured using the contact corneal esthesiometer (Cochet-Bonnet; Luneau, France). The laser scanning CCM (Heidelberg, Germany) was used to quantify corneal nerve fiber density (NFD), nerve branch density (NBD), nerve fiber length (NFL), and DC density. There was a significant reduction in NFD ($P = 0.001$) and NFL ($P = 0.031$) and an increase in DC density ($P = 0.038$) in patients with BD compared to healthy controls, whereas corneal sensitivity ($P = 0.066$) and NBD ($P = 0.067$) did not differ significantly. There was no difference in corneal sensitivity, corneal nerve parameters, or DC density between BD patients with [$n = 18$ (36.7%)] and without a previous history of uveitis ($P > 0.05$ for all). Disease duration [median (IQR), 6.5 (4.0–14.5) years] correlated with corneal sensitivity ($\rho = -0.463$; $P = 0.001$) and NFD ($\rho = -0.304$; $P = 0.034$) and corneal sensitivity correlated with NFD ($\rho = 0.411$; $P = 0.003$) and NFL ($\rho = 0.295$; $P = 0.039$) in patients with BD. CCM demonstrates corneal sub-basal nerve fiber loss and increased DC density, providing a non-invasive ophthalmic means to identify peripheral neuropathy and inflammation in patients with BD.

Keywords: Behçet's disease, corneal confocal microscopy, corneal nerves, corneal sensitivity, dendritic cells

INTRODUCTION

Behçet's disease (BD) is a chronic relapsing vascular-inflammatory disease, typically characterized by oro-genital ulcers, ocular inflammation and cutaneous manifestations. The disease may also involve cardiovascular, pulmonary, articular, gastrointestinal, and neurologic systems (1). Although BD has a worldwide distribution, it is seen more commonly in the Middle East, Far East, and the Mediterranean basin (2).

Neurological involvement in BD ranges from 2.2 to 49% and occurs within the first 10 years of disease (3–6). While brain injury is well documented with parenchymal and vascular involvement (7), there are also studies showing spinal cord and peripheral nerve participation (5, 8–10). An axonal neuropathy has been demonstrated in nerve biopsy and electrophysiological studies in patients with BD (9, 11) and a peripheral neuropathy has also been described in children with BD (12). In a study of 26 patients with BD without neuropathic symptoms, there was electrophysiological evidence of an axonal neuropathy (13). A detailed electrophysiological study of 63 patients with BD showed abnormal nerve conduction and F-wave latencies in 14% (8). In addition, an acute polyradiculoneuritis and mononeuritis multiplex have also been reported in patients with BD (14, 15). Autonomic dysfunction has been described in some (16) but not other (17) studies of patients with BD. A study of 111 patients showed that 19.8% of patients with BD had neuropathic pain, suggestive of underlying small fiber pathology (18).

In relation to eye involvement in BD, most studies have described an optic neuropathy and retinal pathology with maculopathy and retinal neovascularization (19, 20). Studies using optical coherence tomography (OCT) have demonstrated a significant reduction in the thickness of the retinal nerve fiber layer (21), ganglion cell and inner plexiform layers in patients with BD (22), while another study showed an initial increase in the nerve fiber layer in those with a short duration of disease, with thinning in those with a longer duration of disease (23), certainly indicating retinal nerve fiber pathology in patients with BD.

An alteration in corneal biomechanical properties has been demonstrated (24) and central corneal thickness has also been found to be increased in patients with active BD due to inflammation (25). However, there are no studies to date showing corneal nerve involvement in patients with BD. Corneal confocal microscopy (CCM) is a non-invasive ophthalmic imaging technique which allows detailed quantification of the corneal sub-epithelial nerve plexus and dendritic cells (DCs), which are reported to be increased in inflammatory processes (26). We and others have used CCM to demonstrate corneal nerve loss in a broad spectrum of central (27–30) and peripheral (31–34) neurodegenerative conditions and also shown increased DCs in patients with inflammatory neuropathies (35, 36) and multiple sclerosis (29).

To our knowledge, no previous study has used CCM to identify corneal nerve and immune cell alterations in patients with BD. Therefore, the present study has evaluated corneal sensitivity using a contact corneal esthesiometer, and quantified corneal nerve fiber morphology and DC density using CCM in patients with BD.

MATERIALS AND METHODS

Study Subjects

Forty-nine patients (17 males, 32 females) with a diagnosis of BD and 30 healthy control participants (10 males, 20 females) were enrolled in this cross-sectional study undertaken at a tertiary referral university hospital. All patients fulfilled the criteria of the International Study Group for Behçet's Disease

(37). Among patients with BD, 18 (37%) had a previous history of uveitis. Exclusion criteria were active uveitis with topical steroid use, glaucoma, a known history of ocular trauma or surgery, diabetes, other causes of neuropathy, or any other systemic disease that might effect the cornea. The study was approved by the Institutional Review Board and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all participants after explanation of the nature and possible consequences of the study. All patients underwent a complete ophthalmologic examination. Medical records were reviewed for demographic characteristics, the history of previous episodes of uveitis, duration of the disease, and topical or systemic medications.

Corneal Sensitivity Assessment

Central corneal sensitivity was measured using a contact corneal esthesiometer (Cochet-Bonnet; Luneau, France). The esthesiometer is based on the principle of pressure transmitted axially by a nylon monofilament, which was applied with a low pressure perpendicular to the center of the cornea. The filament length was progressively reduced from 6 cm in 5-mm steps until the first response occurred. The longest filament length (cm) resulting in a positive response was verified twice and recorded as a measure of central corneal sensitivity (38).

Corneal Confocal Microscopy

Laser scanning CCM was performed in all subjects using the Rostock Corneal Module/Heidelberg Retina Tomograph III (Heidelberg Engineering, Germany). Three possible examination modes are available in this device (**Figure 1**). In the “section” mode, a single image is acquired and stored at a time. In the “volume” mode there is automatic acquisition of up to 40 images in consecutive focal planes. The “sequence” mode allows a movie which includes up to 100 images at a selected rate of between 1 and 30 frames per second. In this study, the full thickness of the central cornea was scanned using the section mode and 2D digital images were obtained with a lateral digital resolution of 1 $\mu\text{m}/\text{pixel}$, a depth resolution of 2 $\mu\text{m}/\text{pixel}$ and an image size of 400 $\mu\text{m} \times 400 \mu\text{m}$. A previously defined standardized image selection protocol was used (39). Three best-focused nerve plexus images containing the highest, intermediate, and least number of nerve fibers with optimal contrast and without pressure lines or overlapping corneal layers were analyzed from each subject and the average of these results was considered. Automated CCMetrics software, ver. 2.0 (University of Manchester, UK) was used to quantify nerve fiber morphology (40). Three parameters were quantified: corneal nerve fiber density (NFD), the total number of major nerves/ mm^2 ; nerve branch density (NBD), the number of branches emanating from major nerve trunks/ mm^2 ; nerve fiber length (NFL), the total length of all nerve fibers and branches (mm/mm^2) (41). The same images were used to quantify DC density. The number of highly reflective cells with dendriform structures was counted manually and the density was derived as the number of cells/ mm^2 using the proprietary software within the corneal confocal microscope. All the image analyses were performed by a single masked observer. Only one eye of each patient and control participant was included for the

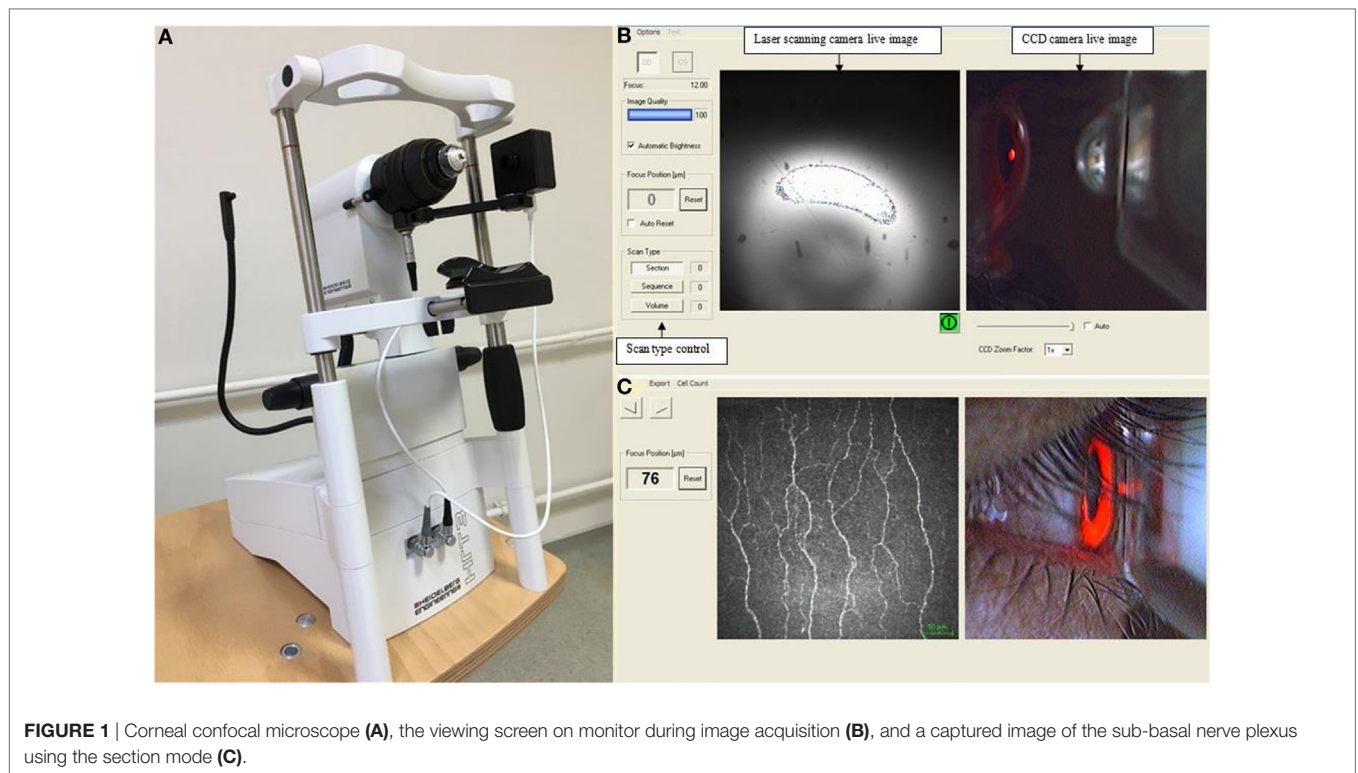


FIGURE 1 | Corneal confocal microscope (A), the viewing screen on monitor during image acquisition (B), and a captured image of the sub-basal nerve plexus using the section mode (C).

analysis as we have previously shown no difference between the right and left eye in healthy control subjects (42) and patients with neuropathy (43). In all except two patients who had undergone cataract surgery in the right eye, right eyes were included.

Statistical Analysis

Statistical analysis was performed using SPSS ver. 21.0 (Chicago, IL, USA) software. Basic descriptive statistics were calculated on all the data and are reported as the mean \pm SD or median (interquartile range), as appropriate. The Pearson χ^2 -test was used to compare categorical variables. Normal distribution of continuous variables was confirmed with the Shapiro–Wilk test. Independent samples *t*-test for normally distributed data and Mann–Whitney *U*-test for non-normally distributed data were used to compare the parameters between the patients with BD and control subjects. The correlations between disease duration, corneal sensitivity, and confocal microscopic parameters were measured using Spearman's correlation coefficient. For all evaluations, a *P*-value of less than 0.05 was considered statistically significant.

RESULTS

The mean ages of the patients with BD and control group were 39.9 ± 11.2 years (range 18–64 years) and 41.2 ± 11.5 years (range 18–61 years), respectively. There were no statistically significant differences between the patients with BD and healthy controls for age ($P = 0.618$) and gender ($P = 0.902$). The median value of the time from initial diagnosis of BD was 6.5 years (IQR 4.0–14.5 years).

Figure 2 illustrates the CCM images of the central corneal sub-basal nerve plexus in a patient with BD and a healthy control subject. NFD ($P = 0.001$) and NFL ($P = 0.031$) were significantly reduced and DC density was increased ($P = 0.038$) in patients with BD compared to controls (Table 1; Figure 3). There was no significant difference in central corneal sensitivity ($P = 0.066$) and NBD ($P = 0.067$) in BD patients compared to controls. There was no difference in corneal sensitivity, corneal nerve morphology, or DC density between BD patients with and without a previous history of uveitis (data not shown, $P > 0.05$ for all).

Twenty patients (41%) with BD were receiving oral colchicine, 12 (25%) were receiving azathioprine, 4 (8%) were receiving both colchicine and azathioprine, 4 (8%) were receiving both cyclosporine and azathioprine, 2 (4%) were receiving infliximab, and the remaining 7 (14%) were not receiving any treatment. Patients receiving colchicine alone or in combination ($n = 24$) and patients not receiving colchicine ($n = 25$) showed no difference for any of the study parameters (data not shown, $P > 0.05$ for all).

Central corneal sensitivity showed a significant positive correlation with NFD ($\rho = 0.411$; $P = 0.003$) and NFL ($\rho = 0.295$; $P = 0.039$) in patients with BD. Disease duration showed a significant inverse correlation with central corneal sensitivity ($\rho = -0.463$; $P = 0.001$) and NFD ($\rho = -0.304$; $P = 0.034$).

DISCUSSION

In this cross-sectional study of 49 patients with Behçet's disease, a decrease in corneal nerve parameters and an increase in dendritic cell density were observed when compared with healthy controls,

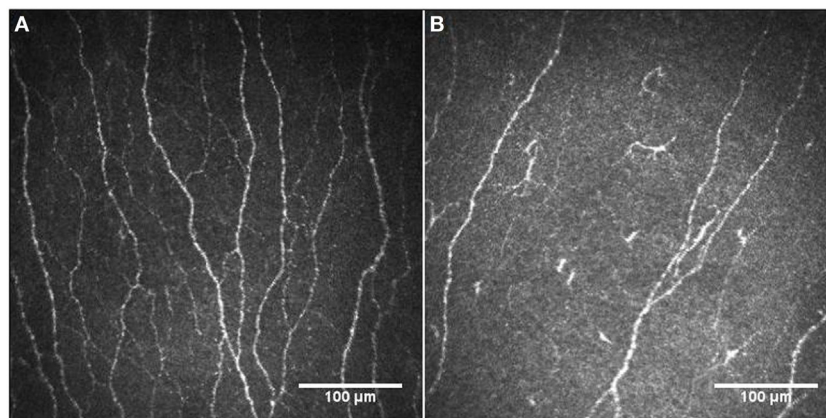


FIGURE 2 | Representative corneal confocal microscopic images of the corneal nerve plexus in a healthy control participant (A) and a patient with Behçet's disease (B), showing reduced nerve fibers and increased dendritic cells.

TABLE 1 | Central corneal sensitivity and corneal confocal microscopic parameters in patients with Behçet's disease and healthy control group.

	Healthy control group (n = 30)	Patients with Behçet's disease (n = 49)	P-value
Central corneal sensitivity (cm, mean ± SD)	5.9 ± 0.2	5.6 ± 0.6	0.066 ^a
Nerve fiber density (fibers/mm ² , mean ± SD)	35.6 ± 10.0	27.7 ± 8.6	0.001 ^a
Nerve branch density (branches/mm ² , mean ± SD)	46.8 ± 24.3	36.9 ± 23.9	0.067 ^a
Nerve fiber length (mm/mm ² , mean ± SD)	18.5 ± 4.1	16.3 ± 4.6	0.031 ^b
Dendritic cell density [cells/mm ² , median (IQR)]	10.1 (4.5–26.3)	19.6 (6.3–46.3)	0.038 ^a

^aMann–Whitney test.

^bIndependent samples t-test.

with no change in central corneal sensitivity. To our knowledge, this is the first study demonstrating corneal nerve loss in patients with BD and it extends the utility of CCM for identifying peripheral neuropathy in BD.

Neurological involvement is known to be a poor prognostic factor in patients with BD (44). In a large cohort of 530 patients with BD, while central nervous system involvement with parenchymal and vascular pathology affected 10.2%, overt peripheral neuropathy was limited to a single patient (7). However, other studies have reported peripheral neuropathy in 2.1–15.4% of patients with BD (3, 8, 9, 45). Indeed, studies using electrophysiological tests have revealed a subclinical axonal neuropathy in patients with BD (8, 9, 13). Namer et al. (11), have also demonstrated nerve fiber loss in a sural nerve biopsy from a patient with BD and signs of peripheral neuropathy. Neuropathic pain assessed using the Leeds Assessment of Neuropathic Symptoms and Signs index was found in 19.8% of patients with BD, indicative of an underlying small fiber neuropathy (18).

Previous studies have assessed ocular neural tissues in patients with BD using OCT and show a reduction in the retinal nerve fiber layer in patients with Neuro-Behçet's disease and uveitis (21, 46). More recently, even patients with BD without ocular disease have shown a significant reduction in the retinal nerve fiber layer, ganglion cell layer, and inner plexiform layer, indicative of sub-clinical retinal nerve degeneration (22). Our findings are consistent with previous clinical, histopathological and electrophysiological studies reporting peripheral nerve degeneration in BD (8, 9, 11, 13, 45). We have previously shown that corneal nerve loss is early and comparable to intraepidermal nerve fiber loss (47) and is related to reduced corneal sensitivity and the severity of neuropathy in patients with diabetes (48). In the present study, while we find no overall reduction in corneal sensitivity, it was associated with reduced NFD and NFL as well as disease duration.

Colchicine has been associated with an axonal neuropathy, especially in the presence of renal insufficiency (49). We show no significant difference in corneal sensation or corneal nerve morphology between patients treated with or without colchicine. This is supported by a study of 29 patients with BD which also reported no difference in neurophysiology between patients treated with or without colchicine (50).

Dendritic cells are known to migrate to the central cornea in inflammatory conditions (51). Stettner et al. (36) have reported an increase in DC density in patients with chronic inflammatory demyelinating polyneuropathy and recently we have shown increased DC density around the central corneal nerve plexus of patients with multiple sclerosis (29). We show that DC density is increased in patients with BD, but was not related to disease duration or corneal nerve parameters. Although this study lacks patients with active uveitis, we found no relationship to a history of uveitis.

The limitations of this study are the relatively small sample size and the cross-sectional nature of the study design which precludes conclusions about the natural history of alterations

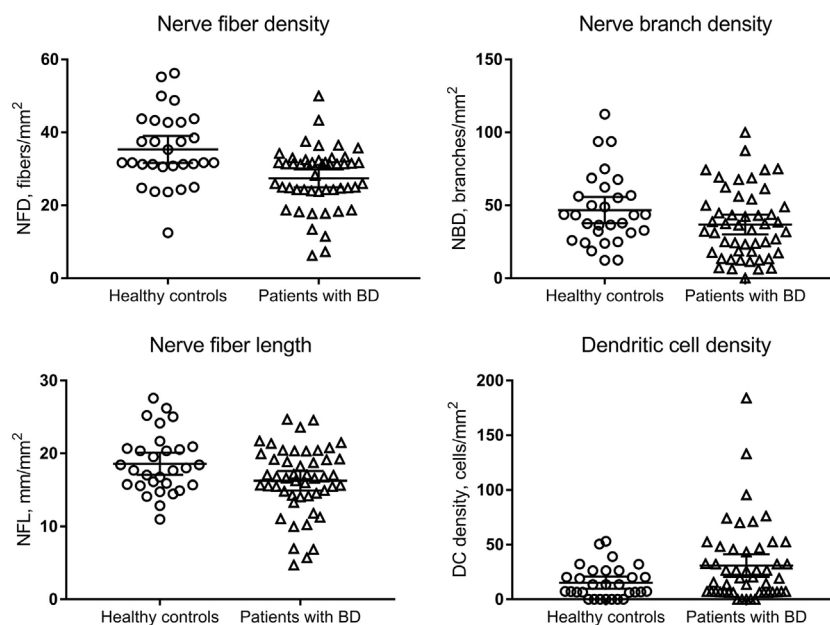


FIGURE 3 | Comparison of corneal subepithelial nerve parameters and dendritic cell (DC) density between healthy control subjects and patients with Behçet's disease (BD). Patients with BD had a lower nerve fiber density (NFD, $P = 0.001$) and nerve fiber length (NFL, $P = 0.031$), and higher DC density ($P = 0.038$) with no difference in nerve branch density (NBD, $P = 0.067$) compared to controls.

in corneal nerves and DCs in BD. We have also not compared patients with and without Neuro-Behçet's disease and active uveitis.

In conclusion, we show that CCM can identify subclinical corneal nerve fiber damage and an increase in dendritic cells in patients with BD. Further longitudinal studies are required to determine the diagnostic and prognostic ability of CCM as an imaging biomarker of axonal degeneration and immune activation in patients with BD.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Declaration of Helsinki. All subjects gave written informed consent. The protocol was approved by the Clinical Research Ethics Committee of the Necmettin Erbakan University (2017/844).

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AUTHOR'S NOTE

The preliminary findings of this study were presented at the 51st Annual Congress of the Turkish Ophthalmology Society, October 28, 2017, Antalya, Turkey.

AUTHOR CONTRIBUTIONS

All authors contributed sufficiently for being listed as authors of this article. Design of the work: GB, RM, and AO. Data acquisition: GB and EK. Data analysis and interpretation: GB, EK, GS, and RM. Drafting the manuscript: GB and EK. Revising the manuscript: GS, RM, and AO. Final approval: all authors.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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