Retinal Blood Flow Changes in Patients With Insulin-Dependent Diabetes Mellitus and No Diabetic Retinopathy

A Video Fluorescein Angiography Study

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Purpose. The authors investigated retinal blood flow changes in patients with insulin-dependent diabetes mellitus (IDDM) and no diabetic retinopathy compared to age-matched subjects without diabetes. They also investigated whether blood glucose levels could modulate retinal blood flow in these patients with diabetes and whether this modulation would impact retinal blood flow data used in cross-sectional studies assessing changes in retinal blood flow.

Methods. Retinal blood flow was measured using video fluorescein angiography, and blood glucose levels were manipulated using glucose clamp methodologies with continuous basal insulin replacement. Blood glucose levels were clamped at 100, 200, and 300 mg/dl. Retinal blood flow measurements were performed at each blood glucose level after subjects had been stabilized for an hour at each of the different blood glucose levels.

Results. Retinal blood flow was found to be significantly decreased ($P < 0.01$) in the group of patients with no diabetic retinopathy (19.4 ± 4.6 arbitrary units [AU]) compared to retinal blood flow in subjects without diabetes (28.7 ± 6.4 AU). During glucose clamp adjustment of blood glucose levels, it was found that as blood glucose levels were increased from euglycemia (100 mg/dl) to 200 mg/dl and to 300 mg/dl, retinal blood flow was significantly increased at the 200 mg/dl level (21.5 ± 4.7 AU, $P < 0.05$) and at the 300 md/dl level (25.9 ± 8.8 AU, $P < 0.01$) compared to the 100 mg/dl level (16.3 ± 3.8 AU). In addition, the retinal blood flow at the 100 and 200 mg/dl levels was significantly reduced ($P < 0.01$) compared to nondiabetic retinal blood flow (28.7 ± 6.4 AU).

Conclusions. Retinal blood flow was found to be decreased in patients with IDDM with no diabetic retinopathy, and acute elevations in blood glucose levels resulted in increased retinal blood flow in these patients. The acute modulation of retinal blood flow by blood glucose levels should be considered in cross-sectional studies investigating retinal blood flow changes in patients with diabetes. The results from this study indicate that if blood glucose levels are not accounted for in the analyses, larger populations would have to be studied to demonstrate statistically significant differences between groups with and without diabetes. Invest Ophthalmol Vis Sci. 1996;37:886-897.

Abnormalities in retinal circulation and its regulation have been shown to occur in diabetes mellitus.[1-8] Results from clinical studies[1,2,7,9] have shown that retinal blood flow increases once diabetic retinopathy has reached nonproliferative and more advanced diabetic retinopathy levels and that, after panretinal laser photocoagulation, blood flow decreases. In addition, it has been shown that the regulatory response of the retina to hyperoxia is significantly blunted in patients with diabetes compared to subjects without diabetes.[10] The studies in hyperoxia also demonstrated a weak negative correlation between the magnitude of the retinal hyperoxic autoregulatory response and blood glucose levels.[11]

More recently, studies[12-15] have begun to focus on the mechanisms responsible for these changes, especially in the early stages of diabetes before the development of clinically overt pathology. With the increasing

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emphasis on investigating mechanisms associated with the development of diabetic retinopathy, a noninvasive method for quantitating changes in retinal physiology at the earliest stages of diabetes becomes essential for the rapid assessment of potential therapeutic agents to ameliorate the risk for developing diabetic retinopathy. We have used a video fluorescein angiography (VFA) system coupled with computerized image analysis to quantitate retinal blood flow changes in diabetes.[16-18] An important factor to consider in clinical studies is the level of blood glucose at the time of the blood flow measurements. Indeed, a previous study[11] has shown that acute reductions in plasma glucose levels after insulin injection significantly lowered retinal blood flow in patients with diabetes with no diabetic retinopathy or with nonproliferative diabetic retinopathy. Another study[19] performed by the same investigators showed that, after 5 days of intensive insulin treatment, retinal blood flow was reduced significantly in those eyes that showed no progression of diabetic retinopathy during the 6-month study period. All patients in this study also had nonproliferative diabetic retinopathy.

The current studies were aimed at investigating retinal blood flow changes in the early stages of diabetes, as well how acute changes in blood glucose levels modulate retinal blood flow in patients with diabetes before the appearance of diabetic retinopathy. If the level of blood glucose at the time of measurement has a significant effect on retinal blood flow, blood glucose levels would contribute to the variability of the retinal blood flow measurements and would reduce the sensitivity for detecting retinal blood flow changes secondary to the effects of potential therapeutic agents. In this study, blood glucose levels were manipulated using the glucose clamp methodology,[20] and retinal blood flow was measured using VFA techniques.[16]

METHODS

Patient Population

Patients with type I diabetes mellitus were recruited from the Joslin Diabetes Center population, and informed consent was obtained from all subjects before participation in the study. Study protocols conformed to the tenets of the Declaration of Helsinki and were approved by the Joslin Diabetes Center Review Board. All study patients underwent visual function testing, slit lamp biomicroscopy with intraocular pressure measurements followed by pupil dilation, and indirect ophthalmoscopy. Stereoscopic retinal fundus photographs were obtained from both eyes according to the ETDRS protocol by an ETDRS-certified ophthalmic photographer. Retinal photographs were graded in a masked manner by two independent graders using the ETDRS grading protocol. All patients with diabetes enrolled in this study were graded as having ETDRS level 10 retinopathy (no diabetic retinopathy). Patients with diabetes also conformed to additional study eligibility requirements: They had to be between 18 and 45 years of age, duration of diabetes had to be less than 10 years, they could have no microalbuminurea, and they had to be ocularly and systemically normotensive without medication.

Twelve patients were enrolled in the glucose clamp study group. Glucose clamp study patients were asked to return for repeat VFA measurements to ensure that the retinal blood flow changes measured during the glucose clamp procedure were related to the glucose level changes rather than possible nonspecific effects related to the mechanics of the glucose clamp protocol. Seven patients returned for repeat retinal blood flow measurements within 3 months of completing their glucose clamp protocol. These seven patients were included in a separate study population referred to as the Random Diabetic Patient group, as were five additional patients with diabetes who met the above eligibility requirements and who were selected sequentially from those undergoing regular retinal blood flow measurements during the same period. This group comprised a group with diabetes that was used for comparison with subjects with and without diabetes. The additional patients were included to provide the same number of patients as in the glucose clamp study group. A third study group included 12 age-matched subjects without diabetes who were undergoing retinal blood flow measurements during the same period.

Instrumentation

Retinal hemodynamic parameters were determined from video recordings of fluorescein dye passage through the retinal circulation. The VFA system used for these measurements has been described previously.[16,17] Briefly, the system consists of a Nikon (Tokyo, Japan) NFC-50 fundus camera connected by a C-mount adapter to a Dage–MTI (Michigan City, IN) silicon-intensified target video camera, which is a low light level-sensitive, high-resolution camera system. The video camera output was directly digitized at 30 frames/second and stored using a Recognition Concepts (Carson City, NV) TRAPIX Plus/DataStore system. In addition, the analog video signal was recorded on a Sony (Park Ridge, NJ) U-Matic video tape recorder for archival purposes. The TRAPIX system could store 20,000 digitized images or approximately 11 minutes of real time fluorescein angiogram recordings. This was sufficient capacity to store all the recorded images from the multiple angiograms performed during the glucose clamp protocol. The digitized images were analyzed on a frame-by-frame basis using a Digital Equipment Corporation (Maynard,

MA) VAX 4000/300 computer to determine the retinal circulatory parameters of interest.

Glucose Clamp Study Protocols

The glucose clamp protocol followed for this study was the same as that described by DeFronzo et al[20] and Wolpert et al.[21] Patients arrived on the morning of the study after an overnight fast. A catheter was inserted into the antecubital vein of each patient's arm. The catheter was fitted with a 2-port stopcock. One port was used for the continuous basal infusion of insulin at a rate of 0.2 mU/kg·minute, and the second port was used for the continuous infusion of a 20% dextrose solution delivered at an adjustable rate. The withdrawal port for this catheter was used for the introduction of the bolus injection of sodium fluorescein dye performed during the VFA procedure for retinal blood flow measurements.

A retrograde catheter was inserted into a wrist vein, and the catheterized hand was placed in a hot box at a temperature of 140°F. This provided an arterialized venous blood sample for blood glucose measurements. Blood samples were obtained from this catheter every 5 minutes. The sample was centrifuged, and plasma glucose levels were measured using a Beckman Glucose Analyzer (Beckman Instruments, Fullerton, CA). Adjustments were made to the glucose infusion rate as needed.

The average blood glucose level for the patient group without diabetes at entry to the study was 201.7 ± 51.3 mg/dl. At the basal infusion rate of insulin used here, it took an average of 87 ± 40 minutes to reach a blood glucose level of 100 mg/dl. Patients were maintained at the 100 mg/dl blood glucose level for a period of 60 minutes, after which a video fluorescein angiogram recording was obtained for retinal blood flow determinations. After completion of the VFA, the blood glucose level was elevated to and maintained at 200 mg/dl for 1 hour. At the end of this time, a second VFA recording was obtained, and the blood glucose level was then elevated to and maintained at the 300 mg/dl level for another hour, after which a final VFA recording was obtained. The transition between one glucose level and the next took no more than 10 minutes. At the conclusion of the study, patients' blood glucose levels were returned to normal.

For each hyperglycemic step, the concentration of free insulin was measured from blood samples using a standard double antibody radioimmunoassay. The average insulin levels were not statistically different at any of the blood glucose levels (24.1 ± 9.9 μU/ml at the 100 mg/dl glucose level, 22.1 ± 9.4 μU/ml at the 200 mg/dl level, and 21.7 ± 9.1 μU/ml at the 300 mg/dl level).

Random Retinal Blood Flow Measurements

After routine ophthalmologic examination and retinal fundus photography, all subjects were asked to provide a urine sample for microalbuminurea determination. They were then seated in front of the VFA system, and blood pressure readings were obtained. A catheter was then inserted into the antecubital vein, and blood samples were withdrawn for blood glucose and hemoglobin A1 assays. Patients then positioned themselves in the chin rest of the VFA system retinal fundus camera. A focused 30° image of the retina was obtained with both the disc and macula in the field of view. A short video segment was recorded using the blue excitation light illumination. These images provided the template for the subsequent analysis of recorded video fluorescein angiograms and vessel diameter determinations. Image acquisition and storage sequence were started after insertion of the fluorescein angiography barrier filter into the observation light path and after a rapid (1-second duration) bolus injection of 0.75 ml of 10% sodium fluorescein into the antecubital vein using the arm vein catheter. The rapid injection and small bolus volume of fluorescein dye ensured a sharp dye front in the retinal vasculature. Recorded images were digitized and stored for subsequent analysis. All retinal analyses were performed from the left eye in these studies.

Data Analysis

Digitized and stored VFA images were analyzed densitometrically on a frame-by-frame basis. Sample sites were chosen using primary temporal retinal vessels at a fixed (1.5 disc diameters) radial distance from the center of the optic disc. Measurements were typically made from both the superior and the inferior temporal retinal arteries and veins. At these sites, artery and vein diameters are in the 100- to 200-μm range.

Vessel diameters were determined from images recorded before fluorescein injection at the defined fixed retinal vessel sites. The diameter of each vessel was calculated in units of pixels (video elements) by locating the edge of the vessel from the measured optical density profile using a boundary-crossing algorithm. The difference between half-heights of the optical density profile across the vessel yielded the vessel width at that site. Each vessel width could be measured to within ±0.5 pixels. Vessel diameter measurements were calculated as the average diameter from four distinct sites within a 10-pixel length of the vessel of interest. Average retinal artery and vein vessel diameters calculated for each eye represented the average of the individual diameters of both the superior and the inferior temporal vessels.

Retinal vessel fluorescent intensities were measured from the fixed vessel sites using a measuring window defined by the width of the vessel at that site.

Vessel fluorescence intensity levels (proportional to the computer-generated gray level ranging between 0 and 255) were determined for each VFA frame starting 2 seconds before the appearance of dye in the retinal arteries and ending when recirculation of the dye was complete (approximately 30 seconds). Average retinal artery and vein fluorescence intensities within these sample site windows were determined on a frame-by-frame basis to provide dye dilution curves for each retinal vessel. Changes in fluorescence intensity with time or dye dilution curves are illustrated in Figure 1. For illustration purposes, vessel fluorescences from every fourth frame were plotted; however, analyses used the data from every frame. Retinal artery and vein pair dye dilution curves are shown, after a 0.75-ml bolus injection of 10% fluorescein dye, from a subject without diabetes (Fig. 1a) and a patient with diabetes (Fig. 1b).

The resultant fluorescence data were fit to a lognormal distribution function[22,23] using a least-squares fit algorithm. The lognormal distribution function used here has the functional form:

$$I = I_0 + I_P \exp(-\beta \ln^2(t/t_P))$$

where I_0 represents the background vessel intensity, I_P represents the peak fluorescence intensity, β represents the coefficient of the lognormal exponent and describes the shape of the curve, t_P represents the time at which the peak fluorescence intensity occurred, and ln represents the natural logarithm. The mean retinal vessel circulation time, t_m, is defined as:

$$t_m = t_P \exp(3/4\beta)$$

and the difference in retinal artery and vein mean circulation times defines the retinal mean circulation time (MCT). In Figure 1, the solid lines drawn through the data points represent the lognormal distribution function fit to the data up to the point at which recirculation becomes apparent. Parameters from these fits were used to determine mean vessel circulation times (vertical lines) and the MCT.

Examination of the measured dye dilution curves from the retinal vessels in Figure 1 illustrate the sharply peaked artery and vein dye fluorescence curves from subjects with and without diabetes. In general, recirculation begins to have an effect on the first-passage clearance of dye approximately 10 seconds after the peak fluorescence time. This provides approximately 300 data points for fitting the clearance phase of the dye dilution curve. In this study, the resultant fits to the retinal vessel dye dilution curves were all statistically significant ($P < .0001$; $r^2 > 0.9$). Thus, the well-defined dye dilution curves following the rapid small volume bolus injection of the dye ensures an accurate determination of mean vessel circulation times.

An analysis was performed to see how the coefficient β of the lognormal distribution function affected the determination of mean vessel circulation times. From the relationship above, in the limit as β becomes large, we note that the mean vessel circulation time (t_m) equals the peak fluorescence time (t_P). In other words, the larger the value of β, the more sharply peaked and the less skewed is the dye dilution curve. Figure 2 illustrates the empirical variation of (t_m/t_P) with β. It is evident that t_m/t_P varies slowly with β for values greater than 0.7. For values less than 0.7, there can be very large changes in mean vessel circulation time for very small changes in β. In this case, the curve-fit solution becomes unstable, and the mean vessel circulation time cannot be quantitated with any degree of accuracy. For the subjects without diabetes in this study, the average vessel coefficient β was 2.0 ± 0.3, and for patients with diabetes, the average vessel coefficient β was 1.7 ± 0.3. Thus, for the subjects studied here, β calculated from the vessel dye dilution curves showed that any changes in the shape of the curves had a minimal impact on the determination of mean vessel circulation times.

The shape of the venous dye dilution curve is determined primarily by the distribution of discrete dye transit times through the retinal capillary bed and secondarily by the time at which the recirculation component would be expected to influence the first passage clearance phase. Recirculation, if it occurs early enough during first-passage dye clearance, could prolong effectively the mean venous circulation time. If the recirculation component were subtracted from the first-passage dye clearance, this would shift the mean vessel circulation time closer to the peak vessel fluorescence time. For this reason, the venous peak–arterial peak time difference was determined as an additional retinal hemodynamic parameter in this study. Retinal circulation time data from all the study groups were pooled to investigate whether the MCT was related to the peak fluorescence time difference. Results are illustrated in Figure 3. Least-squares regression analysis showed a significant association between the retinal MCT and the artery–vein peak time difference (slope = 1.2; $r^2 = 0.76$; $P = 0.001$).

Segmental retinal blood flow was calculated using the retinal MCT and vessel diameter measurements. The segmental retinal blood flow is proportional to the sum of the squares of the retinal artery and vein diameters divided by the MCT. It has been theoretically justified that the sum of the squares of the retinal artery and vein diameters is proportional to the perfused vascular volume.[24] The retinal blood flow is defined in arbitrary units (AU) and was calculated as the average of the flows from the superior and inferior

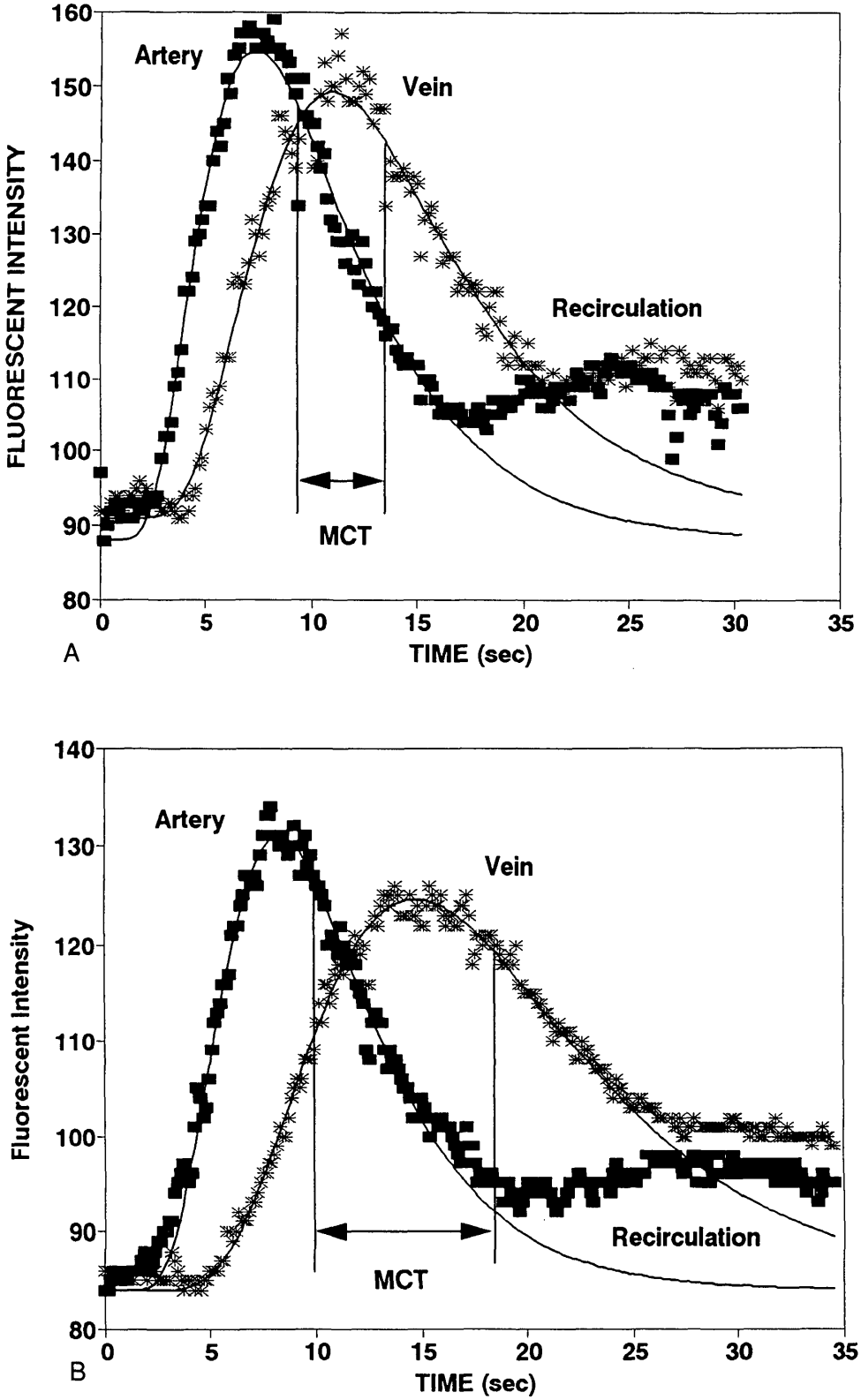

FIGURE 1. (A) Fluorescence dye dilution curves from a temporal retinal artery vein pair of a subject without diabetes. (B) Fluorescence dye dilution curve from a retinal artery vein pair of a subject with diabetes MCT = mean circulation time.

temporal retinal quadrants. All values are quoted as average ± standard deviation.

The group comparisons were performed using the SigmaStat (Jandel Scientific, San Rafael, CA) statistical analysis package. One-way repeated measures analysis of variance (ANOVA) were used to compare the same patients at different blood glucose levels. Group comparisons were performed using one-way ANOVA. Populations were tested for normality using the Kolmogorov–Smirnov test for normality and the Levene Median test for equality of variance. If the distributions failed the normality or equal variance test, then the Kruskal–Wallis ANOVA on ranks was performed. All pairwise multiple comparisons were performed using

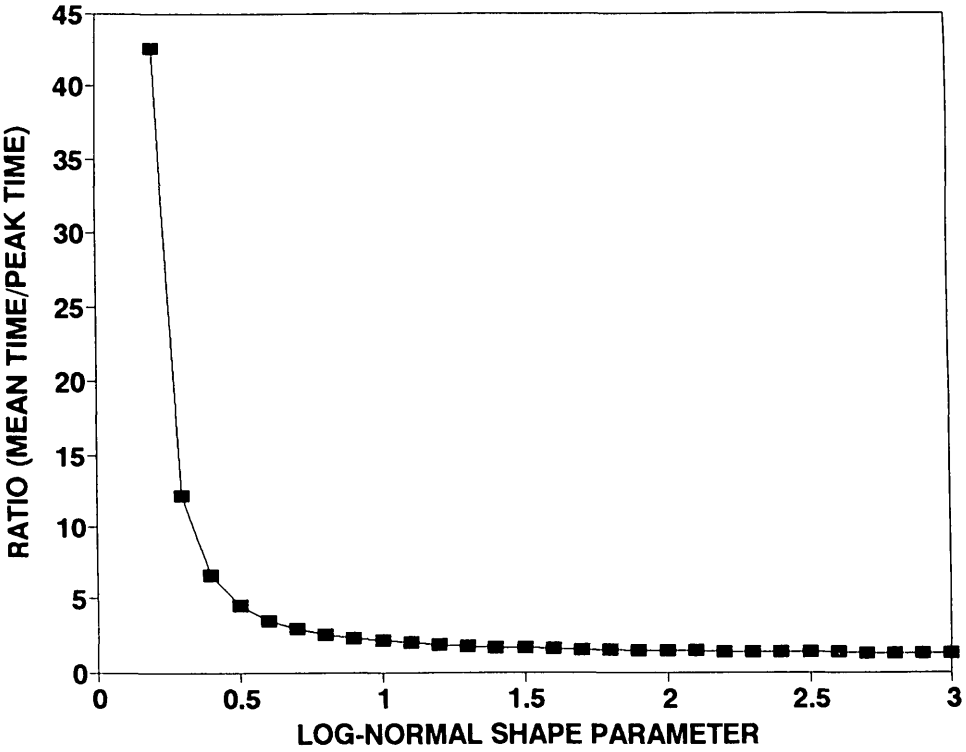

FIGURE 2. Dependence of the ratio of mean vessel circulation time(t_m) to peak fluorescence time (t_P) on changes in the coefficient of the exponent (β) of the lognormal distribution function.

the Student–Newman–Keuls test. Values of $P < 0.05$ were considered statistically significant.

Reproducibility

Repeated measurements were made from a subject without diabetes during a 12-month period; the VFA procedure was repeated five times during this period. The average temporal retinal MCT for these measurements was 3.86 ± 0.2 second, and the average temporal retinal artery–vein fluorescence peak time difference was 3.69 ± 0.05 second. This represents a coefficient of variation for MCT of 5.2% and for peak difference of 1.4%. The average temporal retinal blood flow was 30.6 ± 2.2 AU, with a coefficient of variation of 7.2%. The coefficient of variation for artery measurements was 3.6% (5.6 ± 0.2 pixel); for veins, it was 3.3% (7.5 ± 0.25 pixel).

RESULTS

Study group characteristics are summarized in Table 1. A one-way ANOVA for the three groups showed no significant differences in age, mean blood pressure, or intraocular pressure. There were no significant differences in duration of diabetes, hemoglobin A1, and baseline blood glucose values between the patients with diabetes who had glucose clamp and the random group of patients with diabetes. There was a significant ($P < 0.0001$) elevation in hemoglobin A1 and blood glucose level comparing the patient group with diabetes and the patient group without diabetes.

Retinal hemodynamic parameters from the glucose clamp study group, the random patient group with diabetes, and the group without diabetes are summarized in Table 2. One-way repeated measures ANOVA showed significant differences ($P < 0.001$) among the MCTs at the different glucose levels in the glucose clamp group, and multiple comparisons showed that the retinal MCT at the 100 mg/dl level was significantly ($P < 0.01$) prolonged compared to the MCT at the 200 mg/dl level and at the 300 mg/dl level. The MCTs at the 200 and 300 mg/dl levels were not significantly different. The artery–vein fluorescence peak time differences also showed significant ($P < 0.001$) differences at the different glucose levels, and multiple comparisons showed significant ($P < 0.01$) prolongation at the 100 mg/dl level compared to the 200 mg/dl and 300 mg/dl levels, whereas there was no statistical difference between the 200 and 300 mg/dl levels. These data demonstrate shortening of the average temporal retinal MCT as blood glucose levels were raised from 100 mg/dl to 300 mg/dl.

There were no significant differences in either arterial or venous diameters at any blood glucose level. Sensitivity for detecting a significant change in retinal vessel diameters can be evaluated using the variances in vessel diameter measurements. In this case, for 12 patients in each group at the $P < 0.05$ level and a power of 0.8, the minimal detectable vessel diameter change was 12% of the mean for arteries and veins.

Mean circulation time and vessel diameter measurements were used to calculate temporal retinal blood flow. One-way repeated measures ANOVA

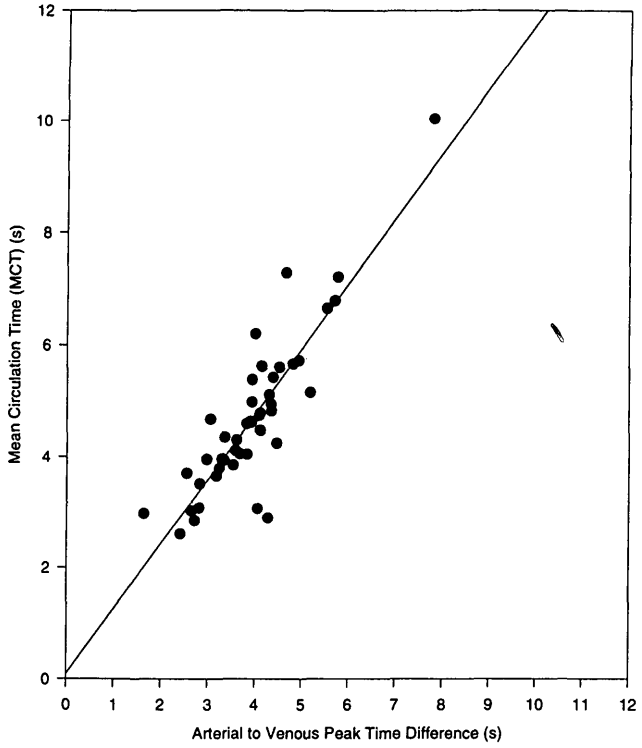

FIGURE 3. Association between mean circulation time and arterial-to-venous peak time differences. Solid line represents the regression fit to the data.

showed that retinal blood flow was affected significantly ($P < 0.001$) by glucose levels. Multiple comparisons showed that the flow at the 100 mg/dl level was significantly lower ($P < 0.01$) than the flows both at the 200 mg/dl level and at the 300 mg/dl level. There was no significant difference in flow between the 200 and 300 mg/dl levels. Thus, in parallel with the shortening of the retinal MCT and artery–vein fluorescence peak time difference, as blood glucose levels were raised from 100 to 300 mg/dl there was an increase in average temporal retinal blood flow.

The results at the different levels of blood glucose were compared to results obtained from the random group with diabetes and the group of patients without diabetes using the one-way ANOVA. There was a significant ($P < 0.01$) difference in MCT and artery–vein fluorescence peak time difference between groups. Multiple comparisons showed that the average temporal MCT at the 100 mg/dl glucose clamp level was significantly ($P < 0.01$) prolonged compared to that of the 300 mg/dl group and to the group without diabetes, but it was not significantly different from that of the random group with diabetes or of the group at the 200 mg/dl glucose clamp level. The temporal MCT was prolonged significantly ($P < 0.05$) at the 200 mg/dl glucose clamp level compared to the group without diabetes. Additionally, the average MCT for the random group with diabetes was prolonged significantly ($P < 0.01$) compared to the group without diabetes. In contrast, there was no significant difference between the retinal MCT at the 300 mg/dl glucose clamp level compared to the group without diabetes, whereas at this level there was a significant ($P < 0.05$) shortening of the MCT compared to the random group with diabetes. The retinal artery–vein peak fluorescence time differences changed in parallel with the MCT changes at the same levels of significance.

There were no significant differences between groups, at any glucose level, in arterial or venous diameters. One-way ANOVA demonstrated significant ($P < 0.001$) differences in blood flow between groups, and multiple comparisons showed, in parallel with the MCT changes, significantly lower average retinal blood flow in the patients with diabetes at the 100 ($P < 0.01$) and 200 mg/dl ($P < 0.05$) glucose clamp levels compared to the group without diabetes. Retinal blood flow also was significantly lower ($P < 0.01$) in the random group with diabetes compared to the group without diabetes. Blood flow at the 300 mg/dl level, though still lower than that for the group without diabetes, was not statistically significant. Retinal blood flow at the 300 mg/dl glucose level, however, was significantly ($P < 0.05$) greater than the retinal blood flow of the random group with diabetes (Fig. 4).

Data from the subset of seven patients who returned for random retinal blood flow measurements after undergoing the glucose clamp procedure were compared using the one-way repeated measures ANOVA. Average blood glucose measurements for this group at the time of the random retinal blood flow measurement (232.2 ± 70.4 mg/dl) were not signifi-

TABLE 1. Characteristics of Study Populations (12 Patients in Each Group)

	Age (years)	*Duration (years)*	*Hemoglobin A1 (%)*	*Blood Glucose (mg/dl)*	*Mean Blood Pressure (mm Hg)*	*Intraocular Pressure (mm Hg)*
Diabetic patients at start of glucose clamp	29.3 ± 8.6	4.6 ± 2.6	9.6 ± 1.2*	201.6 ± 50.6*	79.0 ± 5.2	13.3 ± 1.9
Random diabetic patient group	28.9 ± 5.9	5.1 ± 3.1	9.8 ± 1.8*	208.1 ± 79.7*	82.5 ± 7.0	12.3 ± 1.2
Nondiabetic subjects	30.4 ± 6.7		6.2 ± 0.6	92.7 ± 9.6	82.6 ± 5.6	13.6 ± 2.9

* $P < 0.0001$ versus nondiabetic group.

TABLE 2. Retinal Hemodynamic Parameters for the Study Groups (12 Patients in Each Group)

	Artery/Vein Peak Time Difference (seconds)	*Temporal MCT (seconds)*	*Arterial Diameter (pixels)*	*Venous Diameter (pixels)*	*Temporal Retinal Blood Flow (arbitrary units)*
Glucose clamp at 100 mg/dl	4.4 ± 0.8*	5.6 ± 1.1*	5.8 ± 0.7	7.6 ± 0.8	16.3 ± 3.8*
Glucose clamp at 200 mg/dl	3.8 ± 0.8†	4.5 ± 0.8†	5.8 ± 0.6	7.8 ± 0.8	21.5 ± 4.7*
Glucose clamp at 300 mg/dl	3.5 ± 0.8	3.8 ± 1.2	5.8 ± 0.7	7.7 ± 0.7	25.9 ± 8.8
Random diabetic group	4.3 ± 1.3*	5.1 ± 1.8*	5.7 ± 0.6	7.8 ± 0.8	19.4 ± 4.6*
Nondiabetic group	3.1 ± 0.7	3.4 ± 0.7	5.9 ± 0.5	7.9 ± 0.6	28.7 ± 6.4

MCT = mean circulation time.
* $P < 0.01$ versus nondiabetic group.
† $P < 0.05$ versus nondiabetic group.

cantly different from those measured at entry to the glucose clamp procedure (182.6 ± 20.1 mg/dl). Increased variance in the random measurements was associated with the fact that we did not require retinal blood flow measurements of these patients after overnight fasting. Repeated measures ANOVA showed a significant ($P < 0.05$) difference in blood flow between the groups, and multiple comparisons demonstrated that the retinal blood flow at the 100 mg/dl glucose level (16.3 ± 4.1 AU) was significantly ($P < 0.05$) lower than the retinal blood flow at the 300 mg/dl level (25.2 ± 9.9 AU), as was noted in the larger glucose clamp study group. However, there were no statistically significant differences between the retinal blood flows at the 100 and 200 mg/dl (19.6 ± 4.7 AU) levels or between these retinal blood flows and the retinal blood flow at the random visit (20.7 ± 4.3 AU). Blood flow at the 300 mg/dl level, though greater than that at the random visit, did not reach statistical significance. Thus, the retinal blood flow at the 200 mg/dl glucose clamp level was comparable to the blood flow at the random visit (average blood glucose level of 232.2 ± 70.4 mg/dl), indicating that the stress of the glucose clamp procedure did not exert a nonspecific effect on retinal blood flow measurements.

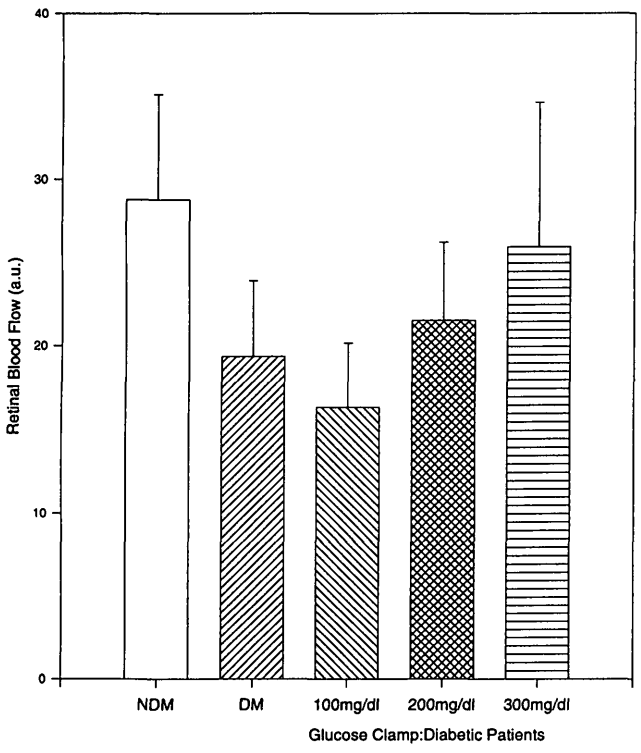

FIGURE 4. Average retinal blood flow for the different study groups. NDM = subjects without diabetes; DM = random group with diabetes.

DISCUSSION

Results from this study demonstrate that the average retinal blood flow measured from the random group with diabetes (no diabetic retinopathy) was significantly lower (32%) than the average retinal blood flow of the group without diabetes and is in agreement with our preliminary results.[14] These results are also in agreement with a recent study using laser Doppler velocimetry in which a 33% reduction in retinal blood flow was measured in patients with diabetes with no diabetic retinopathy compared to patients without diabetes.[19] We also showed that acute changes in blood glucose can modulate retinal blood flow in patients with IDDM with no diabetic retinopathy. Retinal blood flow was lowest at the 100 mg/dl glucose level and increased as blood glucose levels were raised to 200 and 300 mg/dl. However, retinal blood flow values at both the 100 and 200 mg/dl levels were significantly lower (43% and 25%, respectively) than retinal blood flow from the group without diabetes and the retinal blood flow at the 300 mg/dl level. Although the blood flow at the 300 mg/dl level remained lower than nondiabetic blood flows, the difference was not statistically significant. Thus, retinal blood flow increased in response to acute elevations in blood glucose levels; however, long-term retinal complications and their association with chronically elevated blood glucose levels,

concomitant cellular metabolic abnormalities, and retinal blood flow alterations are not yet fully understood.

Retinal blood flow measurements made were based on the use of fluorescein dye and indicator dilution methodologies. The excitation light used to cause the fluorescence of the fluorescein dye was absorbed strongly by hemoglobin, which restricted the penetration depth of the light into the vessel lumen. Thus, the fluorescences were weighted toward the blood flowing in the vessel periphery, leading to a sampling of the slower blood flow velocities. Retinal circulation times measured using fluorescein dye compared to indocyanine green (ICG) dye, for example, with which fluorescence excitation is achieved using infrared light (greater penetration with sampling of faster center line flows), tends to be longer than those measured using ICG. Blood flows measured using fluorescein dye tend to be lower than those measured using ICG and those measured using laser Doppler velocimetry, which sample the center line or maximal red blood cell velocities. A comparison study between fluorescein dye and ICG dye indicator dilution methodologies is being conducted.

Retinal blood flow measurements, for this study, were made on the assumptions that the indicator was an intravascular dye and that the sum of the squares of the artery and vein diameters were proportional to the perfused vascular volume.[24] For the younger subjects without diabetes and the younger patients with diabetes with no diabetic retinopathy, these assumptions were valid. First, in the patients with diabetes, clinical evidence indicated that the retinal vasculature was intact with no signs of capillary nonperfusion. The sum of the squares of the major retinal arteries and veins reflected the proportionality between this parameter and the perfused vascular volume. Second, the sodium fluorescein dye used as the indicator in this study was not considered a true intravascular dye because some dye was able to permeate the vascular endothelial cell tight junctions into the retinal tissue, possibly affecting the measured dye dilution curves. Theoretical dye dilution studies[25] indicate that a freely diffusible dye, such as tritiated water, can cause a prolongation of the mean vessel circulation time compared to intravascular tracers, such as creatinine. However, the diffusivity of the tracer has little effect on the peak tracer concentration appearance time in the vessel.

The diffusivity properties of fluorescein dye have been used to investigate blood retinal barrier permeability changes using vitreous fluorophotometry. These studies[26,27] showed no significant differences in blood retinal barrier permeability to fluorescein between patients without diabetes and patients with diabetes but no diabetic retinopathy. In addition, after intravenous injection, 80% to 85% of the dye was rapidly bound to serum proteins,[28,29] binding was independent of fluorescein concentration,[29] and there was no significant difference in binding between patients with and without diabetes.[30] Plasma-bound fluorescein will not diffuse from the retinal vessels, limiting the total concentration of freely diffusible fluorescein and minimizing any effects associated with the extravascular diffusion of the dye on the measured dye dilution curves. Results from previous studies[27,31] showed retinal permeability to fluorescein in the range of 2 to 6 $\times 10^{-7}$ cm/second and fluxes in the rabbit retinal vessels[32] in the range of 10^{-10} gm/ml · minute per millimeter. Given that only 20% of this dye is free to diffuse across the retinal vessels, and taking into account the fluorescein flux values above, one can see that during the period of first dye passage through the retinal circulation (20 to 30 seconds), the contribution to the vessel dye concentration from diffusion effects has little or no impact on the characteristics of the measured dye dilution curve. Thus, in the study populations used here, over the time course of the first passage of dye through the retinal circulation, sodium fluorescein approximated a true intravascular dye tracer. Additionally, because there were no differences between groups with and without diabetes concerning dye permeability or fraction of free fluorescein, there was no systematic bias imparted to the data when group comparisons were performed.

Prolongation of the retinal MCT in patients with diabetes may be associated with dye recirculation broadening of the first-passage clearance phase. Analysis of the retinal MCT involved fitting a lognormal distribution function to the data points before the appearance of the recirculation phase. Parameters from these fits were used to determine mean vessel circulation times and the segmental retinal MCT. Inspection of the artery and vein curves in Figure 1 shows that in the patient with diabetes, the MCT and the artery vein peak time difference were prolonged. Prolongation of circulation times was associated primarily with changes in the venous curves. Arterial curves from patients with and without diabetes were comparable with respect to shape and mean arterial circulation times. Venous curves for the patients with diabetes, however, tended to be broader with prolonged mean venous circulation times compared to those of the subjects without diabetes. In addition, the time difference between arterial dye appearance and the appearance of dye in the corresponding vein was approximately 1 second longer in the subjects with diabetes compared to the subjects without diabetes. These differences suggest that the mean transit time through the retinal capillary bed connecting the artery to the vein shifted toward longer transit times in the subject with diabetes, giving a prolonged retinal MCT and a reduced segmental retinal blood flow. Alternatively, one could postulate that the longer mean ve-

nous circulation times seen in patients with diabetes is a result of recirculation interference in the broader venous curves of the patients with diabetes. Considering the curves illustrated in Figure 1, and taking into account the 1-second prolongation of dye appearance times in the patient with diabetes, one would expect the venous recirculation to have an effect approximately 1 second later than the appearance of the arterial recirculation phase. This would affect the final 1 to 2 seconds of the venous clearance phase data used in the lognormal distribution fitting algorithm. The time course of the curves was approximately 15 seconds, or 450 data points. Recirculation could affect the final 1 to 2 seconds of this curve, or 30 to 60 data points. Data points potentially compromised by recirculation would contribute only approximately 10% of the total weight of the curve fit. It is unlikely that recirculation plays a prominent role in the prolongation of the retinal MCT.

In the final analysis, if any recirculation effects were to be subtracted from the original artery and vein curves, mean vessel circulation times would shift to values closer to the artery and vein peak fluorescence times. Results from this study demonstrate that the difference between artery and vein peak fluorescence times was prolonged significantly in the groups with diabetes compared to the group without diabetes and that there was a significant association between this circulatory parameter and the retinal MCT. Thus, prolongation in retinal circulation times in patients with diabetes with no diabetic retinopathy is a reflection of slower perfusion and prolonged transit times through the capillary bed.

Results from this study also demonstrated that retinal blood flow in patients with no diabetic retinopathy was significantly lower (32%) than in the control group without diabetes. Reduction in retinal blood flow was associated primarily with prolongation of the retinal MCT because there were no significant differences in vessel diameters comparing the group without diabetes to patients with diabetes. This suggests that the reduction in retinal blood flow is associated with increased resistance to flow at the microcirculatory level rather than at the level of the major retinal vessels. An earlier study[33] measured vascular dilation of retinal vessels of patients with diabetes. However, closer examination of those data indicated that in patients with diabetes of short duration and, hence, presumably no diabetic retinopathy, retinal artery and vein diameters were comparable to those of subjects without diabetes. It was only in patients with diabetes of longer duration (>10 years) that vascular dilation became marked. In more recent laser Doppler velocimetry studies by Grunwald et al[9] and Patel et al,[7] no significant change in vessel diameter was observed in patients with type I diabetes mellitus with no diabetic retinopathy. In these studies, the respective authors obtained similar results because they both measured no significant difference in blood flow between patients with diabetes with no diabetic retinopathy and subjects without diabetes, although the diabetic subjects tended to have lower retinal blood flows. Once the level of retinopathy had progressed to the early nonproliferative diabetic retinopathy stage, an increase in venous dilation and a significant increase in retinal blood flow was measured at all levels of untreated diabetic retinopathy.[7] This is in contrast to the study by Feke et al[8] demonstrating no significant arterial diameter changes and significantly reduced retinal blood flow in patients with diabetes with no diabetic retinopathy also using laser Doppler velocimetry. Other studies[34] using the fluorescein dye dilution methodology demonstrated reduced retinal blood flow in children with diabetes. Studies[35] in animals have demonstrated decreased retinal blood flow in diabetic dogs using microsphere impaction methodology and in diabetic rats[16,17,36] using the VFA methodology.

Results from the glucose clamp study in patients with IDDM demonstrated that retinal blood flow can be modulated by acute changes in blood glucose levels. These results are in agreement with an earlier laser Doppler velocimetry study[11] in which 12 patients with type II diabetes mellitus (5 of whom were graded as having no diabetic retinopathy) showed significantly increased retinal blood flow at blood glucose levels greater than 300 mg/dl compared to retinal blood flow at blood glucose values between 100 and 125 mg/dl. The patients with no diabetic retinopathy (short disease duration) showed greater reductions in retinal blood flow (20% to 40%) than did patients with diabetic retinopathy. This blood flow reduction in the group of patients with no diabetic retinopathy was comparable to the decrease in retinal blood flow measured in the IDDM glucose clamp study described in this article. In addition, Feke et al[9] also showed that a 50% increase in blood glucose resulted in a 10% increase in retinal arterial blood speed in patients with IDDM. Retinal blood flow has been shown to increase transiently in response to acute blood glucose elevations in minipigs,[37] cats,[38] and dogs.[39]

In patients with diabetes with no diabetic retinopathy, acute elevations in blood glucose resulted in increased retinal blood flow. At blood glucose levels of 100 and 200 mg/dl, the retinal blood flow in the patients with diabetes was significantly lower than that for subjects without diabetes but was not significantly different from that of the random group with diabetes. At the highest blood glucose level, the retinal blood flow was significantly greater than the random group with diabetes and, though still lower than the group without diabetes, was not statistically different. The observed increases in retinal blood flow after acute elevations of blood glucose levels may have to be con-

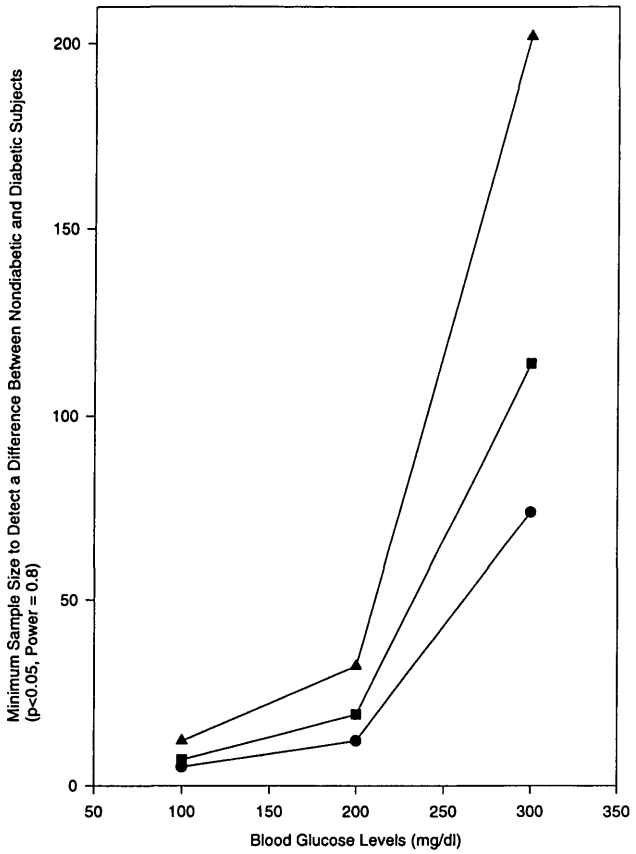

FIGURE 5. Sensitivity analysis to determine minimum sample sizes required to demonstrate significant retinal blood flow differences between subjects with and without diabetes at different blood glucose levels. ● = actual observed difference; ■ = 80% of the observed difference; ▲ = 60% of the observed difference.

sidered in the design of cross-sectional studies aimed at comparing retinal blood flow from patients with diabetes and subjects without diabetes. Blood glucose level at the time of retinal blood flow measurements may contribute to the interpatient variability and average retinal blood flow values. Thus, if study group blood glucose levels tend to be in the 200 to 300 mg/dl range, a larger sample size should be considered to demonstrate any statistically significant changes. This is evident when considering the combined glucose clamp study data at blood glucose levels of 200 and 300 mg/dl. This could be representative of a population of patients with diabetes undergoing retinal blood flow measurement who have blood glucose values in this range. The average retinal blood flow for these patients with diabetes was 23.9 ± 7.4 AU, and the difference between this group and the patients without diabetes approached significance ($P = 0.07$). Statistical calculation using these data shows that a minimum sample size of 34 would be needed to demonstrate statistical significance at the $P = 0.05$ level with a power of 0.8. Figure 5 illustrates results from analyses performed on the data obtained in this study. Minimum sample sizes needed to detect blood flow differences with a power of 0.8 at the $P < 0.05$ level are determined at the different blood glucose levels. The three curves represent minimum sample size calculations based on the actual differences observed between the groups with and without diabetes at the different blood glucose levels and at differences that were 80% and 60% of the observed differences. Thus, if blood glucose values at the time of retinal blood flow measurement are in the 200 to 300 mg/dl range, average retinal blood flow values will be higher than at they are at euglycemic levels. This results in a smaller difference between patients with diabetes and subjects without diabetes; consequently, larger populations may be required to demonstrate significantly reduced retinal blood flow in patients with diabetes.

Key Words

diabetes, fluorescein angiography, glucose clamp, hyperglycemia, retina, retinal blood flow

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