Depth of Penetration of Scanning Laser Doppler Flowmetry in the Primate Optic Nerve

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Objectives: To estimate the measuring depth of the blood flow and to establish the vascular contributions to these measurements with scanning laser Doppler flowmetry (SLDF) of the primate anterior optic nerve.

Methods: Optic nerve blood flow in each eye of 8 monkeys was measured using SLDF before and following surgical occlusion of the central retinal artery (n=4) or posterior ciliary arteries (n=4). The regional blood flow in both eyes was determined using a nonradioactive microsphere method.

Results: The blood flow in the nerve fiber layer (NFL), including the prelaminar region, was measured with microspheres after central retinal artery occlusion; it was significantly reduced (−83%) with no significant change in the combined laminar and retrolaminar regions. The blood flow measured with SLDF had a 51% reduction. After posterior ciliary artery occlusion, the blood flow in the NFL was measured with microspheres and was not significantly affected (+2%); neither was that measured with SLDF (−12%). However, there was a 51% reduction in the laminar and retrolaminar regions when microspheres were used. The mean ± SD tissue thickness of the NFL was 359 ± 16 µm and 353 ± 54 µm in each group.

Conclusions: Scanning laser Doppler flowmetry measures blood flow principally in the NFL of the anterior optic nerve, which is primarily supplied by the central retinal artery. Blood flow in the laminar and retrolaminar regions makes a small contribution to the SLDF measurement, with an NFL thickness between 300 and 400 µm.

Clinical Relevance: Scanning laser Doppler flowmetry is used for the noninvasive evaluation of ocular microcirculation in diseases such as glaucoma. Because of the dual blood flow supply in the optic nerve and the limited penetration power of the laser, the instrument primarily measures the microcirculation on the surface of the optic nerve, which is largely supplied by the central retinal artery rather than the ciliary arteries.


The anterior optic nerve may be anatomically divided into 4 regions: the superficial nerve fiber layer (NFL), the prelaminar region, the lamina cribrosa, and the retrolaminar region. In primates, the central retinal artery (CRA) supplies the superior NFL, whereas the circulation of the posterior ciliary arteries (PCAs) supplies the prelaminar, laminar, and retrolaminar regions. It has been hypothesized that the pathological changes in glaucoma occur primarily in the laminar region and that alterations in the blood supply to this area may contribute to the development of glaucomatous optic neuropathy. Therefore, the ability to measure blood flow within the various regions of the anterior optic nerve, and in particular the laminar region, may be important to further understand the contribution of vascular abnormalities to glaucoma.

Scanning laser Doppler flowmetry (SLDF) is a newly developed technique to provide relative measurements of red blood cell velocity, volume, and flow within a volume of tissue. The potential effective measuring depth into a tissue using SLDF has been estimated at between 300 and 400 µm while the laser light is focused on the tissue surface. Because the thickness of the NFL in the normal optic nerve is close to the estimated measuring tissue depth with SLDF, and the PCAs supply the laminar and retrolaminar regions, SLDF measurements would theoretically derive from the most anterior tissues, supplied predominantly by CRA circulation. However, the blood flow measurements from capillaries at the level of the lamina cribrosa have reportedly undergone imaging with SLDF in both patients with glaucoma and healthy subjects. These
MATERIALS AND METHODS

ANIMALS AND ANESTHESIA

We used 8 adult female rhesus monkeys, weighing 4 to 8 kg and between the ages of 15 and 17 years, in accordance with the Association for Research in Vision and Ophthalmology Statement on the use of animals in ophthalmic and vision research. The animals were anesthetized with pentobarbital sodium. Two femoral arteries were cannulated for blood sampling and blood pressure registration. Another catheter was inserted into the left ventricle via the right axillary artery for later blood flow determination using the microsphere method. The animals were intubated and ventilated mechanically with approximately 32% oxygen. Blood gases were monitored intermittently and maintained at a stable level. A heating pad maintained body temperature.

OCCLUSION OF CRA AND PCAs

Four monkeys underwent CRA occlusion, and the other 4 underwent PCA occlusion. The procedures were performed in 1 eye in each animal with the exception of 1 PCA attempt, in which the occlusion was also performed in the contralateral eye after it could not be done in the first eye. The 7 unmanipulated contralateral eyes remained the controls for the microsphere evaluations. In a lateral decubitus position, the monkeys underwent a lateral orbitotomy to expose the retrobulbar region.

The CRA was identified by observing its entrance into the optic nerve, generally 6 to 8 mm behind the globe. After dissecting the connective tissue underneath the CRA, an 8-0 silk suture was placed around the vessel at a site close to the optic nerve. In the monkeys that underwent PCA occlusion, 2 sutures were used to surround the PCAs at their branch point into the short PCA on the nasal and temporal sides of the optic nerve. With stabilized conditions for blood gases and blood pressure, CRA or PCA occlusion was achieved by tightening the suture.

BLOOD FLOW DETERMINATION

Scanning Laser Doppler Flowmetry

The animal was maintained in a lateral decubitus position, and the pupil was dilated with 1% cyclopentolate hydrochloride (Bausch & Lomb Pharmaceuticals Inc, Tampa, Fla). A rectangular area of the retina (10° × 2.5°) underwent imaging with the Heidelberg Retina Flowmeter (Heidelberg Engineering GmbH, Heidelberg, Germany) at a distance of 2.5 cm between the lens of the instrument and the cornea. This imaged region incorporated the entire temporal optic nerve and the corresponding superior and inferior peripapillary areas. The scans were focused on the surface of the optic nerve rim area at the level of the NFL.

To minimize the source of variability of the SLDF measurement caused by the cardiac cycle, the starting time for each scan was at the end of the QRS complex. The imaging window was set to incorporate the same retinal or optic nerve region. Using these conditions, we measured the blood flow in the optic nerve prior to and after the CRA or PCA occlusion. At each time point, at least 3 images were repeated in each area.

A recently developed SLDF analysis program (SLDF V 3.2; Laboratory for Ocular Perfusion, Department of Ophthalmology, University of Erlangen, Erlangen, Germany) was chosen to analyze the blood flow perfusion. This program allows the computation of averaged blood flow measurements obtained by the user in a specific area of the image. It also eliminates underexposed and overexposed pixels and large vessels in which flow measurements are not accurate. The flow maps are mathematically smoothed by averaging 5 × 5 pixel areas to reduce spatial heterogeneity. Using this program, we analyzed the blood flow of the temporal rim of the optic nerve.

Microsphere Method

Immediately after the last SLDF image was obtained (approximately 30 minutes following the occlusion), the blood flow was measured using the microsphere method. Briefly, a 10-mL microsphere suspension (E-Z Trac Ultraspheres, Los Angeles, Calif) was injected into the left cardiac ventricle within 40 to 60 seconds after a flush of intravenous heparin sodium was administered (500 IU/kg). The suspension consisted of 10⁶ black polystyrene spheres that were 10 µm in diameter. A reference blood sample was collected from a femoral artery catheter continuously for 2 minutes from the start of the injection.

After completion of the collection period, each animal underwent euthanasia. All eyes were then enucleated, and the CRA or PCA occlusion was visually confirmed under a dissecting microscope. The eyes were fixed in 4% formaldehyde, and serial consecutive 90-µm cryosections were cut. The blood flow was determined in 2 regions of the anterior optic nerve: the NFL, including the prelaminar region, and an area including the lamina cribrosa and 1 mm of the retrolaminar region (LRL). These 2 regions were selected based on the approximate blood supply of the CRA and PCAs. The PCA supply-dominated prelaminar region was included in the NFL because the 2 regions could not be distinguished in unstained sections. The microsphere-derived blood flow measurements were determined by comparing microspheres and tissue volume with those in the reference blood sample. Flow values were calculated and expressed in microliters of blood per tissue volume per minute.

Using the same cryosections, the thickness of the NFL (including the prelaminar region in the temporal rim area imaged with the Heidelberg Retina Flowmeter in the 8 eyes that underwent occlusion) was measured to provide an estimation of SLDF penetration depth.

STATISTICS

The difference in the 2 means between the 2 eyes or that before and after the occlusions in the same eyes was evaluated with the paired t test. The data are presented as mean ± SE.

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CRA or PCAs, are compared with blood flow measurements derived with a modified nonradioactive microsphere method in the anterior optic nerve of the primate eye. Using these techniques, we evaluated the measuring depth of SLDF in the anterior optic nerve and the contribution of the CRA and PCAs to the blood flow measurements with SLDF.

**RESULTS**

**BLOOD FLOW AFTER PCA OCCLUSION**

In the LRL region, the mean ± SE blood flow measured with the modified microsphere technique (BF<sub>SPH</sub>) was 0.29 ± 0.08 µL/min/mm<sup>3</sup> in the eyes with PCA occlusion and 0.58 ± 0.05 µL/min/mm<sup>3</sup> in the control eyes. These values demonstrate an approximately 50% decrease in blood flow to the laminar and retrolaminar regions (P = .01). In the NFL, the mean ± SE BF<sub>SPH</sub> in the eyes with occlusion was 0.29 ± 0.08 µL/min/mm<sup>3</sup>, and 0.58 ± 0.05 µL/min/mm<sup>3</sup> in the control eyes; statistical analysis showed no significant difference (P = .74).

The mean ± SE blood flow measured in arbitrary units with SLDF (BF<sub>SLDF</sub>) in the eyes with PCA occlusion was 228 ± 59 preocclusion and 200 ± 37 postocclusion. This decrease of approximately 12% is not statistically significant (P = .50). Table 1 presents the BF<sub>SPH</sub> in the NFL and LRL regions in the 2 eyes of each animal, as well as the BF<sub>SLDF</sub> both before and after PCA occlusion.

**BLOOD FLOW AFTER CRA OCCLUSION**

The mean ± SE BF<sub>SPH</sub> in the NFL with CRA occlusion was 0.15 ± 0.08 µL/min/mm<sup>3</sup>. In the contralateral eyes, the BF<sub>SPH</sub> in the NFL region was 0.78 ± 0.20 µL/min/mm<sup>3</sup>. This represents a decrease of approximately 83% (P = .01). In the LRL region, the mean ± SE BF<sub>SPH</sub> in the eyes with occlusion was 0.36 ± 0.04 µL/min/mm<sup>3</sup>, and 0.44 ± 0.01 µL/min/mm<sup>3</sup> in the control eyes. This is a nonsignificant decrease of 10% (P = .37).

The mean ± SE BF<sub>SLDF</sub> (arbitrary units) was reduced from 223 ± 35 preocclusion to 110 ± 29 postocclusion. This decrease of approximately 51% approaches statistical significance (P = .06). Among the 4 animals, the decrease in BF<sub>SLDF</sub> ranged between 29% and 79%. Table 2 presents the BF<sub>SPH</sub> in the NFL and LRL regions in the 2 eyes of each animal, as well as the BF<sub>SLDF</sub> both before and after CRA occlusion.

**HISTOLOGICAL TISSUE THICKNESS MEASUREMENTS**

The mean ± SE thickness of the NFL (including the prelaminar region in the temporal rim area in the groups

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Table 1. PCA Occlusion: Blood Flow Measured With the Microsphere Method in the NFL and LRL in 2 Eyes of Each Animal and With SLDF Before and After Occlusion

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>BF&lt;sub&gt;SPH&lt;/sub&gt;, µL/min/mm&lt;sup&gt;3&lt;/sup&gt;</th>
<th>NFL</th>
<th>LRL</th>
<th>BF&lt;sub&gt;SLDF&lt;/sub&gt;, AU</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Occlusion</td>
<td>Control</td>
<td>Difference, %</td>
<td>Occlusion</td>
</tr>
<tr>
<td>M42†</td>
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<td>0.99</td>
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<td>0.13</td>
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<tr>
<td>M46</td>
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<td>0.64</td>
<td>−21</td>
<td>0.28</td>
</tr>
<tr>
<td>M55</td>
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<td>0.78</td>
<td>−10</td>
<td>0.46</td>
</tr>
<tr>
<td>M58</td>
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<td>25</td>
<td>0.27</td>
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<tr>
<td>Mean ± SE</td>
<td>0.84 ± 0.17</td>
<td>0.81 ± 0.08</td>
<td>2 ± 12</td>
<td>0.29 ± 0.08</td>
</tr>
</tbody>
</table>

*PCA indicates posterior ciliary artery; NFL, nerve fiber layer (including the prelaminar region); LRL, an area including the lamina cribrosa and 1 mm of the retrolaminar region; SLDF, scanning laser Doppler flowmetry; BF<sub>SPH</sub>, blood flow measured with the microsphere method; BF<sub>SLDF</sub>, blood flow measured with SLDF; and AU, arbitrary units.

†Mean blood flow of all control eyes was used because bilateral occlusion was performed.

Table 2. CRA Occlusion: Blood Flow Measured With the Microsphere Method in the NFL and LRL in 2 Eyes of Each Animal and With SLDF Before and After Occlusion

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>BF&lt;sub&gt;SPH&lt;/sub&gt;, µL/min/mm&lt;sup&gt;3&lt;/sup&gt;</th>
<th>NFL</th>
<th>LRL</th>
<th>BF&lt;sub&gt;SLDF&lt;/sub&gt;, AU</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Occlusion</td>
<td>Control</td>
<td>Difference, %</td>
<td>Occlusion</td>
</tr>
<tr>
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<tr>
<td>M56</td>
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<td>−74</td>
<td>0.39</td>
</tr>
<tr>
<td>M57</td>
<td>0.02</td>
<td>0.54</td>
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<td>0.29</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>0.15 ± 0.08</td>
<td>0.78 ± 0.20</td>
<td>−83 ± 6</td>
<td>0.36 ± 0.04</td>
</tr>
</tbody>
</table>

* CRA indicates central retinal artery; NFL, nerve fiber layer (including the prelaminar region); LRL, an area including the lamina cribrosa and 1 mm of the retrolaminar region; SLDF, scanning laser Doppler flowmetry; BF<sub>SPH</sub>, blood flow measured with the microsphere method; BF<sub>SLDF</sub>, blood flow measured with SLDF; and AU, arbitrary units.
with PCA and CRA occlusion) was 359 ± 16 µm and 353 ± 54 µm, respectively, measured on the cryosections. **Table 3** summarizes the mean percent change of the BFSPH in the NFL and LRL regions in the eyes with occlusion compared with the contralateral eyes in the 2 groups. It indicates the mean percent change of the BFSLDF following occlusion. Occlusion of the PCAs did not significantly alter the BFSLDF or the BFSPH within the NFL. However, there was a significant change of blood flow in the LRL region (51% decrease; *P* = .01) measured with the microsphere method following PCA occlusion. With occlusion of the CRA, the BFSPH decreased significantly (−83%) in the NFL, as did the BFSLDF (−51%). The BFSPH in the LRL was minimally changed (−10%) by CRA occlusion.

**COMMENT**

To estimate the measuring depth of SLDF in the anterior optic nerve of the primate eye, the blood flow before and after CRA and PCA occlusion was determined. Before and after the occlusions, the blood flow change in the same eye was measured with SLDF. The blood flow change in the NFL (including the prelaminar region) and LRL after occlusion was also compared with the contralateral eye using the microsphere method. Because the primary arterial supply of the primate NFL is derived from the CRA, SLDF measurements would be preferentially affected by CRA occlusion if they were predominantly obtained from the NFL; that is, the BFSLDF should exhibit a change parallel to that of the BFSPH in the NFL after the CRA occlusion as well as a minimal change following PCA occlusion. On the other hand, if the measuring depth of SLDF is deeper than the NFL, a less significant change of the BFSLDF after CRA occlusion would be demonstrated, and a change of the BFSLDF would be expected after PCA occlusion.

The results indicate that the BFSPH in the LRL region was less affected by CRA occlusion (−10%). The BFSPH in the NFL was decreased by 83% compared with the contralateral eyes, and blood flow measured with SLDF decreased by 51% compared with the preocclusion values. Because the percentage change of the blood flow was lower in the SLDF measurements, they likely received some contributions from the deeper (more posterior) tissues. It should be noted that the BFSPH in the NFL also includes the prelaminar region, which derives its blood supply from the PCAs and CRA. Although these 2 regions are always separated in written descriptions of the anterior optic nerve, no absolute boundaries separate them in regard to blood flow contribution. In addition, from the results of Table 2, 83% of the blood flow in the “combined” NFL was eliminated after occlusion of the CRA. This means that a maximum of only 17% of the blood flow measurement was derived from the thin prelaminar region. Therefore, any overestimation of the laser penetration induced by combining the prelaminar region would be insignificant.

In the group of eyes that underwent PCA occlusion, the BFSPH and BFSLDF in the NFL were closer (2% vs −12%), whereas there was a larger decrease (−51%) in the LRL region as measured with the microsphere technique. This indicates that the BFSLDF was predominantly derived from the CRA and that the PCA system contributed marginally to the blood flow measurement obtained with SLDF.

Based on this analysis, it can be estimated that the SLDF measures the blood flow in the anterior optic nerve at between 300 and 400 µm in depth, with the laser beam focused on the surface of the temporal rim area. Within this range of depth, a large fraction of the measurement obtained with SLDF is derived from the region where the blood is supplied by the CRA.

In our study, we assumed that the 2 eyes of each animal had similar blood flow rates. This assumption was made to determine the percentage change of the occlusion-induced blood flow using the microsphere method. Although a previous study did not show a significant difference between the 2 eyes in human subjects with a similar laser Doppler technique, 13 the variation of the instrument and the methods used in our study cannot be ignored. In addition, quantitatively different collateralization between CRA and PCA blood supplies to the optic nerve may contribute to the variation. The residual blood flow after CRA occlusion is similar to findings in the studies of Hayreh and Weingeist. 16

This in vivo estimation is close to that of the designed measuring range of the instrument. 4,7 It is also comparable to a recent study that used laser Doppler flowmetry. 17 That study determined the depth of laser penetration in the anterior optic nerve in primates. With an experimental setup similar to ours, PCA occlusion caused a 17% nonsignificant increase in blood flow measured with laser Doppler flowmetry. After CRA occlusion, the blood flow was decreased by 39%, 17 or 32% in an earlier report. 18 Assuming that the blood flow in the NFL and LRL regions before and after occlusion is similar to what we measured with the microsphere method,
the measuring depth of laser Doppler flowmetry is deeper than that of SLDF, as was also demonstrated by Piltz-Seymour.19

The blood flow in the choroid is much higher than that in the retina. If the thickness of the retina is larger than the depth of laser penetration, the choroidal blood flow cannot be measured with SLDF. On the other hand, if the retina becomes thinner, as in patients with glaucoma, even a portion of the measurement derived from the choroid may increase the measured values significantly. In the anterior optic nerve, the blood flow in the NFL is higher than in the deeper region. A thinned NFL may allow the laser to penetrate deeper and result in a lower blood flow measurement, even if the flow rate is normal in the NFL. This may complicate clinical interpretations of the anterior optic nerve blood flow values as the retinal NFL thickness changes in various states of disease. When SLDF is used to evaluate the blood flow in glaucoma, the thickness of the tissues should be taken into account unless a uniform blood flow change in the entire region can be proved. Otherwise, with a decreasing NFL thickness, the normally lower blood flow rates of the posterior regions of the optic nerve may make a greater contribution to the measurements. Similarly, in patients who potentially have a thinner retina, such as those with myopia or peripapillary chorioretinal atrophy, the deeper vessels may be imaged and may contribute to the derived blood flow measurements.6,20

Hematocrit is another factor affecting the laser Doppler flowmetry blood flow measurement, shown in an in vitro experiment.21 In that study, the depth of laser Doppler flowmetry was measured through an erythrocyte suspension medium; it increased approximately 10-fold after the hematocrit was decreased from 36.5% to 5%. In our study, the lack of blood flow in the NFL after CRA occlusion may have facilitated the laser penetration. However, our results did not show a significant increase of the measuring depth after CRA occlusion. The explanation may be that the density of the erythrocytes in the tissue is much lower than in a red blood cell suspension. Also, occlusion of the arterial supply does not necessarily evacuate all of the erythrocytes from the vascular beds. Therefore, the tissue thickness itself, rather than the hematocrit, is the most important factor in the derived measurement of blood flow using SLDF in vivo.

In conclusion, SLDF measurement values of the anterior optic nerve blood flow are derived principally from the NFL. However, deeper capillary beds in the prelaminar and laminar regions also contribute to the SLDF measurements in eyes with an NFL thickness between 300 and 400 μm in which SLDF is focused on the surface of the optic nerve. Because the blood of the NFL in the anterior optic nerve is primarily supplied by the CRA, and the PCAs supply the laminar and retrolaminar regions, the SLDF blood flow measurement in the anterior optic nerve is primarily derived from the retinal arterial supply.