Laser Speckle and Hydrogen Gas Clearance Measurements of Optic Nerve Circulation in Albino and Pigmented Rabbits With or Without Optic Disc Atrophy

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Submitted: July 31, 2014
Accepted: October 10, 2014


Purpose. The purpose of this study was to evaluate the correlation between laser speckle flowgraphy measurements of mean blur rate (MBR) and hydrogen gas clearance measurements of capillary blood flow (CBF) in the optic nerve head (ONH) of albino and pigmented rabbits, with or without chronic ischemia-induced ONH atrophy.

Methods. The ONH MBR and ONH CBF were measured at baseline, 30 and 60 minutes after the intravenous administration of endothelin-1 (ET-1) (10⁻¹⁰ mol/kg) in six albino and six pigmented rabbit eyes. The ONH MBR and ONH CBF were also measured in nine pigmented rabbit eyes that underwent the intravitreal administration of ET-1 (20 pmol) twice per week for 4 weeks to provoke chronic ischemia-induced ONH atrophy.

Results. In the group that received intravenous ET-1, average measurements of ONH MBR and ONH CBF at all time points were correlated in both the albino (r = 0.88, P < 0.001, n = 18) and pigmented rabbits (r = 0.85, P < 0.001, n = 18), with no intrarabbit correlations (P = 0.524). The ONH MBR and ONH CBF were also correlated in the model of chronic ischemia-induced ONH atrophy (r = 0.78, P = 0.013, n = 9). Pooled ONH MBR and ONH CBF measurements in both the intravenous and intravitreal groups were also highly correlated (r = 0.87, P < 0.001, n = 45), with no significant intergroup differences in the relationship between ONH MBR and ONH CBF (P = 0.138).

Conclusions. Regardless of the presence of fundus pigmentation or ONH atrophy, ONH MBR and ONH CBF were highly correlated, suggesting that MBR in the ONH tissue is usable for interindividual and intergroup comparisons.

Keywords: mean blur rate, laser speckle flowgraphy, capillary blood flow, optic nerve head, endothelin-1

The noninvasive evaluation of ocular ischemia in living eyes is an important goal of ongoing efforts to understand retinal diseases, such as diabetic retinopathy, and axonal diseases, such as glaucoma. These diseases are among the main causes of blindness worldwide,¹³ but their pathogenesis remains unclear. Recently, reports have indicated that decreased ocular blood flow, especially reduced optic nerve head (ONH) microcirculation, may play an important role in the pathogenesis of glaucoma.⁵,⁶ Therefore, it would be very useful to establish a quick, easy, and noninvasive method to measure ONH microcirculation suitable for large-scale clinical studies in order to investigate the possible involvement of compromised blood flow in the ONH in glaucoma.

Laser speckle flowgraphy (LSFG) is a promising candidate technology for this purpose. It uses the laser speckle phenomenon to enable the in vivo quantification of circulation in the ONH, choroid, and retinal vessels and can reveal the circulatory condition of these tissues separately.⁷,⁸ LSFG-NAVI, a new version of this technology, was approved as a medical device in Japan in 2008. It introduced a new measurement parameter, mean blur rate (MBR), as a quantitative index of retinal blood cell (RBC) velocity.⁹,¹⁰ Until the introduction of MBR, LSFG measurements were mainly used to monitor changes in ONH or choroid circulation over time in a single site in the same eye.⁷ This use of LSFG was prompted by the fact that in addition to RBC velocity, the laser speckle signal is also influenced by laser beam reflectance and target tissue absorption.⁹ However, recent studies have shown that MBR is closely correlated to hydrogen gas clearance-measured capillary blood flow (CBF) in the ONH of albino rabbits,¹¹ especially in comparison to normalized blur (NB), a quantitative index of RBC velocity used in earlier versions of the LSFG device.¹² The ONH MBR in rhesus monkeys with or without experimentally induced glaucoma has also been reported to be closely correlated with microsphere-determined CBF.¹³ These recent studies raise the possibility that MBR values can be used to...
compare ONH circulation not only in a single site in an individual eye, but also between eyes.

Thus, to more precisely determine the potential of using LSFG measurements to make interindividual comparisons of ONH circulation, we used LSFG to measure MBR and the hydrogen gas clearance technique to measure CBF concurrently, in the ONH of albino and pigmented rabbits and investigated the effect of fundus pigmentation on the relationship between MBR and CBF. We also studied the effect of ONH atrophy on the MBR-CBF relationship in pigmented rabbit eyes that had received repeated intravitreal injections of endothelin-1 (ET-1) in order to model chronic ischemia-induced ONH atrophy.\(^{14,15}\)

**Methods**

**Animals**

Fifteen male pigmented Dutch rabbits and six male Japanese albino rabbits (2.0–2.5 kg; Kitayama Labes, Ina, Japan) were used. All MBR and CBF measurements were carried out in animals anesthetized by the subcutaneous injection of 0.8 mg/kg urethane at 0.4 g/mL (Nakalai, Kyoto, Japan). All experimental procedures conformed to the ARVO (Association for Research in Vision and Ophthalmology) Statement for the Use of Animals in Ophthalmic and Vision Research and were conducted with approval from the Experimental Animal Management Committee of the Tohoku University School of Medicine.

**Experimental Protocol**

Human ET-1 (Peptide Institute, Inc., Osaka, Japan) was dissolved in 0.1% aqueous acetic acid to obtain a 10\(^{-4}\) mol/L solution. For intravenous administration, the concentration was then adjusted to 10\(^{-6}\) mol/L by dilution with saline, and for intravitreal administration, it was diluted with balanced saline solution. The MBR and CBF were simultaneously measured in the ONH tissue as described below in the eyes of six Japanese albino and six pigmented rabbits before and 50 and 60 minutes after the intravenous administration of the prepared ET-1 at a dose of 10\(^{-10}\) mol/kg. Relative changes in MBR and CBF (expressed as percentages of the baseline) were determined as the quotient of posttreatment values divided by baseline pretreatment values.

Mean blur rate measurements in the ONH tissue were also made in a separate group of nine pigmented rabbits before a course of intravitreal injections of ET-1, at a dose of 20 pmol, twice a week for 4 weeks. Concurrent measurements of MBR and CBF in the ONH were carried out, according to the procedure described above, 4 weeks after the final intravitreal injection and after confirmation that chronic ischemia-induced ONH cupping was present in optical coherence tomography (OCT) scans.\(^{14,15}\)

**Measurement of MBR in the ONH**

The pupil of each rabbit was dilated with one drop (approximately 40 μL) of 0.4% tropicamide ophthalmic solution (Mydri-M; Santen Pharmaceutical Co., Ltd., Osaka, Japan). The MBR values in the ONH tissue were measured with the LSFG-NAVI Analyzer device (Softcare Ltd., Fukutsu, Japan), which consists of a fundus camera equipped with a diode laser (wavelength: 830 μm) and a charge-coupled device (CCD) image sensor (750 × 360 pixels). Average MBR was measured in a square area of at least 15 × 15 pixels in the ONH that was free of visible surface vessels and was adjacent to the placement of the hydrogen electrode (as described below) in the ONH.\(^{11,12}\) Recorded values for MBR at each experimental time point were averages of five successive measurements.

**Measurement of CBF in the ONH**

Capillary blood flow was measured in the ONH with the hydrogen gas clearance technique, as previously described,\(^{11,12}\) concurrently with ONH MBR. Briefly, after pupil dilation, a hydrogen electrode (catalog no. OA211-013, platinum needle with a 0.7-mm-long, 0.1-mm-diameter Pt-Ir tip; Unique Medical Co., Ltd., Tokyo, Japan) was inserted into the lower, avascular zone of the ONH through the vitreous body with a pars planar approach. The animals then inhaled 5 L/min of 10% hydrogen gas for 5 minutes through a mask, and a hydrogen clearance flow meter (model MDH-D1; Unique Medical Co., Ltd.) was used to measure the hydrogen concentration half-life and determine CBF. To confirm the repeatability of CBF measurements in the ONH, three measurements separated by 15-minute intervals were made before the intravenous administration of ET-1. The criterion for measurements to be considered reliable was a coefficient of variance for CBF within 0.1. Concurrent measurements of ONH CBF and ONH MBR were then carried out before and 30 and 60 minutes after the intravenous injection of ET-1. In eyes with ischemia-induced ONH atrophy, three ONH CBF measurements were taken, as described above. During this examination, five successive ONH MBR measurements were also taken.

**Measurement of ONH Cupping After Repeated Intravitreal Injections of ET-1**

At baseline and 4 weeks after intravitreal ET-1, B-scan images of the ONH were obtained with the macular multiscan protocol of the RS-3000 Advance (NIDEK, Inc., Tokyo, Japan) spectral-domain OCT device. The vertical B-scan that was closest to the center of the ONH and did not include any retinal arteries or veins was selected for the analysis. A reference line connecting the edges of Bruch’s membrane opening was drawn using the calipers provided by the OCT software. A vertical line was then manually drawn from the reference line to the deepest surface of the lamina cribrosa, and its length was measured in pixels and expressed as a percentage relative to a baseline, set as 1.0 (100%). The ONH cupping depth was defined as this relative value. The same area was scanned 4 weeks after the intravitreal administration of ET-1. The average of three repeated measurements at each time point was used for the statistical analysis. All of the measurements were made by the same observer (NA).

**Statistical Analysis**

A paired t-test was used to determine the significance of differences between values at baseline and after intravenous or repeated intravitreal ET-1 administration. Correlations between MBR and CBF were evaluated using Spearman’s rank correlation coefficient. The association between MBR and CBF was also investigated using a generalized estimating equation with an exchangeable working correlation matrix and a robust standard error, in order to determine the effect of intrarabbit correlations. We also used an analysis of covariance (ANCOVA) to determine differences among regression lines fitted to the data obtained from each of the three groups (the albino and pigmented rabbits that received an intravenous ET-1 injection and the pigmented rabbits that received repeated intravitreal ET-1 injections). Statistical analyses were performed with JMP software (Pro version 10.0.2; SAS Institute Japan, Inc., Tokyo, Japan). The significance level was set at \( P < 0.05 \). A power
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### Table. CBF and MBR Values After the Intravenous Administration of ET-1

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>30 min</th>
<th>60 min</th>
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</thead>
<tbody>
<tr>
<td>Albino, n = 6</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CBF ml/min/100 g</td>
<td>57.9 ± 16.8</td>
<td>47.5 ± 7.4</td>
<td>43.7 ± 7.1</td>
</tr>
<tr>
<td>MBR, AU</td>
<td>5.8 ± 1.7</td>
<td>4.5 ± 1.2</td>
<td>4.1 ± 0.9</td>
</tr>
<tr>
<td>Pigmented, n = 6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBF ml/min/100 g</td>
<td>71.8 ± 12.6</td>
<td>60.6 ± 14.2</td>
<td>54.9 ± 12.9</td>
</tr>
<tr>
<td>MBR, AU</td>
<td>7.8 ± 1.2</td>
<td>7.0 ± 1.1</td>
<td>6.0 ± 1.5</td>
</tr>
</tbody>
</table>

Paired t-test, compared to baseline. AU, arbitrary unit.
* P < 0.05.
† P < 0.01.

Analysis by G* Power software (v. 3.1.3.) showed that a total sample size of nine was adequate, with the conditions that the effect size was 0.7, the error probability (α) was 0.05, and the power (1 – β) was 0.8.

### Results

#### Relationship Between ONH MBR and ONH CBF Before and After Intravenous ET-1 Administration

In the albino and pigmented rabbits, ONH MBR was statistically similar: 6.5 ± 2.3 and 7.8 ± 1.2 arbitrary units, respectively (mean ± SD; P = 0.136). The ONH CBF was also statistically similar: 57.9 ± 16.8 and 71.8 ± 12.6 ml/min/100 g at baseline, respectively (n = 6; P = 0.256). The ONH MBR and ONH CBF values significantly decreased 60 minutes after intravenous ET-1 injection in both the albino (Table; 24.5%, P = 0.035, and 19.3%, P = 0.038, respectively) and pigmented rabbits (Table; 23.5%, P = 0.001, and 23.1%, P = 0.002, respectively) and 30 minutes after intravenous ET-1 injection in the pigmented rabbits (Table; P = 0.007 and 0.024, respectively). The relationship between ONH MBR and ONH CBF before and after the intravenous administration of ET-1 is shown in Figure 1. Baseline and 30 and 60 minutes after the intravenous injection of ET-1, ONH MBR and ONH CBF were significantly correlated in a pooled group of pigmented and albino rabbits (r = 0.90, P < 0.001, n = 36). The association between MBR and CBF was not significantly different in the albino and pigmented rabbits (CBF by rabbit interaction term, P = 0.524). The relationship between changes in CBF and MBR after the intravenous administration of ET-1 is shown in Figure 2. Changes in MBR and CBF relative to baseline and 30 and 60 minutes after the intravenous injection of ET-1 were also significantly correlated in a pooled group of all rabbits (shown as a regression line, r = 0.74, P < 0.001, MBR = 0.844 × CBF + 0.117, n = 24). White circles indicate albino rabbits and black circles pigmented rabbits.

#### Relationship Between ONH CBF and ONH MBR 4 Weeks After Repeated Intravitreal ET-1 Injections

Four weeks after the cessation of intravitreal ET-1 injections, ONH cupping was significant, with the cup having deepened to 130.6% ± 32.3% (n = 9) of baseline (P = 0.011). The ONH MBR also significantly decreased to 83.2% ± 29.8% of baseline (P = 0.045). As seen in Figure 3, ONH MBR and ONH CBF were significantly correlated 4 weeks after the cessation of intravitreal ET-1 injections (r = 0.78, P = 0.013, n = 9).

#### Analysis of Pooled ONH MBR and ONH CBF Measurements

Pooled ONH MBR and ONH CBF values from the rabbits in all groups were significantly correlated (Fig. 4; r = 0.87, P < 0.001, n = 45), with no significant intergroup differences in the relationship between ONH MBR and ONH CBF (P = 0.138).
DISCUSSION

We used LSFG to measure MBR in the ONH tissue of albino and pigmented rabbits that had received either an intravenous ET-1 injection to provoke acute ischemia or repeated intravitreal ET-1 injections to provoke chronic ischemia-induced ONH atrophy,\textsuperscript{14,15} and we concurrently used the hydrogen gas clearance technique to measure CBF in the same ONH tissue. We then investigated the relationship between measurement values obtained with these two techniques and found that there was a close, linear correlation between them, regardless of fundus pigmentation or the presence of chronic ischemia-induced ONH atrophy. These results indicated that fundus pigmentation and coexisting ONH atrophy did not have a significant effect on the correlation between the results of these two measurement techniques.

There have been many studies of the ocular hemodynamic effects of ET-1.\textsuperscript{11,12,14–20} Intravenous administration has been reported to reduce ONH circulation by approximately 20% in both human subjects and rabbits,\textsuperscript{12} a comparable result to that obtained here (i.e., a 24.5% CBF reduction in albino rabbits and a 23.5% reduction in pigmented rabbits). Our findings in albino rabbits also agree with those of Takahashi et al.,\textsuperscript{11} who showed that ONH MBR and ONH CBF were closely correlated in albino rabbits that had inhaled CO\textsubscript{2} or received intravenous ET-1 injections. Our study expanded on these findings by also measuring ONH MBR and ONH CBF in pigmented rabbits and revealed that fundus pigmentation had little effect on measured values of ONH MBR. Furthermore, Wang et al.,\textsuperscript{13} in a study of ocular blood flow reduction in rhesus monkeys with experimental laser-induced glaucoma, found that measurements of ONH MBR made with LSFG and ONH blood flow rate made with the microsphere technique were highly correlated. Although the laser-induced glaucoma model of ONH atrophy used in Wang’s study\textsuperscript{13} has inherent histologic differences from the intravitreal ET-1–induced chronic ischemic model of ONH atrophy\textsuperscript{14,15} used here, Wang’s results were consistent with our finding that the relationship between ONH MBR and ONH CBF was not significantly different in pigmented rabbits with chronic ischemia-induced ONH atrophy and normal albino or pigmented rabbits. Taken together, the results of the current and previous studies suggest that ONH MBR can be used to noninvasively and quantitatively estimate changes in circulatory conditions, irrespective of fundus pigmentation or changes related to ONH atrophy, and that ONH MBR values can be used to make interindividual and intergroup comparisons of circulatory conditions in the ONH tissue.

Earlier versions of the LSFG technology measured blood velocity with a quantitative index and NB, and they had a smaller image sensor (100 × 100 pixels) that could only measure a 0.42 × 0.42-mm area at a 30-degree angle of view and a 0.62 × 0.62-mm area at a 45-degree angle of view in the
eyes of rabbits.\textsuperscript{12,21} By contrast, ONH MBR is measured with a newer version of the LSFG technology that scans a much larger area covering the entire ONH (750 × 360 pixels).\textsuperscript{5,10} An earlier LSFG study used partial data (from an area of the ONH less than 0.1 × 0.1 mm) to show that the correlation of NB in ONH tissue and ONH CBF was not as high as ONH correlation between the change rate of ONH NB and ONH CBF.\textsuperscript{14} Although we measured an area similar to that in the previous report, we could easily select the most suitable area, that is, one free of surface vessels, because of the wide scanning ability of the newer LSFG devices. Furthermore, older LSFG versions did not have an ordinary CCD camera with eye-tracking capability and were thus unable to analyze the blur rate of the speckle field. Newer LSFG devices can also produce blood flow maps of the ocular fundus with good spatial resolution.\textsuperscript{22} Thus, the new version of LSFG measures a larger ONH area and reduces measurement error. We speculate that blood flow maps from the newer LSFG device will be less affected by regional variations, resulting in a better correlation between unprocessed MBR and CBF values in the ONH.

In principle, LSFG, including both the new and older versions, is affected by laser speckle signal bias due to the influence of laser beam reflectance and target tissue absorption. The reflectance of the retina is thought to be similar in different human ethnicities, although the absorption of the retinal pigment epithelial cell layer has been reported to differ depending on fundus pigmentation, with a reported absorption rate of approximately 5% in Caucasians and 30% in Africans.\textsuperscript{23} However, the fact that the ONH is a pigment-free tissue might explain our observation that ONH MBR was not affected by fundus pigmentation. It must therefore be noted that the results obtained here may not apply to MBR measurements obtained in pigmented tissues, such as the choroid.

The current study had several limitations related to its use of an animal model. The current findings for rabbit eyes cannot be directly applied to human eyes because of specific histologic and structural differences. Additionally, this study’s experimental animal model of chronic ischemia-induced ONH atrophy caused by repeated intravitreal ET-1 injection\textsuperscript{11,12} is histologically different from glaucomatous ONH atrophy or other forms of ONH atrophy in human eyes, which may limit the applicability of the current results to human subjects. Furthermore, although we assumed in our analysis that our LSFG and hydrogen gas clearance measurements represented blood flow in the same region of the ONH tissue, the measurements were in fact obtained from adjacent regions, not exactly the same area. Specifically, LSFG measures a square area of the ONH, while hydrogen gas clearance measures blood flow around the hydrogen electrode’s insertion point into the avascular zone of the ONH. However, we believe that the current data provide information that will be useful in interpreting ONH MBR measurement results in human eyes, as the invasive hydrogen gas clearance technique cannot be applied in human subjects.

In conclusion, we compared concurrently obtained LSFG measurements of ONH MBR and hydrogen gas clearance measurements of ONH CBF in a group of albino and pigmented rabbits, as well as a group of pigmented rabbits with chronic ischemia-induced ONH atrophy. We found that MBR and CBF measured in the same ONH simultaneously had a close, linear correlation, regardless of the presence of fundus pigmentation or ONH atrophy. Since fundus pigmentation or ONH atrophy had no significant effect on the relationship between MBR and CBF, MBR measurements in the ONH may be a useful way to perform interindividual and intergroup comparisons of circulatory conditions in the ONH.

Acknowledgments

We thank Takuhiro Yamaguchi, PhD, Department of Biostatistics, Tohoku University Graduate School of Medicine, Sendai, Japan, for his help with the statistical analysis.

This paper has not been presented previously.

Supported in part by a JST grant from Japan Society for the Promotion of Science KAKENHI (Tokyo, Japan) Grants-in-Aid for Scientific Research (B) Grant 26293372 (TN), Scientific Research (C) Grant 26462629 (HK), and Exploratory Research Grant 26670751 (TN).

The principal investigators, Naoko Aizawa and Fumihiko Nitta, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the analysis. The authors alone are responsible for the content and writing of the paper.

Disclosure: N. Aizawa, None; F. Nitta, None; H. Kunikata, None; T. Sugiyama, None; T. Ikeda, None; M. Araie, None; T. Nakazawa, None

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