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Abstract: Diabetic retinopathy (DR) may evolve from retinal neurovascular dysfunction and oxidative stress. We therefore investigated the effects of euglycaemic (6 mmol/l) and hyperglycaemic clamps (15 mmol/l) with insulin infusions (6 pmol/kg/min), with or without vitamin C (2 g intravenous), on pattern electroretinogram (PERG) and flicker-induced vasodilation responses in 12 adults with type 1 diabetes. The PERG ratio (0.8° vs. 7° checks) increased by (mean [95% C.I.]) 0.31 (0.03 – 0.59) after hyperglycaemia with vitamin C. Venule maximum dilations were absolutely increased by 1.7 (0.5 – 3.0) % after euglycaemic clamps. Arteriole maximum dilations were non-significantly larger with hyperglycaemia and vitamin C (P = 0.07 by ANOVA). Insulin, and possibly vitamin C, may improve retinal neurovascular function in type 1 diabetes.

Diabetic retinopathy (DR) is a potentially blinding complication of diabetes mellitus. Among people with a 20-year history of type 1 diabetes, roughly 92% will have some level of DR and 18% will have vision-threatening DR (1). DR may evolve in part as a consequence of sustained retinal neurovascular dysfunction, which in turn may be exacerbated by poor glycaemic control and oxidative stress.

Retinal flicker-induced vasodilation (2-7) and PERG responses (6-9) are potential sensitive markers of DR. Based on previous work (2-20), we hypothesised that flicker-induced vasodilation would improve and be impaired by euglycaemic and hyperglycaemic clamps, respectively, in people with type 1 diabetes. We simultaneously predicted that PERG responses, markers of ganglion cell function, would be stable across glucose conditions.

Twelve people with type 1 diabetes aged ≥ 18 years and no significant history of ocular or systemic disease were recruited. Smokers and those with known mild non-

proliferative DR or worse were excluded. The protocol followed the tenets of the Declaration of Helsinki and was approved by the St. Vincent's Hospital Melbourne Human Research Ethics Committee D (096/13). Written informed consent was obtained from all participants.

Participants were studied on two mornings at least two weeks apart. Six participants were randomised to receive vitamin C at their first visit. Double-blinding was used. We asked participants to cease all supplements from two weeks before their first visit. Participants fasted from midnight before each visit and were given individualised insulin advice. PERG and flicker-induced vasodilation responses were measured, in order: at baseline; after one hour of insulin and 30 minutes of euglycaemia; and after 30 minutes of hyperglycaemia. Two-field colour fundus photography, centred on the macula and optic disc, was done for both eyes with a Canon CR-2 digital non-mydriatic camera (Canon, Melville, NY, USA) with a viewing angle of 45°. DR was assessed by independent graders according to the Early Treatment Diabetic Retinopathy Study severity scale (21).

We used a two-stage euglycaemic and hyperglycaemic clamp based on the technique of DeFronzo and colleagues (22). After baseline tests, one 20 gauge cannula was inserted into the antecubital vein of each arm: one for infusions and one for blood samples. Insulin (Actrapid, Novo Nordisk Pharmaceuticals, Baulkham Hills, NSW, Australia) was infused at 6 pmol/kg/min. Glucose 25% was titrated to achieve euglycaemia (6 ±1 mmol/l). A computer algorithm guided glucose infusion rates (23). Blood glucose levels were measured every 10 minutes with the YSI STAT Plus (YSI Incorporated, Yellow Springs, OH, USA). After euglycaemic tests, intravenous vitamin C (2 g in 100 ml sodium chloride 0.9%; Biological Therapies, Braeside, VIC, Australia) or placebo (100 ml sodium chloride 0.9%) was infused over 10 minutes and the glucose rate was re-titrated to achieve hyperglycaemia (15 ±1 mmol/l). After insulin and glucose infusions were ceased, participants were provided with a meal and observed for 45 minutes.

PERG studies were performed on the left eye according to the ISCEV standard (24). DTL fibre electrodes were positioned along the lower eyelid margin. Gold cup skin electrodes were positioned over the lateral canthus (reference) and central forehead (ground). Signals were amplified by the Espion E^3 Electroretinography System (Diagnosys LLC, Lowell, CA, USA) with a sampling rate of 1.2 kHz and band-pass filter of 0.625 to 100 Hz at a digital resolution of 32 bits.

Participants were seated 57 cm from a 21-inch Cornerstone p1650 cathode ray tube monitor (Cornerstone, Fremont, CA, USA) connected to a Pattern Stimulus Generator (Diagnosys LLC, Lowell, MA, USA) with a frame rate of 100 Hz. Stimuli were stationary black and white checks with a mean luminance of 54 cd/m^2, and contrast of 100%. Steady-state responses with 0.8° and 7° checks were recorded from 50 sweeps of one second with a reversal rate of 8.33 Hz. Second harmonic (2F) amplitudes were extracted from eight cycles of 120 msec. using the discrete Fourier transform. The PERG ratio was defined as the ratio of 2F amplitudes with 0.8° and 7° checks. 2F amplitudes with smaller checks are more affected in glaucoma (25), a disease of retinal ganglion cells, and the PERG ratio is a better predictor of glaucoma incidence than individual 2F amplitudes (26).

Flicker-induced retinal vasodilation responses were measured in the dilated right eye with the Dynamic Vessel Analyzer (DVA; IMEDOS Systems UGI, Jena, Germany). Tropicamide 1% (Bausch & Lomb, Macquarie Park, NSW, Australia) was used for mydriasis. The fundus was examined with a viewing angle of 30° under green light with an average luminance of 130 cd/m^2. A unique fundus region was selected for eye tracking. We analysed an arteriole and venule segment within one half to two disc diameters from the optic disc margin. The same segments were used for repeated tests. Each test consisted of 50 seconds of constant light, followed by three cycles of 20 seconds of diffuse luminance flicker at 12.5 Hz and 80 seconds of constant light. Pre-flicker diameters (MU) and maximum dilations (%) were extracted for each vessel based on the average of three periods of 100 seconds (30 seconds pre-flicker, 20 seconds of flicker and 50 seconds post-flicker).

Conditions were compared by ANOVA. Post-hoc comparisons for significant differences used Student's *t*-tests with Sidak's adjustments for unbalanced levels. Reproducibility and reliability were assessed from one-way ANOVA estimates of the within-subjects standard deviation (SD) and intraclass correlation coefficient (ICC) (27). Data were analysed in STATA version 12.1 (StataCorp LP, College Station, TX, USA). Two-tailed $P < 0.05$ was considered significant.

Participants were (median [interquartile range]) aged 24 (20.5 – 30) years, with diabetes durations of 10.5 (3.8 – 17) years and an even number of males and females. Three participants had photographic signs of mild non-proliferative DR in at least one eye, despite documentation of no DR in the preceding year.

Functional parameters for each condition are described in **Table 1**. PERG parameters were stable, except for the PERG ratio, which increased after hyperglycaemia with vitamin C compared with baseline by (mean [95% C.I.]) 0.31 (0.03 – 0.59). Venule maximum dilations were 1.7 (0.5 – 3.0) % larger after euglycaemic clamps compared with baseline. Arteriole maximum dilations were non-significantly larger after hyperglycaemia with vitamin C. Pre-flicker vessel diameters were unaffected by any condition. Other than plasma glucose levels, no factor changed significantly from euglycaemia to either hyperglycaemia arm. Similarly, no significant differences were found between vitamin C and placebo at hyperglycaemia.

The reproducibility and reliability of functional parameters at baseline were estimated from their within-subjects standard deviation (SD) and intraclass correlation coefficient (ICC), respectively, expressed in **Table 2**. Flicker responses were generally reproducible within participants but more variable between participants. The reliability of the PERG ratio was better than individual second harmonic amplitudes, while flicker responses had excellent reliability.

Our study demonstrates that retinal venule dilations during flicker robustly improve during euglycaemic clamps in type 1 diabetes. The absence of hyperglycaemic effects during insulin infusions suggests that insulin improves retinal blood flow regulation and this is mediated by more than simple glucose-lowering. Retinal ganglion cell function is generally stable in the short-term. Antioxidants such as vitamin C might protect retinal neurovascular function in type 1 diabetes, but more evidence is required to prove this hypothesis.

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Table 1. Participant factors grouped by glucose conditions (*n = 12*)

Parameter	Baseline	Euglycaemia	Hyperglycaemia		*P*
			Vitamin C	Placebo	
Glucose, mmol/l	8.4 (3.6)	6.1 (0.8)**	15.0 (1.9)***	14.4 (0.8)***	< 0.001
Insulin, pmol/l	65 (24)	334 (63)***	330 (58)***	338 (75)***	< 0.001
Systolic blood pressure, mmHg	120 (11)	115 (7)	113 (10)*	112 (9)*	0.005
Diastolic blood pressure, mmHg	66 (7)	62 (3)	60 (5)*	61 (5)*	0.005
PERG					
0.8° 2F amplitude, μV	1.4 (0.5)	1.3 (0.4)	1.6 (0.3)	1.4 (0.5)	0.18
7° 2F amplitude, μV	1.3 (0.4)	1.1 (0.2)	1.2 (0.3)	1.2 (0.3)	0.45
PERG ratio	1.1 (0.3)	1.2 (0.3)	1.4 (0.6)*	1.2 (0.3)	0.03
Flicker responses					
Arteriole pre-flicker diameter, MU	125 (19)	125 (21)	122 (19)	123 (21)	0.12
Arteriole max dilation, %	3.2 (2.4)	3.6 (2.4)	4.4 (3.5)	3.7 (2.3)	0.07
Venule pre-flicker diameter, MU	143 (19)	142 (19)	141 (18)	140 (18)	0.22
Venule max dilation, %	3.3 (3.4)	5.0 (4.1)**	4.7 (2.8)	5.0 (3.7)*	0.002

Data are means (SD). Data for baseline and euglycaemia used the mean for each participant. *P <0.05, **P < 0.01, ***P < 0.001 vs. baseline.

Table 2. Reproducibility and reliability of parameters at baseline (n = 12)

Parameter	Between SD	Within SD	ICC (95% C.I.)
PERG			
0.8° 2F amplitude, μV	0.4	0.5	0.40 (0.00 – 0.89)
7° 2F amplitude, μV	0.3	0.3	0.41 (0.00 – 0.89)
PERG ratio	0.3	0.2	0.75 (0.50 – 1.00)
Flicker responses			
Arteriole pre-flicker diameter, MU	19.1	2.3	0.99 (0.97 – 1.00)
Arteriole max dilation, %	2.3	1.2	0.79 (0.57 – 1.00)
Venule pre-flicker diameter, MU	18.3	5.6	0.91 (0.82 – 1.00)
Venule max dilation, %	3.2	1.9	0.74 (0.48 – 1.00)

Data are estimates from one-way ANOVA models.