Confocal Microscopy of Corneal Sub-basal Nerve Plexus: A Quantitative and Qualitative Analysis in Healthy and Pathologic Eyes

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ABSTRACT

PURPOSE: To validate corneal sub-basal nerve plexus examination by in vivo corneal confocal microscopy.

METHODS: Five parameters of corneal sub-basal nerve plexus in 250 human eyes (nerve fiber length, number of fibers, number of beadings, branching pattern, fiber tortuosity) acquired using in vivo corneal confocal microscopy (Confoscan 4.0; NIDEK Co Ltd) were analyzed. The first operator repeated the parameter analysis twice, performing the 2 evaluations 4 weeks apart. The second operator analyzed the cases once.

RESULTS: Intraoperator reproducibility of nerve fiber length, number of fibers, and number of beadings (intraclass correlation coefficient [ICC] = 0.96, 0.96, and 0.93, respectively) and interoperator reproducibility (ICC = 0.94, 0.95, and 0.87, respectively) were very good. Intraoperator reproducibility for branching pattern was good (ICC = 0.74), whereas interoperator reproducibility was very good (ICC = 0.81). Reproducibility of fiber tortuosity was good both at intra- and interoperator levels (ICC = 0.69 and 0.60, respectively).

CONCLUSIONS: Corneal confocal microscopy with the NIDEK Confoscan 4.0 represents an in vivo, noninvasive, and reproducible diagnostic technique for the analysis of sub-basal corneal nerve plexus. Methods used to analyze quantitative and qualitative variables were highly reproducible. [J Refract Surg. 2009;25:S125-S130.]

The cornea is one of the most densely innervated tissues in the body, abundantly supplied by sensory nerve fibers.1 Nerve bundles enter the cornea at the periphery in a radial manner parallel to the corneal surface. The nerve bundles lose their perineurium and myelin sheaths approximately 1 mm from the limbus and continue into the cornea surrounded by Schwann cell sheaths, and then subdivide several times into smaller branches. Stromal nerve trunks move from the periphery toward the corneal center and eventually turn 90°, proceeding toward the corneal surface and penetrating Bowman’s layer.2 After penetrating Bowman’s layer, the large nerve bundles divide into several smaller bundles, which turn another 90° and continue parallel to the corneal surface, between Bowman’s layer and the basal epithelial cell layer, creating the corneal sub-basal nerve plexus. The corneal sub-basal nerve plexus is characterized by local axon enlargements, or beadings, which are accumulations of mitochondria and glycogen particles located at the periphery of the bundle.3

The corneal sub-basal nerve plexus is involved in different systemic diseases (eg, diabetes mellitus)4-10 and eye conditions (eg, keratoconus, neurotropic keratitis, herpetic infections, corneal dystrophies, amyloidosis, dry eye), and can be affected by corneal surgery (epithelial wound healing, LASIK, photorefractive keratectomy [PRK], corneal transplant).11-22 Therefore, it is essential to find a valid, standardized, and reproducible method to analyze corneal sub-basal nerve plexus changes.

PATIENTS AND METHODS

PATIENTS AND CONFOCAL MICROSCOPY

A total of 250 eyes from 250 individuals referred to the Department of Ophthalmology at the University of Padova...
were studied between December 2006 and June 2007. Ninety-six eyes were healthy, whereas 154 were pathologic (eg, diabetes, dry eye, herpetic keratitis, contact lens intolerance, corneal dystrophies). Corneas characterized by significant opacities, which prevented correct visualization of the corneal sub-basal nerve plexus, were excluded.

Corneal confocal microscopy was performed using the Confoscan 4.0 (NIDEK Co Ltd, Gamagori, Japan), a scanning slit confocal microscope equipped with the Achromplan nonapplanating 40× H11003 immersion objective lens (Zeiss Inc, Jena, Germany) designed for full-thickness examination of the cornea. Confoscan 4.0 is equipped with a z-ring adapter system, which is an optomagnetic sensor that makes the examined eye integral with the tip of the confocal microscope during the examination. Therefore, with the z-ring sensor, the confocal microscope tip position is fixed in regard to the examined cornea, compensating for eye movements along the z-axis.

Each examination was performed according to a standard procedure, described previously. Briefly, before the examination, a drop of topical anesthetic 0.4% oxybuprocaine chlorohydrate (Novesina; Novartis Farma, Varese, Italy) was instilled in the lower conjunctival fornix of the eye. One drop of 0.2% polyacrylic gel (Viscotirs Gel; Ciba Vision Ophthalmics, Venezia, Italy) was applied onto the objective tip as an immersion fluid. The patient was seated in front of the microscope with chin rest and forehead support while fixating with the examined eye on a bright blue target inside the instrument to minimize eye movement during the examination. The objective lens was moved toward the eye until the gel contacted the cornea. When the stroma appeared on the monitor, a recording button was pressed and a micrometric motor-driven system automatically completed the alignment. The focal plane is automatically moved to reach the anterior chamber and begins recording several scans of the entire cornea. The central cornea was examined in all cases. The z-ring was used in all cases. Illumination intensity was kept constant for all cases. The standard dimension of each image was 340×255 µm (area 0.132 mm²), with an optical section thickness of 5.50 µm. The overall examination took 2 to 3 minutes. No individual experienced any visual symptoms or corneal complications as a result of corneal confocal microscopy.

**CORNEAL SUB-BASAL NERVE PLEXUS ANALYSIS**

In each case, the best-focused frame of the sub-basal nerve plexus was chosen. The same magnification was used to analyze all 250 images. All blurred, oblique images were discarded. The five parameters of the corneal sub-basal nerve plexus analyzed were the nerve fiber length, number of fibers, number of beadings, branching pattern, and fiber tortuosity. Each parameter was analyzed two times by the first operator; the second masked measurements were performed 4 weeks later on all images. Of the 250 images examined previously, 100 were randomly selected and analyzed by a second masked operator.

Nerve fiber length was calculated using the NeuronJ program, an image processing computer tool, to outline nerve fibers from each corneal sub-basal nerve plexus frame (Fig 1). Nerve fiber length for each image was calculated as the total length of the nerves (in micrometers) divided by the area of the image (0.132 mm²) and expressed as micrometers per square millimeter (µm/mm²). Number of fibers was manually calculated and defined as the total number of principal nerve trunks per image. Anastomosis (perpendicular, short nerve link) and nerve branches were not consid-
Number of beadings was defined as the number of well-defined, hyperreflective points compared with the nerve trunk, protruding slightly from both sides of the nerve fiber, manually calculated over 100 μm of one fiber. Each fiber to be examined was randomly chosen by the first operator from the best-focused fibers. The same standard magnification was kept for all images during counting. The first operator selected, using two crossbars, a section of nerve during the first calculation (Figure 2). The second operator counted the number of beadings on the same segment selected by the first operator.

Branching pattern and fiber tortuosity were calculated using, respectively, the Midena et al. score and Oliveira-Soto and Efron score. Branching pattern was defined as the highest number of branching considering only 1 fiber per image. For branching pattern, the grading system ranged from 0 to 3, as reported previously. Grade 0 is characterized by no corneal sub-basal nerve plexus fiber branching; grade 1 shows 1 fiber presenting 1 or more direct branchings from the major nerve trunk; grade 2 shows 1 fiber presenting 1 branching originating from a branching of principal nerve trunk; and grade 3 shows 1 fiber presenting 1 branching originating from a grade 2 fiber branching (Figure 3). The score system proposed by Oliveira-Soto and Efron was used for fiber tortuosity. This score considers simultaneously frequency and amplitude of changes in the nerve fiber direction. Fiber tortuosity values ranged from 0 to 4. Grade 0 corresponds to almost straight fibers (an aspect typical of stromal nerves and rarely, if ever, observed in sub-basal nerve plexus); grade 1 corresponds to slightly tortuous nerve fibers; grade 2 to moderately tortuous nerve fibers, with frequent and small amplitude changes of direction; grade 3 to tortuous nerve fibers, with severe amplitude of direction changes; and grade 4 to very tortuous nerve fibers, characterized by abrupt and frequent changes in direction (Figure 4).

**Statistical Analysis**

The analysis of 250 images evaluated by the first operator and the analysis of 100 images evaluated by the second operator were performed using SAS 9.1 software (SAS Institute, Chicago, Ill) with a personal computer. The statistical description of nerve fiber length, number of fibers, and number of beadings was made through the customary methods used to analyze continuous quantitative parameters (numerosity, media, standard deviation, range, and median). Branching pattern and fiber tortuosity, expressed as a discrete quantitative and qualitative parameter, respectively, were analyzed using frequency distribution. Intra- and interoperator reproducibility of corneal sub-basal nerve plexus analysis was calculated for all parameters. The first operator measured the full images set twice, whereas the second operator analyzed 100 randomly selected images from the whole set. Intraclass correlation coefficient (ICC) was used to express reproducibility of the continuous quantitative measurements. Both intra- and interoperator reproducibility were calculated for each parameter. Reproducibility level was very good for ICC ≥ 0.80; good for 0.60 ≤ ICC < 0.80; quite good for 0.40 ≤ ICC < 0.60; and insufficient for ICC < 0.40. To reach a valid and reproducible analysis method, ICC has to be 0.60. Cohen’s kappa was considered the reproducibility of qualitative and discreet quantitative variables; its reproducibility level was the same used previously for ICC. Both ICC and kappa have a confidence interval (CI) of 95%. For the reproducibility evaluation, Bland and Altman’s graph was used.

**Results**

Mean age of the examined individuals was 50.7 ± 16.9 years. No difference in intra- and interoperator parameter reproducibility was found between healthy and pathologic corneas. For nerve fiber length, both intra- and interoperator reproducibility were very good (ICC = 0.96, CI = 0.93-0.98; and ICC = 0.94, CI = 0.72-0.98, respectively). Also, for number of fibers, both intra- and interoperator reproducibility was very good (ICC = 0.96 and 0.95, respectively; CI = 0.95-0.97 and 0.93-0.97, respectively). Intra- and interoperator reproducibility for beadings count was very good (ICC = 0.93, CI = 0.91-0.94; and ICC = 0.87, CI = 0.82-0.93, respectively). For branching pattern, intraoperator reproducibility results were good (κ = 0.74, CI = 0.67-0.81), whereas interoperator reproducibility was very good.
Intraoperator reproducibility for fiber tortuosity was good ($\kappa = 0.69, \text{CI} = 0.60-0.77$), whereas interoperator reproducibility was quite good ($\kappa = 0.60; \text{CI} = 0.46-0.75$).

**DISCUSSION**

A good intra- and interoperator reproducibility represents the basis to correctly analyze corneal sub-basal nerve plexus parameters, as any other biologic parameters. Previous reports on corneal sub-basal nerve plexus analysis are characterized by significant variability in the number and definition of parameters examined. $4-7,9,10,15-17,21-25,30-33$ This variability reflects subjectivity in examining some (not all) specific corneal sub-basal nerve plexus parameters that are evaluated in different corneal diseases. Our approach was to analyze all known parameters of corneal sub-basal nerve plexus with the current methods. Therefore, this is the first study aimed at analyzing both the intra- and interoperator reproducibility of all corneal sub-basal plexus parameters, considering a qualified dataset and accurate reproducibility indices.

In this study, intra- and interoperator reproducibility for nerve fiber length was high (0.96 and 0.94, respectively) and better than reported previously. $10,31,32$ Grupcheva et al$^{31}$ reported a 93% repeatability (second measurement 3 months apart) and an interobserver variation within 12%. Malik et al$^{10}$ and Mehra et al$^{32}$ found that number of fibers showed a 12% intraoperator variation coefficient. Castillo et al$^{16}$ reported 93% intraoperator reproducibility and 10% interobserver variation. To quantify the number of beadings, reproducibility counting was performed on the same nerve segment to avoid bias introduced by the intrinsic variability in beading along the length of a nerve fiber and among different fibers. Reproducibility was high (0.93 and 0.87, respectively, as previously reported. $10,31$ For branching pattern, reproducibility was good for both intra- and interoperator measurements (0.74 and 0.81, respectively). Branching pattern reproducibility has not been analyzed previously. For fiber tortuosity, both inter- and intraoperator reproducibility were good, but confidence intervals were large. As a consequence, interoperator variability was high, suggesting that fiber tortuosity, using this score, is the most operator-dependent parameter.

Kallinikos et al$^{19}$ used a software program described previously to evaluate tortuosity in corneal sub-basal nerve plexus images of patients with diabetes. The average tortuosity score was mathematically calculated for all nerve fibers, not for nerve branches, and a final score was reported. We did not consider Kallinikos et al’s method because the proposed algorithm does not accurately quantify fibers characterized by high tortuosity. Moreover, the Oliveira-Soto and Efron score sys-

Figure 3. Branching pattern. A) Grade 0, no branching. B) Grade 1, 1 or more fibers presenting one direct branch from the principal trunk. C) Grade 2, 1 or more fibers with 1 branch originating from a principal trunk. D) Grade 3, 1 or more fibers with 1 branch originating from a grade 2 fiber.
tem is a fast and reproducible method that can be used in clinical practice. The higher reproducibility found for nerve fiber length, number of fibers, number of beadings, and branching pattern is a consequence of corneal sub-basal nerve plexus morphologic features, such as high reflectivity, which greatly improves visual detection and the beading count. This study demonstrates that it is possible to obtain valid measurements of corneal sub-basal nerve plexus parameters using corneal confocal microscopy, an in vivo, noninvasive, and reliable technique for qualitative and quantitative evaluation of corneal sub-basal nerve plexus. Full automatic quantification of these parameters will allow operator-independent calculation; a simple, faster evaluation; and widespread application in clinical practice.

REFERENCES


Figure 4. Grading scales for tortuosity provided by Oliveira-Soto and Efron.25 A) Grade 1, the nerve fibers appear slightly tortuous. B) Grade 2, the nerve fibers appear moderately tortuous, with frequent but small amplitude changes in the direction of fibers. C) Grade 3, the nerve fibers appear quite tortuous, with frequent and severe amplitude changes in the direction of fibers. D) Grade 4, the nerve fibers appear very tortuous, with abrupt and frequent changes in the direction of nerve fibers.