Measurement of Regional Choroidal Blood Flow in Rabbits and Monkeys Using Fluorescent Microspheres

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Objective: To develop a quantitative measure of regional variation in choroidal blood flow (ChBF).

Methods: Five million 15-µm fluorescent microspheres were injected into the left ventricles of 4 rabbits and 3 monkeys. The fixed globes were bleached, flat mounted, and photomicrographed. After image analysis to locate each microsphere, regional densities and blood flow were determined.

Results: Regional variation in ChBF was clearly evident. In the rabbit, a high density of spheres was seen in the visual streak. This was surrounded by a middle peripheral area of low sphere density and a far peripheral region of moderately high density. In the monkeys, sphere density was markedly greater in the macula compared with the periphery. Contour plots produced lines of constant flow that were oval and extended farther nasally than temporally in the monkeys. The ratio of central to peripheral ChBF was much greater in the monkeys than in the rabbits.

Conclusion: Quantitative assessment of regional ChBF can be performed using a modification of the fluorescent microsphere impaction method.

Clinical Relevance: This method of determining regional ChBF will be useful for studying the vascular effects of pharmacologic agents and for characterizing animal models of human disease involving the outer retina.


Choroidal Blood Flow (ChBF) accounts for 85% of the circulation to the eye.1-3 Knowing how it is affected by disease and pharmacologic intervention is critically important. However, measurement of ChBF is problematic because its vasculature is partially hidden from view by the overlying retinal pigment epithelium (RPE). Numerous methods have been developed, none of which is completely satisfactory for all experimental purposes. A brief review of some of these methods follows.

Total ChBF can be determined invasively by cutting the vortex veins and measuring output.4 A noninvasive method is color Doppler ultrasonic flowmetry of the ophthalmic artery,5,9 which serves as an estimate of ChBF given that 85% of its flow serves the choroid. Laser interferometry10-12 and laser Doppler flowmetry13 are also noninvasive techniques, but ChBF measurement is limited to the fovea. Radioactive krypton desaturation,14 hydrogen clearance,15 and calorimetric methods16 have also been used. Choroidal blood flow in localized areas has been estimated using fluorescein dye–filling curves17 and indocyanine green dye.18 A recently described novel means of studying ChBF in small portions of the choroid uses laser rupture of dye-laden liposomes.19,20

Except for direct measurement at the vortex veins, all of these methods are limited in that they are approximations of ChBF, show only relative blood flow, or look at selected small regions of the choroid. One additional, highly accurate means of determining blood flow is the use of arterially injected nonrecirculating labeled microspheres. The density of spheres in any given tissue is proportional to the blood flow through that tissue at the time of injection. An absolute blood flow determination can be made by simultaneously withdrawing a reference sample of arterial blood. Multiple injections using differentially labeled spheres is also possible, permitting the technique to be used to determine acute changes in blood flow in response to changes in various physiologic variables, such as blood pressure, oxygenation, and pharmacologic intervention.

The use of radiolabeled microspheres to measure blood flow in nonsurvival ex-
periments was introduced by Rudolph and Heymann in 1967. Since then, it has become the accepted standard for determining arterial flow to capillary beds in various organs, including the choroids of the cynomolgus (Macaca fascicularis) monkey1,2,3 and several other species. More recently, fluorescent microspheres have been used. In 1993, Austin et al and Glenny et al described quantitative comparisons of the 2 types of microspheres and found that they give similar results in terms of blood flow. The technique using fluorescent microspheres has since been shown to be at least as reliable as that using radiolabeled microspheres, and without the health and environmental risks or costs of tissue disposal and storage.

In the present study, we investigated the use of fluorescent microspheres to determine regional differences in blood flow of the choriocapillaris. Buckberg et al analyzed 7 areas of the choroid (mean±SD diameter, 15.5±0.42 µm) (Molecular Probes, Eugene, Ore) were injected in 23 seconds into the left ventricle via a catheter inserted into the right femoral artery of 4 New Zealand Black rabbits, a rhesus (Macaca mulatta) monkey, and 2 cynomolgus (M fascicularis) monkeys. Each animal’s left ventricular and aortic pressure was monitored during the injection procedure using a physiologic recorder (WindoGraf 930; Gould Instrument Systems, Valley View, Ohio). The stock concentration of microspheres was 1 million per milliliter, with excitation and emission peaks of either 505 and 515 nm, respectively (yellow-green fluorescent), or 580 and 650 nm (red fluorescent). The left femoral artery was also catheterized and used for obtaining reference blood samples. For the reference samples, a syringe pump (Harvard Apparatus Inc, Holliston, Mass) was fitted with a 10-ml syringe containing 0.6 ml of heparin sodium (1000 USP U/mL) and was used to aspirate the arterial blood at a rate of 1 ml/min for 2 minutes. The reference sample was withdrawn simultaneously with the fluorescent microsphere injection. Immediately after the fluorescent microsphere injection, a 3-ml volume of heparinized saline (20 U/ml in 0.9% sodium chloride solution) was injected to flush the fluorescent microspheres through the catheter. After aspiration, the syringe and the connecting catheter were rinsed twice with 3 ml of 2% Tween 80. The blood plus the rinse solution were deposited in a 50-ml plastic collection tube that was weighed before and after to determine the weight of the blood plus rinse solution. The arterial blood in the catheter was drawn into the reference sample syringe, and the contents were...

METHODS

MICROSPHERE INJECTION

Institutional guidelines regarding animal experimentation were followed. Five million fluorescent polystyrene microspheres (mean±SD diameter, 15.3±0.42 µm) (Molecular Probes, Eugene, Ore) were injected in 23 seconds into the left ventricle of a rabbit through a catheter inserted into the right femoral artery of 4 New Zealand Black rabbits, a rhesus (Macaca mulatta) monkey, and 2 cynomolgus (M fascicularis) monkeys. Each animal’s left ventricular and aortic pressure was monitored during the injection procedure using a physiologic recorder (WindoGraf 930; Gould Instrument Systems, Valley View, Ohio). The stock concentration of microspheres was 1 million per milliliter, with excitation and emission peaks of either 505 and 515 nm, respectively (yellow-green fluorescent), or 580 and 650 nm (red fluorescent). The left femoral artery was also catheterized and used for obtaining reference blood samples. For the reference samples, a syringe pump (Harvard Apparatus Inc, Holliston, Mass) was fitted with a 10-ml syringe containing 0.6 ml of heparin sodium (1000 USP U/mL) and was used to aspirate the arterial blood at a rate of 1 ml/min for 2 minutes. The reference sample was withdrawn simultaneously with the fluorescent microsphere injection. Immediately after the fluorescent microsphere injection, a 3-ml volume of heparinized saline (20 U/ml in 0.9% sodium chloride solution) was injected to flush the fluorescent microspheres through the catheter. After aspiration, the syringe and the connecting catheter were rinsed twice with 3 ml of 2% Tween 80. The blood plus the rinse solution were deposited in a 50-ml plastic collection tube that was weighed before and after to determine the weight of the blood plus rinse solution. The arterial blood in the catheter was drawn into the reference sample syringe, and the contents were...
deposited in a 50-mL conical polypropylene centrifuge tube. The syringe was then rinsed twice as described previously herein.

GLOBE PREPARATION

The animals were humanely killed and their eyes were enucleated, placed in 4% paraformaldehyde overnight at 4°C, and stored in 0.1M phosphate buffer (pH 7.6) at 4°C. After the anterior segments of the eyes were removed, the retinas were carefully dissected away from the underlying RPE and choroid. A 1.5-mm trephine was then used to free the retina at the optic nerve. The anterior cut edge of the RPE/choroid was then tacked to the underlying sclera with cyanoacrylate glue. This last step was done to help prevent shifting of the RPE/choroid when it was being flattened (see later in this article). The posterior poles were incubated in 0.25% potassium permanganate at 37°C for 30 to 60 minutes. They were then placed in 5% oxalic acid for 5 minutes, flattened with 8 radial cuts, laid out on a layer of nonhardening modeling clay (sclera against the clay), covered with 2.5% hydroxypropylmethylcellulose, and pressed between 2 glass slides. Contact tape was wrapped around the 2 ends of the slides to hold them together.

ChBF DETERMINATION

The total number of microspheres in the samples was calculated by manually counting the spheres in 40-µL aliquot samples from the well-agitated (by means of sonication and vortexing) collection tube. The aliquots were placed on large microscope slides and coverslipped. An epifluorescence microscope (Olympus BH2; Olympus America Inc, Melville, NY) was used to visualize the spheres. A sufficient number of aliquots were used so that at least 400 microspheres were counted per sample. Assuming that the liquid in the collection tube was 1 mL/g, the total number of microspheres in the reference sample (S_r) was calculated as

$$S_r = S_a (V_a/V_L)$$

where S_a is the total number of microspheres in the aliquots, V_a is the volume of liquid in the collection tube, and V_L is the total volume of all of the aliquots used for counting.

The microspheres in the flattened and bleached globes were photographed using a digital camera attached to an epifluorescence microscope. Approximately 50 micrographs were needed to image the entire specimen using a ×2 objective lens. A single high-resolution image composed of the 50 micrographs was produced using Adobe Photoshop 7.0 (Adobe Systems Inc, San Jose, Calif.). The cartesian coordinates of each microsphere were then determined using NIH ImageJ v1.31 (National Institutes of Health, Bethesda, Md). Approximately half of the total number of spheres could be detected automatically using ImageJ, and the remaining half had to be identified manually (because they were touching other spheres) using the ImageJ crosshair tool. The data were processed using a customized program in MATLAB v6.5.1 (The MathWorks, Natick, Mass), written by one of us (J.N.V.H.), which tabulates and plots the number of microspheres in bins of any size (0.25-mm² bins worked well for this study). Blood flow was calculated using the following formula:

$$\text{ChBF} = R(S_r/S_a)$$

where R is the rate at which blood was withdrawn for the reference sample, in microliters per minute; S_r is the number of spheres per square millimeter of tissue; and S_a is the total number of spheres in the reference sample. These values were then imported into SigmaPlot 2001 (SPSS Inc, Chicago, Ill) along with the cartesian coordinates of the bins. A 3-dimensional smoothing function was used (locally weighted scatterplot smooth [loess from the SigmaPlot 2001 program]), fourth-order polynomial) to produce a contour plot of blood flow in microliters per minute per square millimeter.

UNITS OF MEASURE

In the literature, ChBF is often expressed in milligrams per minute, which is a shorthand notation for milligrams per minute per tissue. In other words, the total blood flow through the choroid would be measured in milligrams per minute per choroid. In this article, we assume that the specific gravity of blood is approximately 1.0 and thus substitute microliters for milligrams. Also, we are interested in regional variations in blood flow in what is essentially a 2-dimensional capillary bed (the choriocapillaris). Dividing by area, the units then become microliters per minute per square millimeter. Flow measured in this way is sometimes referred to as flux density, or volume of a fluid per time through a given area.

RESULTS

The Table gives the total ChBF of each eye tested. Although variable among animals, these flows were similar to those reported by other researchers in rabbits and monkeys using nonrecirculating microspheres (see the “Comment” section).

Figure 1 illustrates the appearance of the posterior pole of a rabbit eye (rabbit 4) after it had been bleached.

Table. Characteristics of the 7 Study Animals

<table>
<thead>
<tr>
<th>Studied Eye</th>
<th>Animal Weight, kg</th>
<th>Spheres in Choroid, No.</th>
<th>Spheres in Reference Sample, No.*</th>
<th>Total ChBF, µL/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit 1 Left</td>
<td>3.2</td>
<td>7687</td>
<td>21966</td>
<td>350</td>
</tr>
<tr>
<td>Rabbit 2 Left</td>
<td>3.5</td>
<td>8275</td>
<td>19327</td>
<td>428</td>
</tr>
<tr>
<td>Rabbit 3 Left</td>
<td>3.8</td>
<td>7361</td>
<td>14238</td>
<td>517</td>
</tr>
<tr>
<td>Rabbit 4 Left</td>
<td>3.0</td>
<td>22305</td>
<td>30975</td>
<td>720</td>
</tr>
<tr>
<td>Rhesus 1 Left</td>
<td>9.7</td>
<td>2148</td>
<td>3582</td>
<td>600</td>
</tr>
<tr>
<td>Cynomolgus 1 Right</td>
<td>2.9</td>
<td>4761</td>
<td>5699†</td>
<td>835</td>
</tr>
<tr>
<td>Cynomolgus 1 Left</td>
<td>2.9</td>
<td>5466</td>
<td>5699†</td>
<td>959</td>
</tr>
<tr>
<td>Cynomolgus 2 Left</td>
<td>3.0</td>
<td>10474</td>
<td>8208</td>
<td>1276</td>
</tr>
</tbody>
</table>

Abbreviation: ChBF, choroidal blood flow.

*Calculated total based on counts of multiple 40-µL samples.
†Same reference sample used in ChBF calculations for the right and left eyes.
and flattened between 2 glass slides. The retina had been removed before bleaching. The reddish hue corresponds to residual blood in the choroid. Some choroidal pigment remains in the periphery. Just below (ventral) to the optic nerve (white oval) is the visual streak, in which the pigment around the short posterior ciliary arteries can be seen (small dots). The background shading was digitally created to improve contrast with tissue.

Once photographed using an epifluorescence microscope, the cartesian coordinates of each individual sphere were determined in a semi-automated manner (see the “Methods” section) and plotted (Figure 3). By simple inspection of these plots, it was evident that there was a much greater density of microspheres in the portion of the eye corresponding to the visual streak in all of the rabbit eyes. Further quantitative analysis involved sorting the data into either 0.25- or 1-mm² bins (Figure 4). Blood flow corresponding to each bin was calculated according to the equation $\text{ChBF} = R(S_c/S_r)$ (see the “Methods” section). These data were then smoothed and re-plotted as described previously. No attempt was made to compensate for the missing areas produced by the radial cuts, which gave the smoothed graphs a lumpy appearance in the periphery (Figure 5 and Figure 6). From the smoothed plots, it was apparent that in addi-
tion to the higher blood flow in the visual streak, there were 2 areas of greatly lower blood flow corresponding to the choroid just dorsal to and just ventral to the visual streak. Blood flow was also found to be greater toward the far periphery everywhere except directly dorsal to the optic nerve.

Contour plots using lines of equal ChBF showed that the high- and low-flow areas differed by a factor of approximately 3. The features of these plots were relatively insensitive to the size of the bins chosen (Figure 7). The monkeys (rhesus and cynomolgus) showed a much different pattern of blood flow than was the case for the rabbits. In these animals, there was a marked concentration of microspheres in the central macula and a much reduced density in the periphery (cynomolgus 2) (Figure 8). This was especially evident in the smoothed plots (Figure 9). Examination of the contour plots revealed that the lines representing equal ChBF were not simply concentric circles but...
rather fell off more slowly in the nasal retina (corresponding to the temporal visual field) than they did in the temporal retina (nasal visual field) (Figure 10). In contrast to the rabbit, the high- and low-flow regions differed by a factor of as much as 13.

**COMMENT**

Regional variations in ChBF can be identified and analyzed quantitatively using this new method of detecting the distribution of nonrecirculating fluorescent microspheres. As noted early in this article, it is theoretically possible to accomplish such an analysis by dividing the choroid into numerous small pieces and determining the number of microspheres in each piece (using a scintillation counter for radiolabeled microspheres or a spectrofluorophotometer/particle sorter for fluorescent microspheres). However, this has yet to be done for more than 7 pieces, presumably because it would be difficult to cut the tissue into equal-sized pieces on the order of 0.25 or 1 mm². Also, such a method would be unwieldy. For example, the MATLAB analysis performed for rabbit 4 mathematically divided the choroid into 1950 bins (Figure 4). Having to physically process each piece separately, even with some sort of automation, would be untenable. Furthermore, reanalysis, such as that shown in Figure 7 using different bin sizes, is not possible once the tissue is physically sectioned.

Dividing the choroid into so many bins results in fewer than the traditional 400 microspheres per sample. Nevertheless, use of the loess curve-smoothing function compensates for this because each smoothed value is determined by neighboring bins within a defined span. In other words, several bins are combined in a weighted manner to produce each point on the curve. Of course, 400 microspheres per sample is not an absolute limit. It is only the level at which the statistical variability from random distribution is approximately 10%. Lower numbers are acceptable if the differences to be detected are greater than 10%, which is the case with ChBF because the re-
Regional variations are approximately several hundred percent. Finally, even if 400 were used as a strict limit, it would still be possible to divide a choroid such as that shown in Figure 2 into 55 segments (albeit of different sizes), each with 400 spheres.

The 15-µm microspheres used in this study represent a fairly standard size for investigating ChBF. There is probably a range of sizes that could be used that would give a similar result in terms of the calculated blood flow. The choice has to do with finding the optimal combination of size and total number of spheres injected for any given capillary bed. Large spheres are almost all entrapped but block the larger precapillary arterioles, which causes systemic effects if injected in large numbers. Because they block larger vessels, the initial microspheres to reach a capillary bed block subsequent spheres, thus giving an artifactually low value for blood flow. Small spheres have the advantage that they can be injected in larger numbers (increasing precision), presumably because they do not affect certain capillary beds needed for maintaining cardiovascular stability. If they are too small, the spheres will escape entrapment from the capillary bed of interest, thus giving a value that is below the actual blood flow. Buckberg et al\textsuperscript{35} found little difference in calculated blood flow when they compared microspheres in the 8- to 50-µm range. Specifically addressing the issue of sphere size and calculated blood flow in the eye, Alm et al\textsuperscript{23} performed simultaneous injections of 2 different-sized microspheres (each size with distinctive radio-labels). They found that for the choroid in rabbits, the mean ± SD ratio of 15-µm microspheres to 9-µm microspheres was 1.06 ± 0.02. Similarly, the mean ± SD ratio of 35-µm microspheres to 15-µm microspheres in cynomolgus monkey choroid was 1.02 ± 0.07. In other words, the diameter of the microspheres in the studied range made little difference with respect to calculated ChBF, but size was important for other capillary beds in the eye. The 15:9-µm ratios were more than 2 for the iris and ciliary body in rabbits. The 35:15-µm ratio was less than 1 for the retina and iris in the monkey. They concluded that for the choroid at least, 15 µm provided a reasonable balance between precision and accuracy. The choroid is a high-flow system. In the present study, we injected enough of the 15-µm microspheres to achieve a good statistical sampling but not so many as to affect the cardiovascular stability of the animals.

Total blood flow through the choroid varied considerably among animals (Table). However, such variability has been well described. Alm and Bill,\textsuperscript{24} using the microsphere method for whole choroids, noted that blood flows differed by as much as a factor of 3.5 among cats with similar perfusion pressures. (As in the present study, they carefully monitored the animals’ physiologic variables.) Similarly, Stjernschantz et al\textsuperscript{34} found ChBF that differed by a factor of 4 in rabbits (excluding an outlier animal that would raise the factor to nearly 7). Despite such marked differences in flow among animals, measurement of ChBF has proved useful in part because the fluctuation in flow between eyes and between multiple injections in the same eye is considerably less dramatic (eg, see cynomolgus 1, right and left eyes, in Table).

Although increased ChBF in the visual streak of rabbits and in the macula of monkeys has been observed (at least subjectively) by other researchers, this new method has revealed at least 2 previously undescribed features of ChBF. In the rabbit, there is a region of low flow just ventral to the visual streak centrally and also dorsal to the visual streak above the optic nerve. However, in the other areas of the periphery, including along the axis of the visual streak itself, there is a relative increase in flow. The highest flows are at the nasal and temporal periphery, not at the center of the visual streak. Just why this is so is not obvious. One might speculate that because the rabbit is a prey animal, peripheral vision is more important than central vision. Of course, high ChBF may or may not correlate with good vision. The concentrations of short-wavelength-sensitive cones (S-cones or blue cones) are greatest in the inferior periphery of the rabbit eye.\textsuperscript{37, 40}

The other previously undescribed feature of regional ChBF is in the monkey. The contour lines of equal ChBF reach a peak in the central macula, as has previously been shown either subjectively by microsphere impaction or by limited sampling. However, these lines are not radially symmetrical. Instead, they have an increasingly oval shape toward the periphery, extending more nasally than temporally, which would correspond to the temporal and nasal visual fields, respectively. Unlike in rabbits, the peripheral visual acuity in monkeys is known from behavioral testing and is remarkably similar to that seen in humans,\textsuperscript{40} at least in the central 30°. In humans, the nasal peripheral lines of equal light sensitivity (isopters) of standard Goldmann perimetry extend farther for the nasal field than for the temporal field. Morphologically, similar radial asymmetry of the cone photoreceptors has been found in monkey\textsuperscript{30, 51} and human\textsuperscript{51, 52} retinas. Another interesting feature of the ChBF in monkeys is the much greater range (by a factor of as much as 13) between the central macula and the periphery compared with the rabbit (by a factor of 3) even though total circulation may be similar (see previously in this article).

A priori, it is not necessarily the case that regional variations in ChBF should reflect visual sensitivity as measured in photopic conditions. Mammalian retinas are duplex, containing rods and cones that each have different physiologic characteristics and functions. Furthermore, these characteristics change with light adaptation, the rods being more metabolically active in dark adaptation.\textsuperscript{35, 54} Therefore, is ChBF a reflection more of the cone retina or of the rod retina? One might guess that the ChBF variation should follow the variations in oxygen consumption of the outer retina. Although extensive regional studies of comparative outer retinal oxygen consumption have not been performed, Yu and Cringle\textsuperscript{55} did a 2-point comparison using oxygen-sensitive electrodes in rabbits. They found that the oxygen demands of the outer retina were greater in the visual streak than in the vascularized portion of the retina just dorsal to the visual streak (where we found a nadir in ChBF). However, this simple relationship may not hold in the monkey. Ahmed et al\textsuperscript{36} did a similar 2-point analysis in a pig-tailed macaque (Macaca nemestrina) in which they looked at outer retinal oxygen consumption in the fovea compared with the parafoveal region (about halfway between the fovea and the
Correspondence: The effects of variations in ChBF among the animals studied. Using this method, it may be possible to study regional variations in ChBF in response to the light-dark cycle. The effects of drugs and animal models of retinal disease on regional ChBF may, for the first time, be measurable as well.

Submitted for Publication: May 15, 2005; final revision received September 7, 2005; accepted October 23, 2005.

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Financial Disclosure: None.

Funding/Support: This study was supported by grants RO1 EY014041 (Dr Nork) and P30 EY016665 from the National Eye Institute, National Institutes of Health; the Walter H. Helmerich Chair (Dr Nork); Research to Prevent Blindness; the Retina Research Foundation; and the Wisconsin Lions.

Acknowledgment: We thank Carrie Buenger and Derrick Woodward for providing technical assistance.

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