EXTENDED REPORT

Twelve hour reproducibility of choroidal blood flow parameters in healthy subjects

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Aims/background: To investigate the reproducibility and potential diurnal variation of choroidal blood flow parameters in healthy subjects over a period of 12 hours.

Methods: The choroidal blood flow parameters of 16 healthy non-smoking subjects were measured at five time points during the day (8:00, 11:00, 14:00, 17:00, and 20:00). Outcome parameters were pulsatile ocular blood flow as assessed by pneumotonometry, fundus pulsation amplitude as assessed by laser interferometry, blood velocities in the opthalmic and posterior ciliary arteries as assessed by colour Doppler imaging, and choroidal blood flow, volume, and velocity as assessed by fundus camera based laser Doppler flowmetry. The coefficient of variation and the maximum change from baseline in an individual were calculated for each outcome parameter.

Results: None of the techniques used found a diurnal variation in choroidal blood flow. Coefficients of variation were within 2.9% and 13.6% for all outcome parameters. The maximum change from baseline in an individual was much higher, ranging from 11.2% to 58.8%.

Conclusions: These data indicate that in healthy subjects the selected techniques provide adequate reproducibility to be used in clinical studies**.** Variability may, however, be considerably higher in older subjects or subjects with ocular disease. The higher individual differences in flow parameter readings limit the use of the techniques in clinical practice. To overcome problems with measurement validity, a clinical trial should include as many choroidal blood flow outcome parameters as possible to check for consistency.

In recent years the number of studies investigating ocular blood flow regulation has increased. However, it is not uncommon for contradictory results to be reported in this field. One example relates to the level of choroidal perfusion in diabetes. Using different methods, evidence for reduced,[1–3] unchanged,[4] or increased[5 6] choroidal blood flow has been presented. The reason for this problem may be related to the validity of the methods, the reproducibility of the techniques, and inadequate use of the systems. It has previously been pointed out that there is no ''gold standard'' method available for the assessment of choroidal blood flow in animals or humans.[7] Hence, any result obtained has to be interpreted with caution and bearing in mind the limitations of the selected techniques.

Another serious problem is that many studies that have been performed suffer from inadequate sample size and study design. This is obviously dependent on the reproducibility of the selected method, which needs to be thoroughly investigated before a study is performed. The aim of the present study was to compare the reproducibility of a number of blood flow parameters associated with choroidal haemodynamics in the course of a 12 hour period and to investigate a potential diurnal variation of these variables. In addition, we provide an estimate of sample sizes required for each parameter in a study using paired analysis. Finally, a short overview of the most important limitations of each method is given in the discussion section.

METHODS

Subjects

The study protocol was approved by the Ethics Committee of Vienna University School of Medicine and followed the guidelines of the Declaration of Helsinki. Eight female and eight male (mean age 25.2 (standard deviation 3.1) years, range 20–33 years) healthy, non-smoking volunteers participated in this study after signing written informed consent. Each subject passed a screening examination that included medical history and physical examination, 12 lead electrocardiogram, blood pressure, and pulse rate. In addition, an ophthalmic examination was performed in each subject before the study. Inclusion criteria were normal ophthalmic findings, ametropia of less than 3 diopters, and anisometropia of less than 1 diopter. In all subjects the right eye was studied.

Experimental design

All subjects were studied after dilating the pupil with one drop of cyclopentolate (Cyclopentolate 1%, Alcon Ophthalmica, Puurs, Belgium). Measurements started at 8:00 in the morning and were repeated every three hours until 20:00. Accordingly, a total of five measurements were done. All ocular haemodynamic parameters were measured at each measurement cycle in the seated position. The techniques were performed in a predetermined order (laser Doppler flowmetry (LDF), laser interferometry, colour Doppler imaging (CDI), pneumotonometry, applanation tonometry, blood pressure, and pulse rate).

Laser doppler flowmetry

Choroidal blood flow was assessed with laser Doppler flowmetry according to Riva *et al*.[8] In the present study a commercially available system was used (Oculix 4000, Oculix

Abbreviations: CDI, colour Doppler imaging; DSPS, Doppler shift power spectrum; EDV, end diastolic flow velocity; FPA, fundus pulsation amplitude; IOP, intraocular pressure; LDF, laser Doppler flowmetry; PA, pulse amplitude; PCA, posterior ciliary artery; POBF, pulsatile ocular blood flow; PSV, peak systolic flow velocity; RBC, red blood cell

Sarl, Arbaz, Switzerland). The principles of laser Doppler flowmetry have been described in detail by Bonner and Nossal.[9] Briefly, the vascularised tissue is illuminated by coherent laser light. Scattering on moving red blood cells (RBCs) leads to a frequency shift in the scattered light. In contrast, static scatters in tissue do not change light frequency but lead to randomisation of light directions impinging on RBCs. This light diffusion in vascularised tissue leads to a broadening of the spectrum of scattered light (Doppler shift power spectrum, DSPS). From this DSPS the mean RBC velocity (VEL), the blood volume (VOL), and the blood flow (FLOW) are calculated in relative units. In the present study the laser beam was directed to the fovea to assess blood flow in the submacular choroids.[8]

Fundus pulsation technique

Ocular fundus pulsation was assessed by laser interferometry as described by Schmetterer *et al*.[10] Briefly, the eye is illuminated by the beam of a single mode laser diode ($\lambda = 783$ nm) along the optical axis. The light is reflected at both the front surface of the cornea and the retina. The two re-emitted waves produce interference fringes from which the distance changes between cornea and retina during a cardiac cycle can be calculated. These distance changes are caused by the pulsatile inflow of blood through the arteries and by the non-pulsatile outflow through the veins. The maximum change in corneo-retinal distance is called fundus pulsation amplitude (FPA). In the present study FPA was assessed in the fovea.

Pneumotonometry

Pulsatile ocular blood flow (POBF) was determined with a commercially available blood flow system (OBF System 3000, OBF Labs, Malmesbury, UK; now available through Paradigm Labs, USA). The system measures changes in intraocular pressure (IOP), which are caused by the pulsatile rhythmic filling of the intraocular vessels, with a pneumatic applanation tonometer. The maximum IOP change during the cardiac cycle is called pulse amplitude (PA). Based on a hydrodynamic eye model, the POBF is calculated from the IOP variation with time.[11] This model is based on the assumption that venous outflow from the eye is non-pulsatile. Moreover, the ocular rigidity, which is used to derive ocular volume changes from changes in IOP, is assumed to be standard for all subjects. The calculation of POBF is automatically derived from the five pulses that are closest to each other in IOP beat to beat variation.

Colour Doppler imaging

Peak systolic flow velocity (PSV) and end diastolic flow velocity (EDV) were measured in the posterior ciliary arteries and the ophthalmic artery (CFM 750, Vingmed Sound, Horten, Norway) using a 7.5 MHz colour Doppler probe as described previously.[12] From these parameters the resistance index (RI) was calculated as:

$$RI = (PSV - EDV)/PSV$$

In addition, the mean flow velocity (MFV) was measured as the time mean of the spectral outline over the cardiac cycle. A coupling gel was placed on the lid of the closed right eye and the probe was positioned with minimal pressure. The ophthalmic artery (OA) was measured anteriorly, at the point where it crosses the optic nerve. The sample volume marker was placed approximately 25 mm posterior to the globe. The short posterior ciliary arteries (PCAs) were measured temporal to the optic nerve head. Due to their small size, however, it cannot be determined how many of these vessels contribute to the signal.

Measurement of intraocular pressure

A slit lamp mounted Goldmann Applanation tonometer (Nikon 105, Tokyo, Japan) was used to measure IOP. Before each measurement one drop of 0.4% benoxinate hydrochloride combined with 0.25% fluorescein sodium was used for local anesthesia of the cornea.

Systemic haemodynamics

Systolic, diastolic, and mean blood pressures were measured on the upper arm by an automated oscillometric device (HP-CMS patient monitor, Hewlett Packard, Palo Alto, USA). Pulse rate was automatically recorded from a finger pulse oxymetric device (HP-CMS patient monitor).

Data analysis

Repeated measure ANOVA was used to assess the time effect of choroidal haemodynamic parameters versus baseline using absolute values. Results are given as mean (standard deviation). A p value of <0.05 was considered the level of significance.

In addition, the coefficient of variation was calculated for each parameter using the five consecutive measurements. Based on the variability data sample size calculations for clinical studies are provided. These sample size calculations are based on the following assumptions: a double sided α error of 0.05, a ß error of 0.20, and a placebo controlled two way crossover trial. Sample size calculations are given for expected differences of 10%. The range of maximum per cent change of a particular parameter in an individual subject is also provided.

RESULTS

At all measurements cycles, blood pressure, pulse rate and intraocular pressure (table 1) remained stable throughout the study period indicating constant ocular perfusion pressure. In addition, there was no evidence of diurnal variation in any of the choroidal haemodynamic parameters under study (table 1). The changes in the means of the outcome variables were within 15% for all time points and no time effects were observed.

The coefficients of variation as calculated from the five measurement cycles are presented in table 2. All coefficients of variation of the choroidal haemodynamic parameters were within 2.6% and 13.9%. With both methods assessing pulsatile blood flow, FPA showed less variability than POBF. Generally, the variability of blood velocities was higher in the PCAs than in the OA. For both measurement sites MFV showed a higher reproducibility than PSV or EDV. With laser Doppler flowmetry the variability was higher for VOL than for VEL, indicating that much of the variability in FLOW is due to scattering of VOL data.

For individuals maximum changes from baseline were obviously much higher (table 2). In some parameters changes of up to 60% from baseline were observed. Again, the differences in MFV from baseline were smaller than those in PSV and EDV for both measurement locations.

According to the relatively high reproducibility of all outcome parameters as evidenced from table 2, sample sizes calculated as outlined in the statistical analysis section were considerably low (table 3). All sample size values were rounded up to even numbers to allow for balanced randomisation.

DISCUSSION

The present study indicates that diurnal variations in choroidal haemodynamic parameters during the day in healthy subjects are small. Variability of measurements as assessed in the present study may arise from two distinct possibilities: (1) fluctuations in choroidal blood flow over the

Table 1 Blood pressure, pulse rate, intraocular pressure, and ocular haemodynamic variables for the five measurement cycles during the twelve hours study period. Data are presented as mean (SD)

	8:00	11:00	14:00	17:00	20:00
Systolic blood pressure (mm Hg)	125 (12)	124 (13)	124 (11)	125 (11)	129 (14)
Diastolic blood pressure (mm Hg)	67 (7)	66 (8)	65 (7)	64 (8)	67 (7)
Pulse rate (beats/min)	71 (11)	70 (11)	73 (11)	71 (11)	72 (9)
Intraocular pressure (mm Hg)	11.0 (2.9)	10.5 (2.6)	12.0 (2.8)	11.3 (2.8)	10.3 (1.9)
Pulsatile ocular blood flow (µl/min)	1002 (227)	1078 (222)	1054 (225)	1047 (242)	1026 (230)
Fundus pulsation amplitude (µm)	4.06 (1.28)	4.06 (1.34)	4.09 (1.33)	4.09 (1.22)	4.06 (1.29)
Peak systolic velocity$_{OA}$ (cm/s)	53.0 (14.2)	55.3 (12.2)	56.8 (14.0)	53.5 (13.5)	57.8 (14.0)
End diastolic velocity$_{OA}$ (cm/s)	8.2 (1.8)	8.4 (1.9)	8.0 (1.5)	8.9 (1.8)	9.3 (1.7)
Mean flow velocity$_{OA}$ (cm/s)	18.8 (4.2)	19.4 (5.1)	18.4 (4.2)	19.1 (4.3)	19.8 (4.1)
RI$_{OA}$	0.84 (0.04)	0.84 (0.04)	0.86 (0.03)	0.83 (0.03)	0.84 (0.03)
Peak systolic velocity$_{PCA}$ (cm/s)	11.6 (2.0)	11.1 (1.9)	10.6 (2.0)	10.9 (1.4)	10.8 (1.9)
End diastolic velocity$_{PCA}$ (cm/s)	3.5 (0.8)	3.5 (0.8)	3.4 (0.9)	3.2 (0.6)	3.6 (0.7)
Mean flow velocity$_{PCA}$ (cm/s)	5.5 (0.9)	5.7 (1.1)	5.5 (0.8)	5.4 (0.7)	5.7 (1.0)
RI$_{PCA}$	0.69 (0.06)	0.67 (0.09)	0.67 (0.09)	0.70 (0.07)	0.66 (0.06)
FLOW (AU)	6.8 (1.3)	7.0 (1.3)	7.4 (1.5)	6.5 (1.4)	6.8 (1.5)
VOL (AU)	0.15 (0.04)	0.15 (0.04)	0.16 (0.04)	0.15 (0.04)	0.16 (0.04)
VEL (AU)	0.49 (0.08)	0.49 (0.05)	0.48 (0.07)	0.49 (0.08)	0.50 (0.09)

AU, arbitrary units.

twelve hours study period may be responsible for different blood flow parameter readings; (2) method related variability introducing statistical errors may contribute to the variability of data. In the present study we tried to keep the second source of error as small as possible by including only healthy subjects with previous experience of the employed techniques. In addition, all measurements using a particular method were done by the same experienced observer, avoiding interobserver differences.

Numerous previous studies revealed that intraocular pressure shows a characteristic diurnal variation. However, the diurnal variation is small compared with the variation between day and night.[13] It is most likely the present study was underpowered to detect the small diurnal variations which are in the order of 1–1.5 mmHg in healthy young subjects.[13]

Table 2 Coefficient of variation (CV) and the range of maximum deviation from baseline value in a single individual (MAXD) of the choroidal haemodynamic parameters as calculated from the five measurement cycles between 8:00 and 20:00 (n = 16)

	CV (%)	MAXD
Pulsatile ocular blood flow (µl/min)	10.6 (3.4)	5.4–36.7
Fundus pulsation amplitude (µm)	4.9 (1.2)	3.7–12.0
Peak systolic velocity$_{OA}$ (cm/s)	8.1 (3.0)	5.5–30.5
End diastolic velocity$_{OA}$ (cm/s)	12.3 (4.5)	12.1–58.8
Mean flow velocity$_{OA}$ (cm/s)	7.9 (2.7)	5.0–25.4
RI$_{OA}$	2.6 (1.1)	2.0–11.2
Peak systolic velocity$_{PCA}$ (cm/s)	13.8 (3.0)	16.7–51.3
End diastolic velocity$_{PCA}$ (cm/s)	13.9 (4.9)	15.8–40.5
Mean flow velocity$_{PCA}$ (cm/s)	9.6 (4.3)	5.2–29.7
RI$_{PCA}$	7.5 (2.4)	6.7–22.9
FLOW (AU)	11.5 (3.3)	14.5–57.2
VEL (AU)	8.5 (3.2)	5.1–36.4
VOL (AU)	11.1 (3.9)	9.2–49.2

AU, arbitrary units.

For some, but not for all, haemodynamic parameters measured in the present study variability data have been presented previously. Reproducibility data for FPA reported here are in the same order as provided in our previous studies with shorter measurement intervals.[14 15] Variability of POBF data is also in the same order as reported in our previous studies[15] and those reported by other groups.[16–19] With colour Doppler imaging the present study confirms that variability in blood velocities in the PCAs is higher than in the OA.[15 20–22]

There is no study which specifically focused on reproducibility data for laser Doppler flowmetry in the choroid using the present fundus camera based system. Our study, however, confirms previous data in the optic nerve head using the same device[23] and data from the choroid using a

Table 3 Sample size calculations based on the variability data presented in table 2.

Technique	Parameter	Sample size
Pneumotonometry	Pulsatile ocular blood flow (µl/min)	12
Laser interferometry in the macula	Fundus pulsation amplitude (µm)	4
Colour Doppler imaging in the ophthalmic artery	Peak systolic velocity (cm/s)	8
	End diastolic velocity (cm/s)	14
	Mean flow velocity (cm/s)	8
Colour Doppler imaging in the posterior ciliary arteries	Peak systolic velocity (cm/s)	18
	End diastolic velocity (cm/s)	18
	Mean flow velocity (cm/s)	10
Laser Doppler flowmetry in the macula	FLOW (AU)	14
	VEL (AU)	8
	VOL (AU)	12

Sample sizes are given based on a two way crossover design using pairwise analysis, a double sided α error of 0.05, and a β error of 0.20 in order to detect a 10% difference between treatment groups. All sample sizes are rounded up to even numbers to allow for balanced randomisation.
AU, arbitrary units.

portable confocal system[24] showing that variability in FLOW and VOL is higher than in VEL. Moreover, the reproducibility in the present study is comparable with that reported in previous clinical trails using the same device.[25]

The sample size calculations presented in table 3 indicate that most of the employed methods provide adequate reproducibility to be used in pharmacodynamic studies. One needs, however, to consider that the numbers presented only apply for studies in healthy subjects and cannot necessarily be extrapolated to studies in elderly subjects or patients with eye disease. In our experience variability of most of the employed techniques is considerably higher in such populations and an adequate sample size population always needs to be based on reproducibility data in an adequate random sample of the study population. Our data do, however, also indicate that deviations from baseline values in individuals may be considerable (table 2), which are most likely not related to physiological fluctuations of choroidal blood flow. These large differences of up to 60% obviously limit the applicability of these techniques in clinical routine.

In addition to these considerations regarding the variability of choroidal haemodynamic parameters, the validity of the employed techniques needs to be considered. Both methods which assess only the pulsatile component of choroidal blood flow, pneumotonometry, and laser interferometry, do not provide any information on the steady component of blood flow, although there is a high consistency between the two methods.[15 26 27] Langham *et al* estimated that approximately 80% of total choroidal blood flow is pulsatile.[28] By contrast Krakau *et al* stated that with values of IOP in the normal range the total flow is at least twice the pulsatile flow.[29] This limits interpretation of cross sectional as well as longitudinal studies, because differences may arise either from changes in blood flow or from changes in flow pulsatility. Indeed there is evidence that changes in the blood pressure profile, particularly a change in the ratio of pulse pressure amplitude versus diastolic blood pressure, may considerably alter the ratio between pulsatile and non-pulsatile flow in the choroid without any concomitant change in the choroidal perfusion rate.[30]

With colour Doppler imaging one needs to take into account that only blood velocities are measured. For calculation of total blood flow, diameter values of the selected arteries would be required, which are constrained by the limited resolution of the technique. Hence, extrapolation of such data to blood flow values is critically important to constant diameter throughout the study in longitudinal studies and to comparable diameters between subjects in cross sectional studies. For the ophthalmic artery it needs to be considered that only approximately 25% of the blood flowing through this vessel is supplying the eye[31] and that ocular blood flow and blood velocities in the eye can easily be uncoupled.[32 33] The RI has been used as a measure of distal vascular resistance in the vascular beds of the eye. It has already been correctly pointed out that the RI is also influenced by the proximal vascular resistance.[34] In addition, we have previously shown that RI in the central retinal artery is not a valid measure of vascular resistance in the retina.[35]

Several limitations have to be considered when using laser Doppler flowmetry. Firstly, the fundus camera based system does in principle only allow for local measurements in the subfoveal choroid, because the retina is avascular in this region.[8 36] Secondly, measurements using this system in the peripheral retina appear to be also mainly influenced by choroidal blood flow with very little retinal contribution.[37] The sampling depth of the system and the microvessels which contribute most to the signal remain, however, to be established. In addition, one needs to be careful to compare laser Doppler flowmetry readings between subjects because the absolute values are strongly influenced by the scattering properties of the tissue. This has been correctly pointed out when patients with age related macular degeneration are compared with healthy subjects.[38 39] This does, however, apply to all cross sectional studies where morphological differences between groups may be expected. Finally, laser Doppler flowmetry assesses red blood cell movement, and extrapolation to blood flow data may critically depend on the local haematocrit.

In conclusion, within the power of this study, diurnal variations were not detected. The variability of ocular haemodynamic variables in healthy young subjects is sufficient to allow for within subjects clinical studies to have reasonable sample sizes. Single outliers do, however, limit the use of these systems in clinical practice. As there are some concerns about the validity of all currently available systems, any clinical study should include as many techniques as possible to ensure consistency of data.

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