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Reproducibility of Scanning Laser Doppler Flowmetry in the Rat Retina and Optic Nervehead

Akitaka Tsujikawa,* Junichi Kiryu,* Atsushi Nonaka,*
Kenji Yamashiro,* Yuichiro Ogura† and Yoshihito Honda*

*Department of Ophthalmology and Visual Sciences,
Kyoto University Graduate School of Medicine, Kyoto, Japan;
†Department of Ophthalmology, Nagoya City University Medical School, Nagoya, Japan

Purpose: The purpose of this study is to confirm the reliability of scanning laser Doppler flowmetry in the rat retina and optic nervehead, and the validity of measuring changes of retinal blood flow in rats while breathing 100% oxygen.

Methods: We used a commercially available scanning laser Doppler flowmeter. To ascertain reliability, five consecutive and separate perfusion measurements of 12 eyes of 12 anesthetized pigmented rats were performed. To evaluate the validity of the system, repeated measurements were taken in anesthetized rats breathing room air or 100% oxygen. This series of measurements was repeated three times.

Results: The reliability coefficients of volume, flow, and velocity in the optic nervehead and the retina ranged from 0.80 to 0.83 and 0.77 to 0.82, respectively. After the first exposure to oxygen, the measured values of volume, flow, and velocity were reduced by an average of 20.9–24.0%, 21.2–28.2%, and 19.5–24.5%, respectively. After the values returned to the basal condition, the second and third exposures to oxygen yielded measured values that were reduced by the same amounts as at the first exposure.

Conclusions: Scanning laser Doppler flowmetry provided relatively good reliability in measurements of blood flow in the rat retina and optic nervehead. This study has indicated the possibility of applying this system to the rat retina. **Jpn J Ophthalmol 2000;44:257–262**

Key Words: Blood flow, rat, retina, scanning laser Doppler flowmeter.

Introduction

Various methods have been developed to measure retinal blood flow in humans, including laser Doppler velocimetry,[1,2] laser Doppler flowmetry,[3] dye dilution technique,[4] blue field entoptic phenomenon,[5] laser speckle phenomenon,[6] and scanning laser angiography.[7] There are, however, many ethical and statistical limitations in using humans for research. Animal methods are another way to investigate the pathogenesis of diseases and innovative therapies. The results from animal studies should help bring about better understanding of the pathological processes in humans. Therefore, it would be valuable to establish one method that could be applied to both humans and animals. The rat is a familiar animal model in ophthalmological experiments. However, few methods are available to evaluate retinal blood flow in both human and rats other than the dye dilution technique.[8]

Scanning laser Doppler flowmetry[9] is a relatively new system to evaluate regional blood flow in the retina[10] and the optic nervehead,[11] noninvasively and repeatedly. In this system, laser Doppler flowmetry is applied to a scanning laser system. In many previous studies, relatively good reliability has been obtained in measurements of human retinal blood flow with this method,[10–12] but, at the same time, the limitations of this system have been discussed in many reports.[13–15] So far, scanning laser Doppler flowmetry has been applied to many patients, especially to

Received: May 6, 1999
Correspondence and reprint requests to: Junichi KIRYU, MD, Department of Ophthalmology and Visual Sciences, Kyoto University Graduate School of Medicine, Sakyo-ku, Kyoto 606-8507, Japan

Published by Elsevier Science Inc.
0021-5155/00/$–see front matter
PII S0021-5155(99)00226-9

Jpn J Ophthalmol
Vol 44: 257–262, 2000

glaucoma patients.[16–18] To our knowledge, few reports, however, have described the application of this system to animal research.[19] The purpose of this study is to evaluate the reliability of a commercial scanning laser Doppler flowmeter in the rat retina and optic nervehead and its validity by measuring changes of retinal blood flow in rats when breathing 100% oxygen.

Materials and Methods

Reliability

We used a commercially available scanning laser Doppler flowmeter (Heidelberg Engineering, Heidelberg, Germany). To determine the reliability of scanning laser Doppler flowmetry, we used male pigmented Long-Evans rats (200–250 g). Five consecutive and separate perfusion measurements of 12 eyes of 12 rats were performed. After the animals were anesthetized with intramuscular injection of xylazine hydrochloride (4 mg/kg) and ketamine hydrochloride (10 mg/kg), the pupils were dilated with 0.5% tropicamide and 2.5% phenylephrine hydrochloride eye drops. A contact lens was used to retain corneal clarity throughout the experiment. Each rat was placed on a stereotaxic platform in a prone position. The fundus of a rat was scanned with a scanning laser Doppler flowmeter in a 20° field around the optic nervehead and temporal to the optic disc. To compare several measurements from the same retinal location, we set the region of interest (ROI) squares of 10×10 pixels away from large visible vessels in the retina. Figure 1 shows the 4 ROIs used in this study. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

In general, the measured value combines the true mean value, the biological variability component, and the error component as indicated in Eq. (1).[10,11] We calculated the reliability coefficient $r1$ by estimating the variances of the biological component and the error component. The variances of the two components are defined in Eq. (2). The reliability coefficient $r1$ is defined in Eq. (3).

$$xij = \bar{x} + (\bar{x}i - \bar{x}) + (xij - \bar{x}i) = \mu + ai + eij \quad (1)$$

where xij = measurement value; $\bar{x}$ = mean of all measurement values; $\bar{x}i$ = mean of measurement values of each eye; μ = mean value; ai = biological component; eij = *measuring* error component; I = 1,..., n eyes; j = 1,...m measurements.

$$\Sigma\Sigma(xij - \bar{x})^2 = m(\bar{x}i - \bar{x})^2 + \Sigma\Sigma(xij - \bar{x}i)^2$$
$$ST = SA + SE \quad (2)$$

where ST = variance of measurement value; SA = variance of biological component; SE = variance of error component.

$$r1 = SA/(SA + SE) \quad (3)$$

Validity

To evaluate the validity of the system, we used 10 eyes of 10 male pigmented Long-Evans rats. After being anesthetized, their pupils were dilated with the same agents used in the reliability study. While the rats were breathing room air, their arterial blood pressure and heart rate were monitored with a blood pressure analyzer (IITC; Woodland Hill, CA, USA), and the fundus was scanned with a scanning laser Doppler flowmeter in a 20° field. Then the rat was exposed to 100% oxygen over 5 minutes in a closed cage, in which 100% oxygen flowed at a rate of 6 L per minute. Immediately after the rat was removed from the cage, within 10 seconds, the arterial blood pressure and heart rate were measured and the fun-

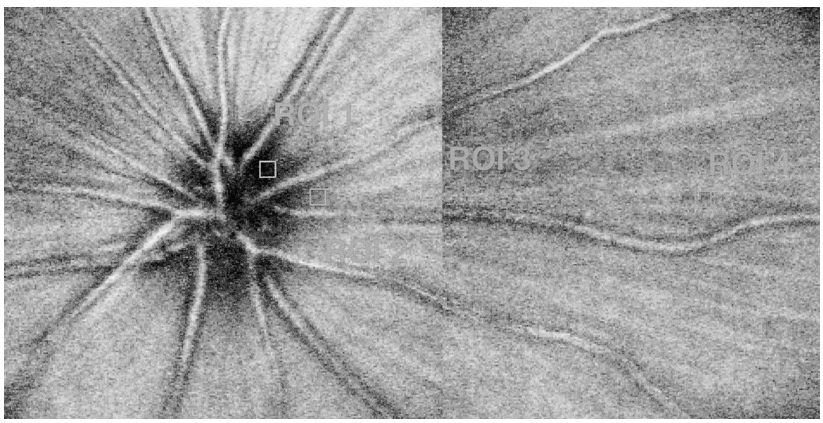

Figure 1. Fundus image of rat retina obtained with use of scanning laser Doppler flowmetry. Regions of interest (ROI) 1–4 represent areas of evaluation of blood flow (10×10 pixels). ROI 1 is on optic nervehead; ROI 2–4 are on retina; ROI 2 is at edge of optic nervehead; ROI 2 and 3 are at a distance of 1 or 3 disc diameters from edge of optic nervehead. All ROI are positioned to spare major retinal vessels.

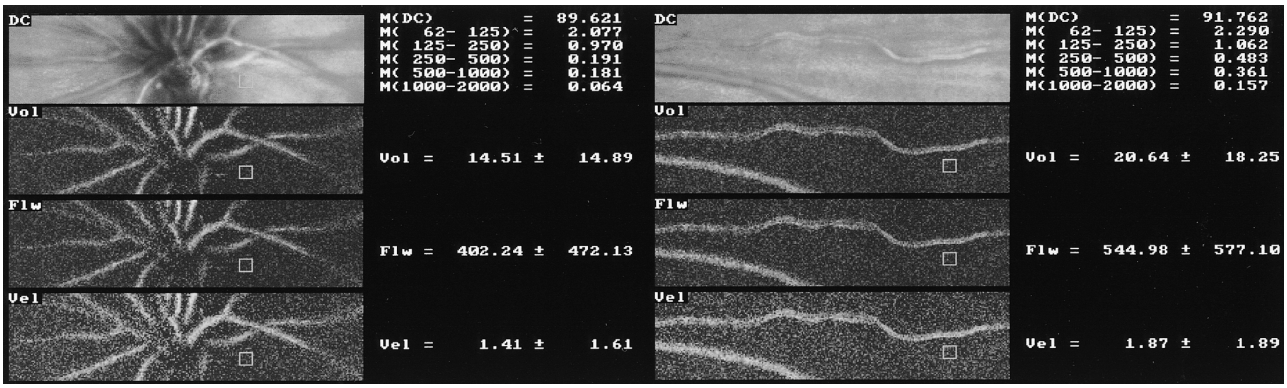

Figure 2. Perfusion map of rat retina. Major retinal vessels were identified with bright colors, because blood flow velocity is very fast. Left: cursor is positioned at region of interest (ROI) 2. Right: cursor is positioned at ROI 4.

dus was scanned with a scanning laser Doppler flowmeter. After the rat breathed room air for 5 minutes, the same measurements were performed. Each series of measurements was repeated three times.

After the experiment, we evaluated the blood flow in the same 4 ROIs mentioned. All values were presented as mean ± SD. Analysis of variance was used to compare measured values, with post-hoc comparisons tested using Scheffe's *F*- test. Differences were considered statistically significant at $P < .05$.

Results

Reliability

Figure 2 shows a perfusion map of a rat retina produced using scanning laser Doppler flowmetry. As the blood flow velocity in the major retinal vasculature was very fast, both retinal veins and arteries appear brightly colored. Table 1 shows the reliability coefficients *r*1 of volume, flow, and velocity for each ROI. The reliability coefficients *r*1 of volume, flow, and velocity of the optic nervehead (ROI 1), which were 0.80 to 0.83, were good. The reliability coefficients *r*1 of the retina (ROI 2, 3, and 4) ranged from 0.77 to 0.82. Most of them were relatively low compared with those of the optic nervehead (ROI 1), but some were not.

Validity

In the basal condition, measured values of volume, flow, and velocity of the optic nervehead (ROI 1) were 20.6 ± 2.6 arbitrary units (AU), 654 ± 124 AU, and 2.19 ± 0.40 AU, respectively. Measured values of volume, flow, and velocity of the retina (ROI 2–4), which were 16.4–21.7 AU, 513–670 AU, and 1.78–2.24 AU, were not uniform ($P = .0033$, $P = .027$, and $P = .032$, respectively). The blood flow of the retina was reduced near the optic disc. Measured values of volume, flow, and velocity of ROI 2 were reduced by 34.0%, 23.5%, and 20.3%, respectively, compared with those of ROI 4, in the normal condition.

Table 2 shows the heart rate and the mean arterial blood pressure of the rats during oxygen exposure. There was no significant change throughout the experiments. With oxygen exposure, measured values of volume, flow, and velocity were significantly reduced ($P < .001$) (Figure 3). After the first exposure to oxygen, the average reductions of measured values of volume, flow, and velocity were 20.9–24.0%, 21.2–28.2%, and 19.5–24.5%, respectively. While the rats were breathing room air after the first exposure to 100% oxygen, measured values returned to those of the basal condition (98.6–100.8%). At the second and third exposures to oxygen, measured values were reduced as much as at the first exposure (74.3–81.6%). While the rats were breathing room air, there were no significant changes of measured values compared with those of the basal condition.

Discussion

In this study, we investigated the reliability and the validity of applying a commercial scanning laser

Table 1. Reliability Coefficients *r*1 of Volume, Flow, and Velocity at Each Measurement Point

Region of Interest	Volume	Flow	Velocity
1	0.82	0.83	0.80
2	0.77	0.81	0.81
3	0.80	0.81	0.82
4	0.77	0.81	0.79

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Table 2. Heart Rate and Mean Arterial Blood Pressure of Rats During Oxygen Exposure

	Air	O_2	Air	O_2	Air	O_2	Air
Heart rate (beats/min)	285 ± 14	290 ± 21	278 ± 14	268 ± 9	290 ± 20	276 ± 19	282 ± 22
MABP (mm Hg)*	97.6 ± 19.7	100.4 ± 13.3	96.0 ± 16.1	90.4 ± 11.7	92.5 ± 14.6	99.5 ± 15.5	90.0 ± 14.6

*MABP = mean arterial blood pressure. There were no statistically significant changes in heart rate or MABP throughout experiment.

Doppler flowmeter to the rat retina and optic nervehead. In most previous studies, a scanning laser Doppler flowmetry measurement of the human fundus in a 10° field was reported to depict an area of about 0.7 mm × 2.7 mm.[17,18,20] In a perfusion map, 1 pixel represented about 10 μm.[9,21] Because the axial length of the rat eye is about one-fourth that of the human eye, the rat fundus is visualized at a higher magnification than that of the human eye. After measurements of blood flow, the rats were sacrificed with an anesthetic overdose, and their eyes were enucleated to calibrate areas of ROI on the perfusion map (pixel^2) with real areas on the retina (μm^2). In this study, in which the rat fundus was scanned with scanning laser Doppler flowmetry in a 20° field, 1 pixel in the perfusion map represented an area of about 5 μm.

Initially, Michelson and Schmauss[11] reported that the reliability coefficients of scanning laser Doppler flowmetry of the optic nervehead in healthy volunteers were 0.84–0.85. Many subsequent reports have indicated good reliability in applying scanning laser Doppler flowmetry to the juxtapapillary retina[13] and the macula.[12] Michelson et al[10] have reported that the reliability coefficients of the juxtapapillary retina were 0.81–0.83. In this study, reliability coefficients of the optic nervehead and retina were 0.80–0.83 and 0.77–0.82, respectively. Similar to previous investigations in humans,[10,11] measurements of the optic nervehead had a tendency to show higher reliability than those of the retina. While good reliability was demonstrated in applying scanning laser Doppler flowmetry to rats, the reliability coefficients were somewhat lower than those found in humans. Simi-

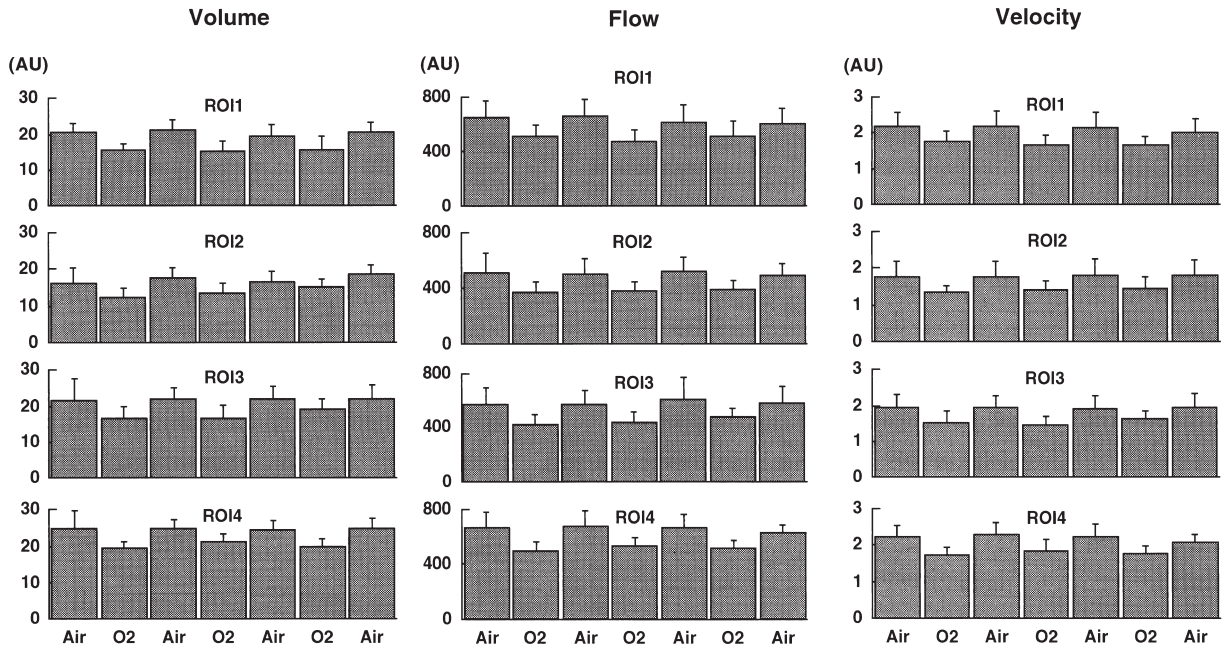

Figure 3. Changes of measurement variables (volume, flow, and velocity) of optic nervehead (region of interest [ROI] 1) and retina (ROI 2–4) during oxygen exposure.

lar to the finding of Michelson et al,[9,10,18] the reliability coefficient in this study was defined as the ratio of biological variation to the sum of biological variation and error variation. In human research, subjects have many biological variations due to sex, age, and physical characteristics. Conversely, the rats used in this study were so homologous that biological variability was minimal, which could account for the underestimation of the reliability coefficients.

In previous studies of healthy volunteers,[12,22] the changes of optic nerve or retinal blood flow during oxygen breathing detected with scanning laser Doppler flowmetry has been described. Langhans et al[22] have reported that blood flow in the optic nervehead and juxtapapillary retina was reduced by 33–37% with oxygen breathing in nonsmoking volunteers. Another study by Strenn et al[12] indicated that the reduction of macular blood flow was 29–33% during 100% oxygen breathing. In our study, the values of flow were reduced by 24% and 23–26% at the optic nervehead and retina, respectively, after oxygen breathing. The changes of the retinal blood flow in rat retina were relatively compatible with the results demonstrated in experiments with humans.[12,22] The reduction of blood flow during oxygen exposure, however, was relatively small, as compared with the values measured by other methods. With use of the dye dilution technique, Takagi et al[8] have reported that retinal blood flow in rats was reduced by 62% with 100% oxygen exposure for 15 minutes. While our results reflect regional blood flow in capillaries, they measured total retinal blood flow.[8] The changes of the total blood flow might not necessarily agree with the changes of the regional blood flow measured by a scanning laser Doppler flowmeter, because there are many anastomoses between retinal arteries and veins or between retinal vessels and choroidal vessels in the rat. Moreover, one limitation of scanning laser Doppler flowmetry may be the large zero off-set,[12] because it cannot be determined in vivo.[15] This off-set can cause an underestimation of the change compared with the basal condition, which could account for the relatively small changes detected with scanning laser Doppler flowmetry during oxygen breathing. This feature would indicate the need for caution in interpreting the results measured by scanning laser Doppler flowmetry.

The limitations in applying scanning laser Doppler flowmetry in humans have been discussed. When applying the method to rats, other problems may arise. Because scanning laser Doppler flowmetry scans with a repetition rate of 4,000 Hz, Doppler frequencies up to 2,000 Hz can be detected with reliability.[9,21] Although it has been reported that Doppler broadenings in the normal retina range from 300 Hz to 800 Hz,[3,10] larger Doppler shifts may occur due to retinal diseases or the administration of an agent. It may not be possible to measure retinal blood flow in all cases. Also, media opacity often degrades the images obtained with scanning laser Doppler flowmetry. To avoid drying of the corneas, the anesthetized rats need to wear a contact lens to retain corneal clarity throughout the experiment. Although the human subject may find fixation on one point difficult for more than 2 seconds without eye movement, fixation does not appear to be a problem in using anesthetized rats. However, the eye of the rat is so small that correction of the refractive error is needed. The ability of the scanning laser Doppler flowmeter is such that it can obtain measurements in eyes from −12 diopters to +12 diopters, which is sufficient in adult-sized rats.

Dye dilution technique,[4,8] which is the other available method to evaluate blood flow in both the rat and human retina, is too invasive to measure blood flow repeatedly. In contrast, scanning laser Doppler flowmetry can be used to perform repeated measurements within short or long time periods.[9,11] Although there may be limitations in applying scanning laser Doppler flowmetry to the rat retina,[12,15,21] this study has indicated the possibility of applying this system to the rat retina.

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan.

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