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Characterising the Time Course of the Dilatory Response of Healthy Retinal Arteries during Flicker-Light Provocation

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Keywords
Retinal vessel reactivity · Flicker light · Dilation · Bi-linear functions · Healthy retinal arteries

Abstract
Introduction: The dilatory response of healthy retinal arterioles to flicker-light (FL) provocation appears to be biphasic. The vessel diameter rapidly increases (acute phase) over 5–10 s, then barely increases thereafter (maintenance phase) until FL cessation. This reaction is usually characterised at a single point by two parameters: maximum dilation (MD) relative to baseline diameter (MD, %) and time to MD (RT, s). This paper describes the biphasic reaction of retinal arteries during FL provocation using a bi-linear function. ***Methods:*** Retinal arterioles from 45 adults were examined during flicker provocation. Each individual time course of arterial diameter change during FL provocation was characterised by a bi-linear equation and compared with MD and RT. ***Results:*** Slopes of the acute phase were 0.506%/s, and the maintenance phase was nearly flat (0.012%/s). The mean time at which the reaction changed from acute to maintenance phase was 7.4 s which is significantly different from RT (16.0 s). Mean dilation at this point (2.987%) was significantly different from MD (3.734%), but it was still 80% of MD in less than half of RT. ***Conclusion:*** Bi-linear fitting parameters better characterises the arterial dilatory response than MD and RT. Further stratification of clinical groups using bi-linear fitting may provide insight of the underlying physiology of vessel dilation for different pathologies.

Introduction

Continuous measurement of retinal vessel diameter, before, during, and after flicker-light (FL) provocation is a non-invasive method of assessing the autoregulation, endothelial function, and reactivity of retinal vessels [1–5]. FL provocation induces increased metabolic activity in the retinal tissue (blood vessels and neural tissue), leading to a cascade of reactions at vascular and neuronal level including nitric oxide synthase (NOS) as well as the release of glutamate (excitatory neurotransmitter) which raises intracellular CA^{2+} which then activates neuronal NOS contributing to retinal arteriolar diameter increase [6]. This reaction is often characterised by two measures: maximum dilation (MD) with respect to the mean diameter over 30 s prior to the first flicker onset[1] and the reaction time (RT), the time between flicker onset and MD. In healthy

[1]MD can also occur after FL cessation.

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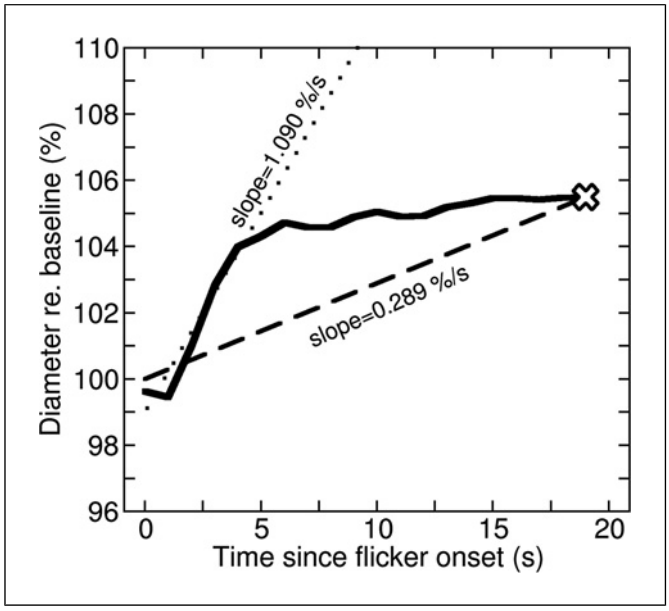

Fig. 1. Mischaracterisation of vessel reaction by MD and RT. (Solid line) Typical arterial vessel reaction curve during FL provocation. White cross denotes RT and MD. Rate of reaction determined using MD/RT (dashed line) not only mischaracterises the vessel reaction by ignoring the plateau but can substantially underestimate the initial rate of reaction (dotted line). Regression line for initial reaction estimated by eye.

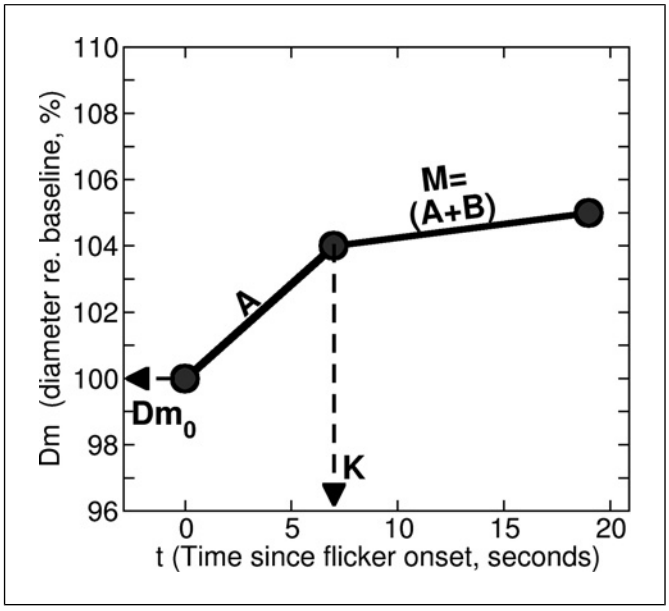

Fig. 2. Illustration of the bi-linear equation. Graphical explanation of the bi-linear equation (equation 1). Dm_0 (%) is the diameter at time $t = 0$; A (%/s) is the slope of the acute phase; K (s), the knee point, is the time at which the slope changes; B is the change in slope from before to after K, though we generally report M, the slope after K, given by A+B.

subjects, reported values for MD lie in the range 3–7.4% depending on what FL protocol and analysis has been applied [7–11], and RT is 14.5–19.3 s [9–16]. Together, MD and RT have been used to characterise the strength (MD) and speed (RT) of the increase in vessel diameter due to FL provocation.

If it is assumed that the change in vessel diameter from FL onset to MD is well characterised by a linear function then the rate of change in normalised arterial diameter, SlopeAD, can be approximated by SlopeAD = MD/RT (%/s). Indeed, previous work [17–19] has reported such a parameter and found for healthy controls a rate of change in normalised arterial vessel diameter of 0.250%/s consistent with a mean RT of 16 s and mean MD of 4% (more recent work [15, 20] shows a steeper reaction in healthy controls of up to 0.5%/s, though no discussion of this difference is offered). In contrast, most published data on the FL response of healthy arteries show that typically, they exhibit a two-stage reaction; an initial rapid increase in diameter (over 5–10 s) followed by a plateau (or very gentle increase) until FL offset [5, 7, 21, 22] (see continuous black line in Fig. 1). The initial rapid increase over 5–10 s followed by a plateau seems incompatible with an average RT of 16 s and suggests that the value for SlopeAD (dashed line) is not a true reflection of the vessel reaction in healthy subjects. Indeed, earlier work [21] in which a linear function was fitted over the first 10 s of flicker found a slope of between 0.367%/s and 0.585%/s (depending on the wavelength of FL), though this analysis does not reveal when or if the vessel reaction changes during FL.

If, for each arterial profile during FL provocation, we determine the best fitting bi-linear equation (Fig. 2), then we can identify three features of the reaction curve: the slope of the acute phase; the point at which the vessel reaction changes from acute to maintenance (the knee point), and the slope of the maintenance phase. In Figure 3a–c, the filled circles connected with dotted lines illustrate bi-linear characterisations of the vessel reaction during flicker (see methods). In these cases, the two-stage nature of the arteriolar reaction has been well characterised by the bi-linear equation. Note that while there is little difference between MD and the dilation at the knee point (D_k), there is no systematic relationship between RT and the time of the knee point, K. We contend that while MD does seem to capture the overall strength of the reaction, RT is not a good measure of its speed. If our hypothesis of a two-stage reaction with an initial phase lasting 5–10 s is correct

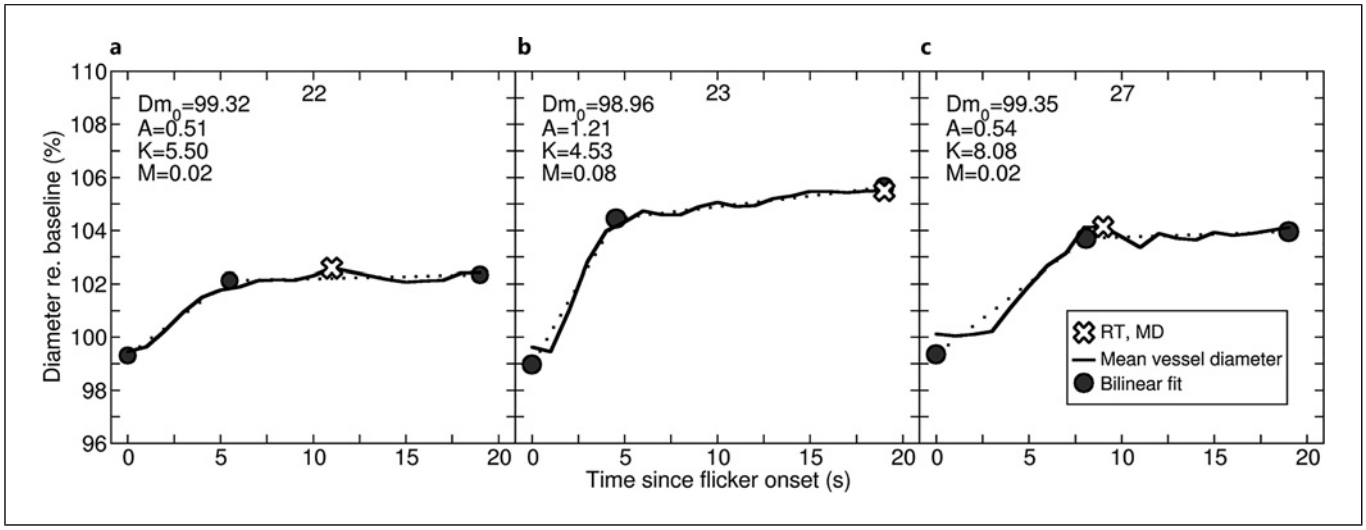

Fig. 3. Example retinal arterial dilatory profiles. Typical normalised arterial diameter reactions to FL (solid line), their bi-linear fit (dotted line and circles), and RT and MD (white cross). In each case, the bi-linear fit is good and illustrates there are two stages to the reaction. In **a** and **b**, RT is a poor descriptor of the reaction curve and does not coincide with the knee point, K. In **c**, RT happens to coincide with K and does provide a good descriptor of the reaction. In all cases, the bi-linear fit is significantly better than a linear fit. Dm_0 = diameter at flicker onset; A = slope of acute phase; K = knee point; M = slope of maintenance phase; D_k = dilation at knee point.

then, over a sample of healthy adults, we would expect K to lie in that range and have a weak or non-existent correlation with RT.

In healthy subjects, the elasticity of the artery is such that the vessel rapidly reaches a high proportion of its maximum dilation. A healthy cardiovascular system – vasodilation and vasoconstriction factors are balanced, with good arterio-venous coupling – can maintain this level of dilation for the remainder of the period of increased metabolic demand. A lack of elasticity, decrease in bioavailability of NO, or a standing level of vessel dilation due to disease (e.g., diabetes mellitus (DM) and cardiovascular disease) could lead to attenuated reactivity [2–26], slower reactivity [10], or possibly an unstable level of dilation during provocation. It is not possible to characterise these different types of vessel dysfunction using sequential diameter response analysis (SDRA) (e.g., MD and RT) alone. Hence, stratification between disease stages and/or groups is not possible based on absolute dilatory capacity MD only, whereas the full dilatory reaction profile might provide further clues into the underlying mechanisms and possible differences between groups. The purpose of this paper was to establish whether we can use a bi-linear equation to effectively characterise the reaction of retinal arteries during FL provocation in a group of healthy individuals, and, if so, to determine the mean duration and mean rate of the initial acute reaction.

Methods

Subjects

Forty-five healthy individuals without systemic or ocular abnormalities were included in this study. Exclusion criteria were presence of connective tissue disease, cancer, stroke, DM, hypertension, atrial fibrillation, stroke, history of any ocular disease, history of neurological diseases associated with loss of visual function, or any type of ocular surgery. Ethical approval was obtained from the Audiology/Optometry Research Ethics Committee at Aston University (ref no: AO2011.03). Written informed consent was received from all individuals taking part in the study. This study has been designed and conducted in accordance with the Declaration of Helsinki. Specific details of the study group can be found in Table 1.

Study Protocol

A full history and examination took place to ensure that subjects were free from any disease as outlined in the exclusion criteria. All subjects were instructed to refrain from consuming caffeinated products, chocolate, drinking alcohol, and smoking on the study day. All subjects underwent the test procedure as outlined below. Intraocular pressure was measured using non-contact tonometry (Keeler Pulsair, Keeler Ltd., UK).

Table 1. Demographic information

			Min	Max
Gender	Male	26	–	–
	Female	19	–	–
Age, years	Mean (st. dev.)	34.4 (11.6)	17	69
IOP, mm Hg	Mean (st. dev.)	13.2 (3.0)	7	20
SBP, mm Hg	Mean (st. dev.)	118.8 (11.8)	87	146
DBP, mm Hg	Mean (st. dev.)	73.5 (9.4)	40	96
MAP, mm Hg	Mean (st. dev.)	88.6 (9.2)	61.7	110.3
OPP, mm Hg	Mean (st. dev.)	45.9 (6.4)	28.1	61.6

IOP, intraocular pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; OPP, ocular perfusion pressure.

Dynamic Retinal Vessel Assessment

To ensure stable BP [27], systemic blood pressure was measured using a digital sphygmomanometer (Digital BP Monitor UA-767EX-C; PMS Instruments) and once full pupil dilation (using 1% tropicamide, Chauvin Pharmaceuticals Ltd., Kingston-Upon-Thames, UK) was reached the mean diameter of a segment of superior temporal retinal artery (>90 AU [28]) was recorded using a Retinal Vessel Analyser (RVA; ImedosSystems GmbH, Jena, Germany). The selected segment was at least 387.5 μm (mean length = 738.9 μm) and was located between 1.5 and 2 disc diameters away from the margins of the optic nerve head. Retinal diameters were measured continuously at a sampling rate of 25 Hz. Stimulation of retinal blood vessels was achieved by interrupting the fundus illumination with an optoelectronic shutter (12.5 Hz) [8]. The baseline diameter of the retinal artery was recorded for 50 s followed by three cycles of 20 s FL provocation each with 80 s recovery time.

Sequential Diameter Response Analysis

We took the normalised diameter readings for each flicker cycle (in 1 s time bins) from the RVA software. Any missing values were filled in using linear interpolation before the recordings were averaged over the three flicker cycles. Values for arterial MD and RT were calculated [8]. Usually, RT and MD are computed over the whole measurement cycle but our main analysis is concerned with the 20 s of FL provocation, so for comparison, we also computed RT and MD over the flicker cycle only: RT_{FL} and $MD_{FL.}$

Two-Stage Reaction Analysis Using a Bi-Linear Equation

Mean normalised vessel diameter during FL was characterised with the following bi-linear equation:

$$\mathrm{Dm}(t) = \mathrm{Dm}_0 + \mathrm{A}t + \mathrm{B}\,\mathrm{I}(t > \mathrm{K}) \quad (1),$$

where I (t>K) = t if t>K, or 0 otherwise, and Dm(t) is the diameter at time, t, since FL onset (seconds), Dm_0 is the estimated vessel diameter (% re-baseline) at FL onset, A is the rate of change in diameter (%/s) during the acute phase, K (s) is the time at which the arterial reaction changes from the acute phase to the maintenance phase (hereafter called the knee point), and B is the change in slope from the acute to the maintenance phase. Specifically, M = A+B is the rate of change in diameter during the maintenance phase. An illustration of the parameters of equation 1 is explained in Figure 2. The best fitting parameters for each flicker reaction were obtained using the Nelder-Mead simplex method [29] by minimising the sum-of-squared errors between measured and fitted values. For each set of arterial diameters, the fitting algorithm was run 100 times with slightly different initial conditions and the parameters with the smallest sum-of-squared errors taken as best fit.

Statistical Analysis

Not all healthy arteries exhibit a strong two-stage reaction, or even any reaction at all, to FL (such individuals may be outliers on a spectrum of healthy reactions, or the lack of a reaction is indicative of an as-yet undiagnosed condition). So, in addition, we fitted the reaction curves with a simple two-parameter linear regression. Since the linear regression is nested inside the bi-linear equation (i.e., B is fixed at 0 in equation 1; hence, K is irrelevant), it is possible to use the nested F test to determine if the extra parameters of the bi-linear fit (B and K) are truly characterising extra features of the vessel reaction or simply overfitting; in general, the greater number of free parameters in an equation the better the resulting fit. In order to impose a conservative criterion on accepting a bi-linear as opposed to a linear fit as a better descriptor of the FL reaction curve, we tested significance at the 1% level. The probability associated with the computed F ratio was obtained using the FDIST function from LibreOffice 3.5.7.2 (LibreOffice foundation). All other parameters, i.e., SDRA-derived RTs and percentage vessel dilation and bi-linearly derived knee points and percentage vessel dilation, were compared using a paired t test where significance was computed at the 5% level.

Table 2. Parameters obtained from bi-linear fitting and SDRA

	All subjects (n = 45)		Subjects with bi-linear fit than linear fit (n = 39)	
	mean	st. dev.	mean	st. dev.
Bi-linear fit				
Dm_0, %	99.560	0.978	99.489	1.017
A, %/s	0.506	0.540	0.620	0.403
K, s	7.375	3.250	7.225	2.627
M, %/s	0.012	0.116	0.026	0.062
D_K, %	2.987	1.627	3.380	1.328
SDRA				
RT, s	16.022	4.784	16.256	3.965
MD, %	3.734	1.797	4.1437	1.547
SlopeAD, %/s	0.244	0.157	0.250	0.107
RT_{FL}, s	14.289	3.935	14.667	3.089
MD_{FL}, %	3.674	1.783	4.081	1.539
$SlopeAD_{FL}$, %/s	0.259	0.152	0.265	0.102

Dm_0, diameter at flicker onset; A, slope of acute phase; K, knee point; M, slope of maintenance phase; D_k, dilation at knee point; RT, reaction time; MD, maximum dilation; SlopeAD, slope of reaction to MD; RT_{FL}, time at which maximum dilation during flicker is reached; MD_{FL}, maximum dilation during flicker; $SlopeAD_{FL}$, slope to MD_{FL}.

Results

The mean parameters from individual fitting the bi-linear equation for each subject and parameters from SDRA can be found in Table 2 (the parameters for all 45 fits and graphs of each fit in comparison with the raw data can be found in online suppl. material; for all online suppl. material, see https://doi.org/10.1159/000541443). The mean parameters over all the 45 bi-linear fits are as follows: acute slope, A = 0.506%/s (SD = 0.540); knee point K = 7.375 s (3.250); maintenance slope, M = 0.012%/s (0.116). The mean dilation at the knee point, D_K, is 2.987% (1.627).

In line with previous work, we find mean RT of 16.0 s (SD = 4.8) and MD of 3.734% (1.797). When computed only during FL duration, RT_{FL} = 14.3 s (3.9) and MD_{FL} = 3.674% (1.783). A paired t test reveals that there are significant differences between the group means for RT and RT_{FL} ($t(44) = 3.474$, $p = 0.001$) and between MD and MD_{FL} ($t(44) = 2.696$, $p = 0.01$). These significant differences reveal that for some participants, a residual dilation is occurring after FL provocation.

As expected (cf. Fig. 1), the slope calculated from dividing MD by RT is much shallower than A. Here, SlopeAD = 0.250%/s (0.107) and $SlopeAD_{FL}$ = 0.265%/s (0.102) which is around 50% of A. These values are consistent with some of the previously measured values of 0.250%/s [17], 0.261%/s [18] but not 0.5%/s [15]. The variability in these measurements is likely down to small n. Values here are consistent with those found by Kotliar et al. [21] who fitted a linear function over the first 10 s of the FL-induced arterial response.

A number of paired-sample t-tests revealed significant differences between the individual bi-linear parameters and their SRDA counterparts. There are highly significant differences between K and RT ($t(\text{df} = 44) = 12.795$, $p < 0.001$), between K and RT_{FL} ($t(\text{df} = 44) = 10.064$, $p < 0.001$), between D_K and MD_{FL} ($t(44) = 8.511$, $p < 0.001$), and between D_K and MD ($t(44) = 9.227$, $p < 0.001$). Although there is a significant difference between D_K and MD, D_K is, on average, 73% of MD despite the fact that K is only 48% of RT; i.e., almost three-quarters of the MD occurs in approximately half the RT.

Of the 45 sets of bi-linear parameters, 39 were significantly better than a linear fit at the 1% significance level (i.e., $F(2, 16)>6.2$). Based on rank order of MD_{FL}, the six fits which did not meet significance were among the eight with smallest MD_{FL}, and generally had RT at the extremes (≤7 s or ≥19 s). There was no systematic association between demographic factors and those individuals who had a better linear fit. For those 39 individuals with a better bi-linear fit, the mean correlation between the fitted function and the data is 0.961; therefore, on average, the bi-linear fit accounts for 92.5% of the variance in the vessel profiles during flicker.

There is a significant moderate correlation between K and RT for all 45 subjects (Pearson's $r = 0.415$, $p = 0.005$). However, once the 6 subjects whose bi-linear fit was not significantly better than their linear fit are removed from the analysis, there is no significant correlation between K and RT (Pearson's $r = 0.196$, $p = 0.232$). That these 6 outliers are responsible for such a substantial change in correlation between K and RT is indicative of their strong linear reaction to FL. Further when the bi-linear parameters genuinely describe the form of the arterial reaction curve, it is clear that K is capturing a change in reactivity.

Discussion

Our results clearly illustrate (visually and numerically) that previous measures of the reaction to FL (MD and RT) in healthy arteries are insufficient to characterise its biphasic dilatory time course; an initial acute reaction (~0.5–0.6%/s) for ~7 s followed by a period where little

change in arterial diameter occurs until FL cessation. While MD in and of itself may still be a useful indication of total dilatory capacity, neither RT nor the derived measure SlopeAD can be considered to be an accurate descriptor of the time course of arterial dilation, since the underlying assumption of those measures is that the arterial dilation follows a linear increase to the point of MD.

Riva and colleagues [30] fitted an exponential function to data from 9 healthy individuals [7, 30] who used 60 s of FL provocation with varying rates of flicker (2–64 Hz). For the data from 8 Hz, the time constant for the exponential fit was 7–12 s, consistent with the value of K here.

The value of K is also consistent with analogous results relating to the time course of ONH blood flow in cat during FL provocation described by Riva and colleagues [31]. They found a rapid increase in blood flow within 10–15 s of FL onset followed by a rapid drop after FL cessation.

The main purpose of fitting the bi-linear equation to the measured diameters is to parameterise the vessel profile data. Another reason is to smooth the data to reduce the effects of noise (e.g., from pulsation of the vessel, eye movements, or uncertainty in the measuring system). Mroczkowska and colleagues [17, 18] smoothed the vessel profile data by fitting a high-order polynomial (15th [17, 18] or 20th [15]) to the complete cycle of measurements (i.e., Baseline + Flicker + Recovery) and using SDRA on the polynomial fit to extract MD and RT [15, 17, 18]. While extracting MD and RT from a smoothed fit is likely to reduce variability in their estimation the extracted parameters still suffer from the limitations described in the introduction, namely, that they do not characterise well the vessel profile. The advantage of the bi-linear equation used here or the exponential function [7, 30] is that parameters of the equation are directly related to identifiable features of the time course of arteriolar dilation.

Since the bi-linear equation is a descriptive rather than a functional model of the time course of FL-induced arterial dilation, it is not possible to ascribe specific parameters of the bi-linear equation to specific physiological processes. It is, however, possible to take what we know of the relevant physiological processes and work out how they might contribute to the shape of the function. Further, we can make predictions about how the various parameters may change in different clinical groups.

The exact time course of retinal vessel dilation to FL is the result of a complex interplay of many processes and components which are still poorly understood. Some authors have analysed the area under the reaction curve [32]. While this provides some measure of "strength" of the reaction, it does not capture the characteristics of the time course beyond that of MD, MC and RT analyses. Trivially, the dilation is dependent on changes in blood flow due to increased metabolic demand, though the specific effects caused by the change in blood flow are dependent on the vessel structure (elasticity, current level of dilation, smoothness of muscle cell walls, health of pericytes), availability of endogenous NO, and the state of neurovascular coupling by glial cell signalling, venous compliance, and endothelial function. The degree to which each of these factors is impaired will depend on the underlying pathology; hence, an analysis of the time course of the reaction (as we do here) may be able to assign specific deviations from normal to specific dysfunctions.

Indeed, Mishra and Newman [33] demonstrated a biphasic arterial dilation to flicker provocation in rats. Although the exact time course of the biphasic dilatory profile in their healthy rat group is different than that observed in humans, it shows a similar rapid initial increase during the initial part of flicker, although that is followed by a moderate decrease in vessel diameter. Despite the differences between rat and human results, the work may provide valuable information on the underlying mechanism of the rapid response to FL provocation; Mishra and Newman [33] further highlight that in their cohort, the reduction in functional hyperaemia (=vessel dilation) was most likely due to disruption in glial cell signalling to the vasculature rather than altered neuronal responses and or a decrease in retinal thickness. The impairment in glial signalling was attributed to a possible increase in NO because the dilatory response was restored through iNOS (inducible nitric oxide synthase) inhibition. We argue that glial cell signalling may account for the majority of the initial rapid FL-induced arterial reaction in humans.

The maximum vessel dilation to FL is likely to be attenuated if the number of photoreceptors has decreased and/or their response impaired. Unfortunately, abnormalities of vascular function and neuronal function are often coupled. For examples, changes in retinal thickness are often age-related [34] and dependent on overall vascular health status, cardiovascular/vascular disease stage and consequently the causes of a weakened response cannot easily be separated; e.g., glaucoma (vascular insufficiency and photoreceptor damage), DM (vascular insufficiency and its impact on photoreceptor metabolism) [35, 36]. Indeed, Gugleta and colleagues [35] highlighted the fact that the location of vascular reactivity measurements in their sample of primary open angle glaucoma (POAG) patients is not the same as the location of ONH damage; therefore, retinal nerve fibre layer thickness

differences at the ONH level cannot explain the reduction of vascular dilation alone. Despite the fact that vascular changes and photoreceptor loss can co-occur, there are a large number of vascular diseases in which they do not (i.e., heart disease, hypertension, arteriosclerosis); hence, a model of the time-dependent vessel response (such as the descriptive equation here) should be better able to tease apart the factors of vessel reactivity affected by CVD (such as changes in acute reaction slope) than is possible with spine point analysis [4] whose parameters are not representative of the whole reaction to FL.

Previous work [35] has found reduced arteriolar and venular MD in POAG compared with healthy controls during FL. The diminished dilation was attributed to a vascular insufficiency in POAG rather than the loss of ganglion cells because there was no correlation between visual field mean defect and MD [35]. An earlier study [37] examining early glaucoma patients which had shown a reduced venous but not arterial dilation to flicker was used to support the conclusion that reduced dilatory response is due to vascular insufficiency rather than ganglion loss. In contrast, a more recent study [38] found similar dilatory capacity in patient with POAG independent of branching level and associated visual field loss. The authors however concluded that for a better understanding of the underlying disease mechanisms and to rule out other vascular abnormalities, the characterisation of the vascular reaction would be a useful measure. The lack of correlation between dilatory response and visual field mean defect and retinal nerve fibre layer thickness does not, however, rule out that ganglion cell loss has not contributed to the reduction of dilation in POAG since the full dilatory profile was not reported in all studies, hence we do not know the shape of the FL-induced reaction. One of the limitations of this particular study identified that the last 2 s of the FL-induced diameter change was averaged to represent the maximal dilatory capacity and that there was considerable overlap of the flicker response between groups. Hence examining the full-time course of FL-induced arterial response, as we attempt here, may provide a better discrimination between groups; correlations may exist between one or more of the bi-linear parameters and ganglion cell loss.

Study Limitations

The present study focussed only on healthy individuals and the retinal arteriolar response to FL provocation. While our sample represented a wide age range of participants, analysis to compare SDRA and bi-linear fit derived parameters was conducted using a pairwise method. Future analyses assessing larger number of participants with and without pathologies will allow for validation and exploration of age (and pathology) dependent changes. While age has not been consistently reported to impact arterial reactivity, this may be different when considering the entire vessel profile reactivity at baseline, during flicker and recovery.

The specific manner in which these physiological deficits affect the biphasic reaction, and hence the bi-linear parameters, remains to be demonstrated. We suspect that in individuals with visual field/ganglion cell loss, the rapidity of the acute dilatory response may be reduced possibly eliminating the biphasic reaction and thus resulting in a linear increasing response during FL. Whereas in conditions such as arteriosclerosis or where vessel elasticity is changed but no ganglion cell loss is present, we would expect to observe a biphasic reaction with the acute phase intact but an overall depression of the vessel diameter reflecting a loss of dilatory capacity.

Statement of Ethics

The study was approved by the Audiology/Optometry Research Ethics Committee at Aston University (ref No. AO2011.03) and adhered to the Declaration of Helsinki. All included participants provided written informed consent. For data analyses, all data were anonymised.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Conceptualisation: R.J.S. and R.H.; data acquisition: R.H.; data analyses: R.J.S.; and manuscript writing: R.J.S. and R.H.

Data Availability Statement

The data that support the findings of this study are not publicly available due to containing information that could compromise the privacy of research participants but are available from the corresponding authors (R.J.S. and R.H.) upon reasonable request.

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