Diabetologia (1993) 36: 1266–1274

Diabetologia

Impaired blood flow and arterio-venous shunting in human diabetic neuropathy: a novel technique of nerve photography and fluorescein angiography

S. Tesfaye[1], N. Harris[2], J. J. Jakubowski[3], C. Mody[4], R. M. Wilson[1], I. G. Rennie[4], J. D. Ward[1]

[1] Diabetes Research Unit, Royal Hallamshire Hospital, Sheffield, UK
[2] Department of Medical Physics, Royal Hallamshire Hospital, Sheffield, UK
[3] Department of Neurosurgery, Royal Hallamshire Hospital, Sheffield, UK
[4] Department of Ophthalmology, Royal Hallamshire Hospital, Sheffield, UK

Summary. New techniques of sural nerve photography and fluorescein angiography which are able to provide an index of nerve blood flow have been developed. Under local anaesthetic, 3 cm of sural nerve was exposed at the ankle using an operating microscope. Without disturbing the epineurium, vessels were identified and photographed at a standard magnification (× 30). These were independently graded by an ophthalmologist not otherwise involved with the study. Fluorescein angiography was then carried out on the exposed nerve. The fluorescein appearance time and intensity of fluorescence were quantified, using computer analysis of digitised images. Thirteen subjects with chronic sensory motor neuropathy, five non-neuropathic diabetic and nine normal control subjects were studied. The mean epineurial vessel pathology score of the neuropathic group was significantly higher than the combined normal control and non-neuropathic diabetic groups ($p < 0.01$). Direct epineurial arterio-venous shunting was observed in six neuropathic and one non-neuropathic diabetic patients and not in any of the normal control subjects. The nerve fluorescein appearance time was significantly delayed in subjects with chronic sensory motor neuropathy (51.5 ± 12 s) compared to both normal (34.7 ± 9 s, $p < 0.01$) and non-neuropathic diabetic subjects (33.4 ± 11 s, $p < 0.025$). The mean intensity of fluorescence at 96, 252 and 576 s, was significantly lower in subjects with chronic sensory motor neuropathy compared with both of the other groups ($p < 0.05$). The epineurial vessel pathology score was significantly related to reduced sural ($p < 0.01$) and peroneal ($p < 0.001$) nerve conduction velocities, elevated vibration ($p < 0.01$) and thermal ($p < 0.001$) perception and the severity of retinopathy ($p < 0.002$). The fluorescein appearance time was significantly related to reduced sural sensory ($p < 0.02$) conduction velocity, elevated vibration ($p < 0.01$) perception and epineurial vessel ($p < 0.002$) pathology score, but it failed to relate to peroneal motor ($p = 0.06$) conduction velocity, thermal ($p = 0.1$) perception and the severity of retinopathy ($p = 0.3$). Intensity of fluorescence was significantly related to fluorescein appearance time (at 96 s, $p < 0.001$; at 576 s, $p < 0.05$) but did not relate to measures of neuropathic severity. These techniques have enabled us to observe that epineurial vessel anatomy is abnormal and that nerve blood flow is impaired in subjects with chronic sensory motor neuropathy. In addition epineurial arterio-venous shunting may be a feature of diabetic neuropathy. These techniques may further be applied to study nerve blood flow in early diabetic neuropathy.

Key words: Diabetic neuropathy, sural nerve, nerve blood flow, epineurial vessel photography, fluorescein angiography, arterio-venous shunting, vasa nervorum.

Evidence for the role of microvascular disease in the pathogenesis of diabetic neuropathy is strong [1]. In streptozotocin-diabetic rats, there is an early reduction in nerve blood flow [2] and oxygen supplementation partially prevents a deterioration in nerve conduction velocity [3]. Furthermore, in a hypoxic environment, normal rats develop electrophysiological and morphological abnormalities similar to those seen in experimental diabetes in the absence of hyperglycaemia [3].

In man, nerve biopsy studies have shown that when there is significant diabetic neuropathy, severe microvascular abnormalities [4–10] are present. In addition, the degree of vessel disease correlates with the severity of nerve damage [6, 10] and the multifocal pattern of nerve fibre loss suggests ischaemia [8, 9]. Newrick et al. [11] demonstrated that sural nerve oxygen tension in vivo is reduced in patients with diabetic neuropathy. More recently we have shown that exercise-induced conduction velocity increment is impaired in diabetic neuropathy suggesting impaired nerve blood flow [12], and a relationship between nerve function and transcutaneous oxygen tension has also been reported [13].

However, almost all our current knowledge of diabetic neuropathy stems from studies on animal models and

Table 1. Clinical details of study subjects

	Diabetic neuropathic subjects	Non-neuropathic diabetic subjects	Normal control subjects
n	13	5	9
Age ± SD (years)	56.3 ± 5.6	51.6 ± 7.3	53.4 ± 5
Male : Female	11 : 2	3 : 2	7 : 2
Duration (years)	12.3 ± 10	26 ± 9	–
Type 1 : Type 2	6 : 7	4 : 1	–
HbA_1 (3.3–6.8%)	9.6 ± 2.1	8.9 ± 1.5	–
Creatinine (μmol/l)	88.5 ± 14	91.6 ± 23	88 ± 17
Retinopathy	9PR, 3BR, 1Nil	1PR, 3BR, 1Nil	All Nil
Neuropathy Score[a]	Stage 2, $n = 1$ Stage 3, $n = 12$	Stage 0, $n = 4$ Stage 1, $n = 1$	Stage 0, $n = 9$

[a] Neuropathy score: Stage 0, no neuropathy; Stage 1, asymptomatic neuropathy; Stage 2, symptomatic neuropathy; Stage 3, disabling neuropathy. PR, Proliferative retinopathy; BR, background retinopathy

human nerve biopsy specimens. There are no in vivo studies of nerve blood flow in man. In this paper, we describe a new in vivo technique of visualising epineurial vessels at high magnification. Fluorescein angiography, a technique normally used for visualising retinal vessels and sometimes used for assessing vascularity and predicting viability of tissues [14–20], was applied to provide an index of nerve blood flow. These techniques have enabled us to observe epineurial arterio-venous shunting and impaired nerve blood flow in human diabetic neuropathy.

Subjects and methods

Thirteen subjects with chronic sensory motor diabetic neuropathy, five non-neuropathic diabetic and nine healthy control subjects were studied (Table 1). Both Type 1 (insulin-dependent) and Type 2 (non-insulin-dependent) diabetic subjects participated in the study. Subjects were classified as having Type 1 diabetes on the basis of having a short history (< 2 months) of severe symptoms, marked weight loss, moderate or high ketonuria and insulin requirement within 1 year of diagnosis of diabetes. All subjects gave their informed consent and the study was approved by the local ethical committee.

All the diabetic and normal control subjects underwent the following:
1) Full history and examination which included neuropathic symptom and deficit scores;
2) Ankle pressure index using a Doppler ultrasound stethoscope, model BF4A (Med Sonics, Mountain View, Calif., USA) as described by Yao et al. [21];
3) Median and peroneal motor conduction velocities and median and sural sensory conduction velocities at a skin surface temperature of 33 ± 1 °C and a room temperature of 24 ± 1 °C, using a Dantec 2000 M electrophysiological system with signal averaging facility (Dantec Ltd, Bristol, UK);
4) Vibration perception threshold over the great toe and medial malleolus using the biothesiometer (Biomedical Instrument Co, Newbury, Ohio, USA);
5) Warm thermal discrimination threshold measurement on the dorsum of the foot with a thermo-aesthesiometer (Medical Instruments Dept., VU Hospital, Amsterdam, The Netherlands) using the forced choice method [22];
6) Autonomic function tests of R-R variation at rest, with deep breathing and standing using computer-assisted autonomic function testing [23] and a standard 12-lead ECG;
7) Standard 7-field, wide angle fundal photography taken with dilated pupils, which were graded by an ophthalmologist on a scale from 0 to 2 (0 = normal, 1 = background retinopathy, 2 = proliferative changes or previous laser treatment).

Exclusion criteria included: history of cardiac disease, the presence of significant peripheral vascular disease with either absence of foot pulses or ankle pressure index less than 1, the presence of other neurological disorders or peripheral neuropathies from causes other than diabetes.

Neuropathy staging was carried out using Dyck's neuropathy scoring on scale from 0 to 3, taking into account results of neuropathy symptom and deficit scores, nerve conduction, quantitative sensory testing and autonomic function tests (Stage 0, no neuropathy; Stage 1, asymptomatic neuropathy; Stage 2, symptomatic neuropathy; Stage 3, disabling neuropathy) [24].

Table 1 shows the clinical characteristics of the subjects. Sural nerve epineurial vessel photography was immediately followed by fluorescein angiography in all subjects, but because of technical difficulties two subjects, one neuropathic and one normal control aged 68 and 52 years, respectively did not have fluorescein angiography. One normal control subject aged 59 years did not have sural nerve photography. Despite this, all the groups that underwent each procedure were matched for age and the diabetic groups, for glycaemic control. The duration of diabetes was significantly higher in the non-neuropathic diabetic group compared to the neuropathic group ($p < 0.02$), as we deliberately selected patients who had no neuropathy after a long period of diabetes. All subjects had normal serum creatinine. All of the subjects in the diabetic neuropathy group had chronic sensory motor neuropathy with autonomic features, all but one having Dyck's neuropathy score of 3 (severe disabling neuropathy). One subject in the non-neuropathic diabetic group had asymptomatic neuropathy (Table 1).

Photography of epineurial vessels in sural nerve

Subjects were admitted to hospital as out-patients to undergo sural nerve epineurial vessel photography in a neurosurgical operating theatre. The procedure, which had been randomly allocated took approximately 90 min, and was performed by the same neurosurgeon who had no previous knowledge of the subjects' clinical state.

At the start of the procedure blood glucose was measured in all diabetic subjects and was found to be 7–11 mmol/l. A venflon catheter was inserted into the cephalic vein and a 5% glucose infusion delivered throughout the procedure to avoid hypoglycaemia. After 1% lignocaine skin infiltration, a linear incision approximately 4 cm long was made 1 cm posterior to the lateral malleolus. With the aid of an operating microscope (Wild M690; Leica Heerbrugg AG, Heerbrugg, Switzerland) the sural nerve was identified and 3 cm of the nerve was exposed. Extreme care is needed to ensure that the

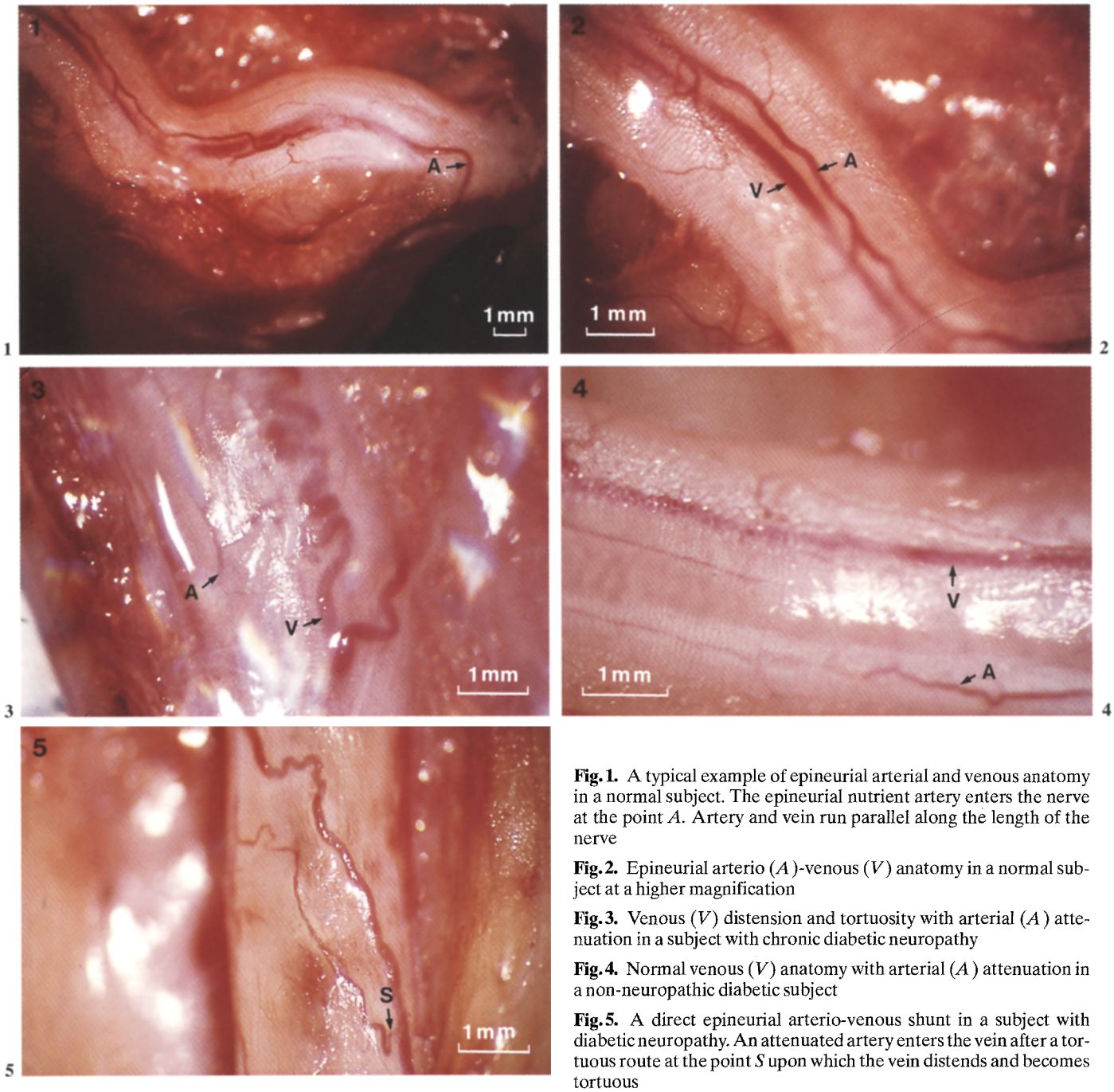

Fig. 1. A typical example of epineurial arterial and venous anatomy in a normal subject. The epineurial nutrient artery enters the nerve at the point *A*. Artery and vein run parallel along the length of the nerve

Fig. 2. Epineurial arterio (*A*)-venous (*V*) anatomy in a normal subject at a higher magnification

Fig. 3. Venous (*V*) distension and tortuosity with arterial (*A*) attenuation in a subject with chronic diabetic neuropathy

Fig. 4. Normal venous (*V*) anatomy with arterial (*A*) attenuation in a non-neuropathic diabetic subject

Fig. 5. A direct epineurial arterio-venous shunt in a subject with diabetic neuropathy. An attenuated artery enters the vein after a tortuous route at the point *S* upon which the vein distends and becomes tortuous

epineurium and entering vessels are not traumatized or damaged which requires considerable expertise as the nerve is only 2–3 mm in diameter. Nerve surface temperature was then measured using a Novo digital thermometer (CP Instruments, Bishop's Stortford, Herts., UK). A 2-cm longitudinal length of the nerve was photographed at a standard magnification of × 30 using a Leitz 35 mm camera. These standard photographs of epineurial vessels were graded by an ophthalmologist who was unaware of the subjects' clinical state and was not involved in any other part of the study. Each of the following features of the arteries and veins (a–e) were graded on a scale from 0 to 3 (0 normal, 1 mild, 2 moderate, 3 severe abnormality) as follows:

Arteries: a) calibre b) degree of tortuosity c) severity of arterio-venous shunt (if present)
Veins: d) calibre e) degree of tortuosity

These were added to give separate scores of the arterial (a + b + c) and venous (d + e) pathology which were in turn combined to give the epineurial vessel pathology score (EVPS) with a maximum possible score of 15.

Sural nerve fluorescein angiography

Fluorescein angiography was then carried out on the exposed nerve at the standard magnification of × 30. One millilitre per 20 kg body weight of 20% sodium fluorescein (minimum 3.5 ml) was administered intravenously over 5 s. Nerve fluorescence was recorded for 15 min using a television camera incorporated to one arm of the operating microscope. Initially this was carried out using a Sony (AVC-3250 CES/K, Japan) television camera, but in later studies this was re-

Table 2. Results of epineurial vessel photography

Age (years)	Duration (years)	HbA_1 (3.5–6.8%)	Retina	FAT	Arterial			Venous		Total
					↓Calibre	Tortuosity	A-V Shunt	↑Calibre	Tortuosity	(EVPS)
Normal control subjects										
58	–	–	0	34	1	1	0	1	1	4
54	–	–	0	44	1	1	0	1	0	3
42	–	–	0	46	0	0	0	1	1	2
54	–	–	0	23	0	0	0	1	0	1
50	–	–	0	39	0	0	0	0	0	0
52	–	–	0	–	0	0	0	0	0	0
54	–	–	0	25	0	0	0	0	0	0
58	–	–	0	38	0	1	0	0	0	1
Non-neuropathic diabetic subjects										
51	13	10.4	2	40	1	0	1	1	1	4
45	27	8.9	1	27	0	1	0	0	0	1
48	27	10.3	1	22	0	0	0	0	1	1
50	39	7.3	0	26	2	1	0	0	0	3
Chronic sensory motor neuropathy										
50	16	13.3	2	32	2	1	1	2	0	6
47	25	10.2	2	40	1	0	2	1	0	4
68	28	8.7	2	–	2	0	2	2	2	8
60	32	10.0	2	34	1	0	0	1	1	3
69	6.0	8.0	2	65	3	1	0	1	1	6
56	10	12.0	2	56	0	1	0	0	1	2
60	15	7.0	2	65	1	3	3	0	3	10
62	1.5	11.3	1	48	1	3	2	1	0	7
54	4.0	11.9	2	64	2	0	1	3	3	9
58	2.5	8.1	1	42	1	0	0	0	1	2
43	4.5	5.9	0	49	2	1	0	0	1	4
55	12	10.2	2	53	1	0	0	3	3	7
62	1.0	8.3	1	70	1	1	0	2	3	7

Retina: 0 = Normal, 1 = Background, 2 = Proliferative retinopathy or previous laser treatment. Arterial and venous pathology scores: 0 = normal, 1 = mild, 2 = moderate, 3 = severe abnormality. FAT, Fluorescein appearance time; EVPS, epineurial vessel pathology score

placed by high sensitivity Newvicon television camera (Panasonic WV1460, Matsushita, Japan). The pictures were recorded on a Sony U-Matic video recorder with a date and time signal superimposed. A yellow-green 540 nm barrier filter (Baird Atomic Inc., Bedford, Mass., USA) was placed in front of the camera to remove the non-fluorescent background illumination. The nerve was illuminated using the operating microscope light source fitted with a blue 480 nm exciter filter (Baird Atomic). Fixed settings were used for the camera aperture and the intensity of microscope light source.

Data analysis

The time taken for the fluorescein to first appear in the epineurial vessels after the injection (fluorescein appearance time) was determined to the nearest second, using the time display on the video recording. The fluorescein appearance time was unaffected by the choice of video camera, however, the high sensitivity Newvicon camera allowed quantitative measurements of nerve fluorescence using computer analysis of the video images. A sequence of 48 video frames was acquired and digitised from the video recording at 12-s intervals from the start of the injection of fluorescein. This was carried out using a Dell 286 computer fitted with a AT Vista video graphics adaptor (True Vision Inc., Indianapolis, Ind., USA) with an 8 bit, 256*256 pixel resolution. The computer images were colour-coded and show the change in intensity from frame one at the start of the injection.

The intensity of fluorescence was measured from two regions of 16*16 pixels on opposite side of the nerve and the mean value calculated. The regions were selected from areas which were furthest away from the epineurial vessels and these were analysed at frames 8, 21, 31 and 48 (corresponding to 96 s, 252 s, 372 s and 576 s after the injection). At this magnification (× 30), any slight movement of the foot will produce significant movements of the nerve within the field of the microscope. Therefore, at each frame the position of the regions was checked and if necessary realigned, using the position of epineurial vessels and the edge of the nerve as a guide.

Statistical analysis

Statistical analysis was carried out using SPSS/PC + Release 5 (SPSS Inc., Chicago, Ill., USA). All data were analysed using the Mann-Whitney U-test and Spearman's rank correlation.

Results

Photography of epineurial vessels in sural nerve

Figure 1 shows a typical example of epineurial arterial and venous anatomy in a normal subject. The epineurial nutrient artery enters the nerve as shown and runs parallel to the vein along the length of the nerve. Figures 2–4 show examples from each group taken at the standard magnification (× 30). The diameter of the sural nerve is between 2–3 mm.

The results of the epineurial vessel photography are summarised in Table 2. The quality of the sural nerve photography of one non-neuropathic subject was not considered satisfactory. The scores for both the arterial and

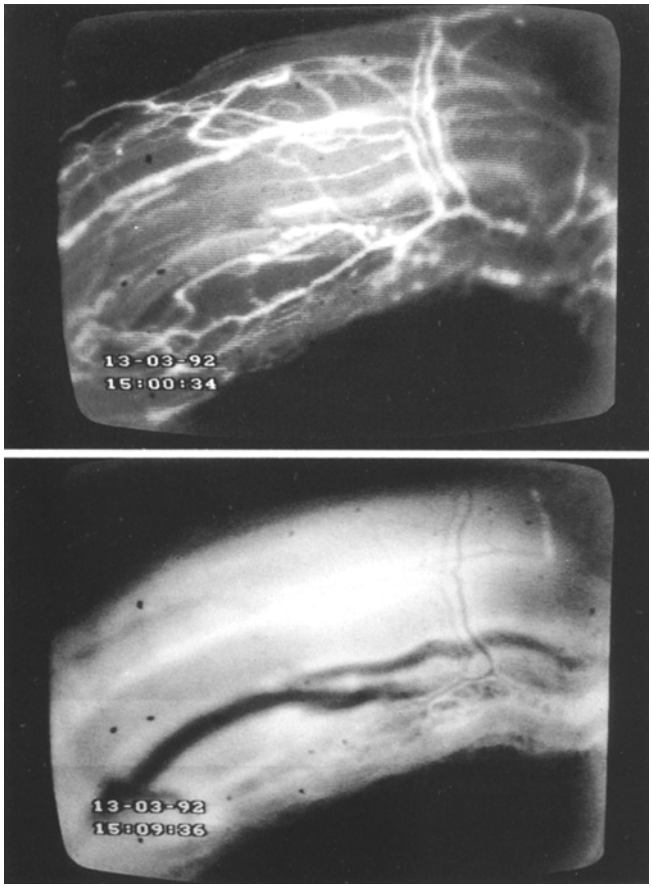

Fig. 6. Sural nerve fluorescein angiogram of a normal subject. *Top panel* taken at appearance of fluorescein. *Bottom panel* taken 576 s after injection

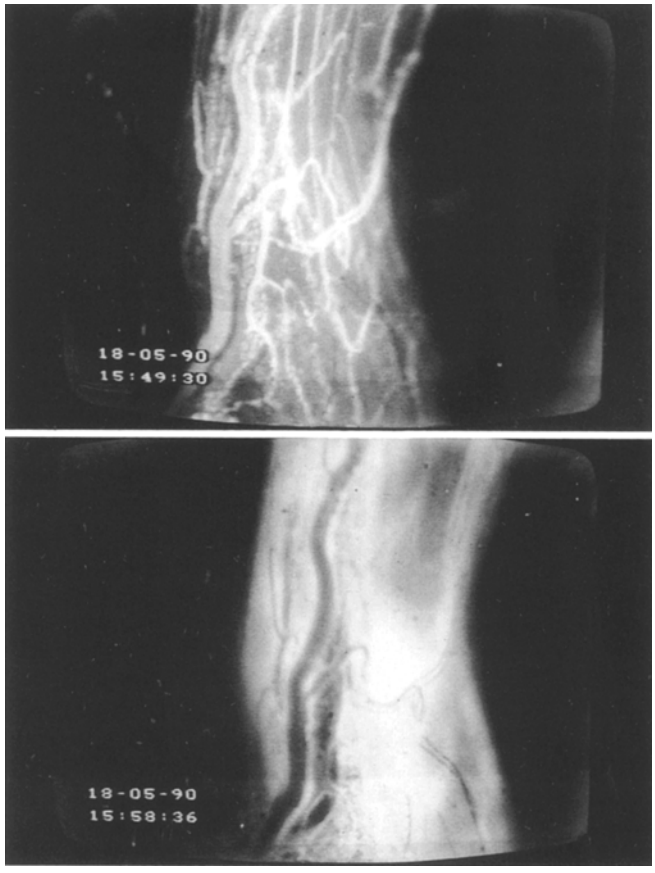

Fig. 7. Sural nerve fluorescein angiogram of a non-neuropathic diabetic subject. *Top panel* taken at appearance of fluorescein. *Bottom panel* taken 576 s after injection

venous pathology were significantly higher in the neuropathic group compared to normal control subjects ($p < 0.01$). Arterial attenuation was observed in all but one of the neuropathic subjects. There was some degree of venous tortuosity in ten of the neuropathic subjects, five of whom had moderate to severe venous distension (Fig. 3). There was no significant difference in epineurial vessel pathology score between the normal control and the non-neuropathic diabetic subjects. The mean vessel pathology score of the neuropathic group was significantly higher ($p < 0.01$) than that of the combined normal and non-neuropathic diabetic groups. Arterio-venous shunting was observed in six of the patients with neuropathy and one non-neuropathic subject and not in any of the normal control subjects. Figure 5 shows an example of a direct epineurial arterio-venous shunt in a neuropathic subject.

Sural nerve fluorescein angiography

In the normal subjects fluorescein first appears in the epineurial arteries between 23 and 46 s (34.7 ± 9 s) after injection. Once this occurs, the fluorescein spreads rapidly and fills almost all of the epineurial vessels within a few seconds. Photographs taken immediately following the appearance of fluorescein are shown in Figures 6, 7 and 8 (top panel). From this point onwards the fluorescein starts to enter the nerve body which begins to glow. Photographs taken at 576 s after injection are shown in Figures 6, 7 and 8 (bottom panel).

The fluorescein appearance time was significantly delayed in neuropathic subjects (51.5 ± 12 s, $n = 12$) compared to both normal control (34.7 ± 9 s, $n = 8$, $p < 0.01$) and non-neuropathic diabetic subjects (33.4 ± 11 s, $n = 5$, $p < 0.025$). The procedure was repeated on two of the subjects. In the first (normal control) subject the appearance time was unchanged after 23 months. In the second subject (neuropathic group) the fluorescein appearance time increased from 54 to 64 s after 29 months. During this time the patient developed proliferative retinopathy and further neuropathic symptoms.

The intensity of nerve fluorescence for each of the three groups is summarised in Table 3 and is shown graphically in Figure 9. There was no significant difference in the mean intensity between the normal and non-neuropathic diabetic subjects although the mean intensities were generally higher in the latter group. The mean intensity was significantly lower in the neuropathic group compared to the non-neuropathic group at all the frames measured (96 s, $p < 0.02$; 252 s, $p < 0.01$; 372 s, $p < 0.03$; 576 s, $p < 0.02$). It was also significantly lower compared to the normal control group at three frames (96 s, $p < 0.04$; 272 s, $p < 0.03$; 372 s, $p = 0.055$; 576 s, $p < 0.04$).

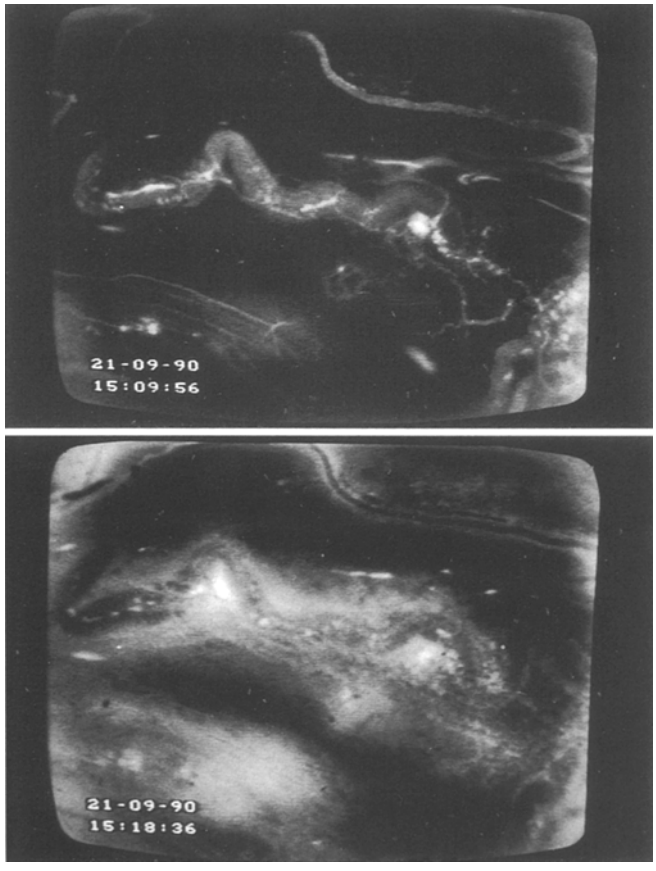

Fig. 8. Sural nerve fluorescein angiogram of a diabetic neuropathic subject. *Top panel* taken at appearance of fluorescein. *Bottom panel* taken 576 s after injection

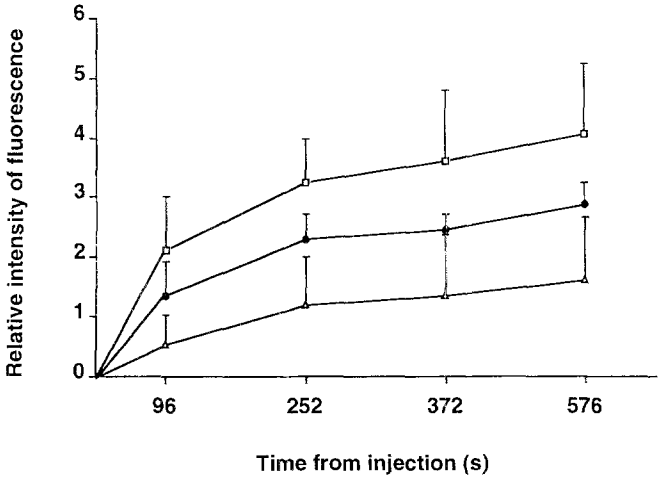

Fig. 9. The change in relative nerve intensity with time in the subjects with chronic sensory motor neuropathy (△), non-neuropathic diabetic (□) and normal control subjects (●)

Correlations between clinical details, microangiopathy, fluorescein appearance time, intensity of fluorescence and neuropathic severity (Table 4)

Only fluorescein appearance time was related to age ($r = 0.42; p < 0.05$). Arterial, venous and epineurial vessel pathology scores were related to elevated levels of HbA_1

Table 3. Results of the fluorescein angiography

Retina	Age (years)	FAT	Mean intensity 96 s	252 s	372 s	576 s
Normal control subjects						
0	54	44	0.71	1.83	1.97	2.37
0	59	28	1.2	2.05	2.46	3.11
0	42	46	1.65	2.88	2.6	3.24
0	54	23	1.91	2.59	2.64	2.73
0	50	39	2.1	2.68	2.81	3.32
0	58	38	0.51	1.85	2.29	2.49
Non-neuropathic subjects						
2	51	40	2.5	3.3	3.76	3.82
1	45	27	1.18	2.02	1.56	2.14
1	48	22	3.1	3.67	3.75	4.02
0	50	26	2.9	4.18	5.29	5.78
1	64	52	0.88	3.1	3.71	4.56
Chronic sensory motor neuropathy						
2	60	34	1.49	2.42	2.95	2.87
2	56	56	0.21	0.56	0.17	0.11
1	62	48	0.23	0.63	1.32	1.79
2	54	64	0.44	1.85	1.8	1.94
1	58	42	0.78	1.58	1.94	2.64
2	55	53	0.01	0.01	0.0	0.35

Retina: 0 = Normal, 1 = Background, 2 = Proliferative retinopathy or previous laser treatment. FAT, Fluorescein appearance time

($p < 0.05$, $p < 0.05$, $p < 0.01$ respectively). Duration of diabetes failed to relate to epineurial vessel pathology score, fluorescein appearance time or intensity of fluorescence. Both arterial and venous pathology scores were related to reduced peroneal motor ($p < 0.01$, $p < 0.01$ respectively) conduction velocity, elevated vibration ($p < 0.01$, $p < 0.01$ respectively) and thermal ($p < 0.03$, $p < 0.01$ respectively) perception. Epineurial vessel pathology score was significantly related to reduced sural sensory ($p < 0.01$) and peroneal motor ($p < 0.001$) conduction velocities, elevated vibration ($p < 0.01$) and thermal ($p < 0.001$) perception and the severity of retinopathy ($p < 0.002$). The fluorescein appearance time was significantly related to reduced sural sensory ($p < 0.02$) conduction velocity and elevated vibration ($p < 0.01$) perception but it was not related to peroneal motor ($p = 0.06$) conduction velocity, thermal ($p = 0.1$) perception and the severity of retinopathy ($p = 0.3$). Significant correlations were observed between fluorescein-appearance time and arterial ($r = 0.57$, $p < 0.02$), venous ($r = 0.59$, $p < 0.01$), and epineurial vessel ($r = 0.59$, $p < 0.002$) pathology scores. We were unable to demonstrate significant correlations between intensity of fluorescence and measures of neuropathic severity, but fluorescein appearance time was related to intensity at 96 s ($r = -0.75$, $p < 0.001$), 252 s ($r = -0.47$, $p < 0.02$), 372 s ($r = -0.56$, $p < 0.02$) and at 576 s ($r = -0.52$, $p < 0.05$).

There were no significant difference in nerve temperature of the normal control (mean 29.1 ± 1.4 °C), non-neuropathic (mean 30.0 ± 1.6 °C) and neuropathic (mean 29.7 ± 1.9 °C) groups.

Table 4. Correlation between vessel pathology score, fluorescein appearance time, intensity of fluorescence and clinical details and measures of neuropathic severity, expressed as Spearman's rank correlation coefficient *(r)* and degree of significance *(p)*

	Age	Duration of diabetes	HbA_1	Retinopathy severity	SSCV	PMCV	VPT	TDT
Arterial pathology score	$r = 0.36$ NS	$r = 0.42$ $p < 0.05$	$r = 0.40$ $p < 0.05$	$r = 0.35$ NS	$r = -0.36$ NS	$r = -0.41$ $p < 0.01$	$r = 0.56$ $p < 0.01$	$r = 0.43$ $p < 0.03$
Venous pathology score	$r = 0.4$ $p < 0.05$	$r = 0.22$ NS	$r = 0.4$ $p < 0.05$	$r = 0.48$ $p < 0.02$	$r = -0.46$ $p < 0.02$	$r = -0.59$ $p < 0.01$	$r = 0.52$ $p < 0.01$	$r = 0.58$ $p < 0.003$
Total epineurial vessel pathology score	$r = 0.24$ NS	$r = 0.32$ NS	$r = 0.51$ $p < 0.01$	$r = 0.57$ $p < 0.002$	$r = -0.52$ $p < 0.01$	$r = -0.61$ $p < 0.001$	$r = 0.49$ $p < 0.01$	$r = 0.65$ $p < 0.0003$
Fluorescein appearance time	$r = 0.42$ $p < 0.05$	$r = -0.03$ NS	$r = 0.24$ NS	$r = 0.2$ NS	$r = -0.45$ $p < 0.02$	$r = -0.38$ $p = 0.06$	$r = 0.52$ $p < 0.01$	$r = 0.33$ NS
Intensity at 96 s	$r = -0.53$ $p < 0.05$	$r = 0.28$ NS	$r = -0.32$ NS	$r = -0.48$ NS	$r = 0.43$ $p < 0.07$	$r = 0.34$ NS	$r = -0.39$ NS	$r = -0.27$ NS
Intensity at 372 s	$r = -0.22$ NS	$r = 0.17$ NS	$r = -0.29$ NS	$r = -0.46$ NS	$r = 0.29$ NS	$r = 0.26$ NS	$r = -0.18$ NS	$r = -0.2$ NS

SSCV, Sural sensory conduction velocity; PMCV, peroneal motor conduction velocity; VPT, vibration perception threshold; TDT, thermal discrimination threshold

Discussion

Novel techniques of sural nerve photography and fluorescein angiography have been developed. Similar studies have been performed in animal models [25] and human nerve biopsy specimens in vitro, but this is the first time these techniques have been applied to human nerve in vivo. Capillary basement membrane thickening which is found in a variety of tissues in diabetes [26] has also been demonstrated in endoneurial vessels in diabetic neuropathy [4–7, 10]. There are, however, few studies on the state of epineurial nutrient arterioles. In focal neuropathies of acute onset the case for epineurial vessel disease is strong. Occlusion of epineurial arterioles has been demonstrated in proximal motor neuropathy [27]. Furthermore, it has been postulated that the most likely cause of the circumscribed lesion found in palsy of the third cranial nerve is the occlusion of epineurial vessels [28, 29]. Korthals et al. [30] measured the epineurial arteriolar wall components of sural nerve biopsy specimens and found that intimal area and numbers of intimal nuclei were significantly greater in diabetic subjects compared to control subjects although no direct relationship was found between the increase in intima and severity of nerve fibre degeneration.

Our results demonstrate that epineurial arteriolar and venous anatomy is significantly abnormal in subjects with chronic diabetic neuropathy compared to normal control subjects. In neuropathic subjects there was arteriolar attenuation with venous tortuosity and distension. Similar abnormalities have been described in the conjunctiva and nailbed in diabetes [31]. Using the operating microscope, at very high magnifications, individual erythrocytes can be visualised moving from the arterial to the venous system. The presence of active epineurial arterio-venous shunts was confirmed by the movement of fluorescein from the arterial to the venous side and by the sudden changes in venous calibre to distension and consequent tortuosity. We observed direct epineurial arterio-venous shunting in six neuropathic subjects with diabetes and one non-neuropathic diabetic subject but this abnormality was not found in any of the normal subjects. These results strongly suggest that epineurial arterio-venous shunting may be a feature of diabetic neuropathy. In addition, two subjects with Type 1 hereditary motor and sensory neuropathy who have undergone sural nerve photography had normal arterial and venous anatomy (epineurial vessel pathology score = 0 and 1 respectively), one subject having mild venous tortuosity.

Fluorescein angiography is widely employed in clinical practice, particularly to study retinal vessels [32]. Dermal fluorometry has been used as an objective measure of limb perfusion in the pre-operative assessment of lower-extremity amputation level [14, 33, 34]. Subsequently, a modified perfusion fluoremeter has been developed as a new method of assessing capillary blood flow [35]. Qualitative visual or quantitative fluorometric assessment of tissue fluorescence after intravenous injection of sodium fluorescein provides an index of fluorescein delivery that correlates with vascularity and viability in skin flaps [17–19] and ischaemic bowel [15, 16]. Fluorescein flowmetry, as a technique of measuring changes in intestine blood flow correlates well with that obtained by electromagnetic flowmetry ($r = 0.86$) and ^{133}Xenon clearance technique ($r = 0.94$) [36]. However, although nerve fluorescein angiography has been used in animal models [25], this is the first time this technique has been applied to study human nerve perfusion in vivo.

Applying the technique to human nerve allows the calculation of an index of epineurial vessel and nerve trunk perfusion. The fluorescein appearance time is the time taken from intravenous injection of fluorescein to its appearance in epineurial vessels. In the absence of peripheral vascular disease, the bulk of the appearance time would be due to the distal resistance arterioles as, according to Poiseuille's Law the rate of blood flow is directly proportional to the fourth power of the radius of the vessel. Thus, the diameter of a blood vessel plays by far the greatest role of all factors including cardiac output and heart rate in determining the rate of blood flow.

Therefore, the fluorescein appearance time can be assumed to give a measure of epineurial vessel blood flow. This is strengthened by the significant correlation between appearance time and epineurial vessel pathology score. The fluorescein appearance time was also significantly related to reduced sural conduction velocity and elevated vibration perception. The fluorescein appearance time (23 s) was exactly the same in one normal subject who underwent nerve fluorescein angiography for a second time after 23 months. But in one neuropathic subject the appearance time increased from 54 to 64 s after 29 months during which he developed further severe disabling neuropathic symptoms and proliferative retinopathy. The intensity of fluorescence was measured to provide an index of nerve trunk perfusion with time. It would seem reasonable to assume that fluorescein enters endoneurial vessels having passed through the perineurial barrier. Whether it then leaks into the endoneurial compartment is speculative. Intensity was measured away from epineurial vessels to get an indication of nerve trunk perfusion. The mean intensity of fluorescence was markedly reduced in the diabetic neuropathic group compared to the other groups but there was no significant difference between the normal and non-neuropathic diabetic groups, although the mean intensity was higher in the latter. The increase in intensity of fluorescence in the non-neuropathic subjects compared to normal control subjects may represent a similar phenomenon as the glomerular hyperperfusion [37] and increased retinal blood flow observed in uncomplicated diabetes [38]. The fluorescein appearance time was significantly related to the intensity of fluorescence throughout the period of video recording but the correlation became weaker with time. This finding and the fact that intensity did not relate to measures of neuropathic severity suggests that intensity may be a measure of both flow and leakage of fluorescein. The delay in fluorescein appearance time and the marked reduction in the intensity of nerve fluorescence in subjects with chronic diabetic neuropathy suggest poor nerve blood flow. This is not surprising as the epineurial vessels supplying the neuropathic nerve are clearly abnormal.

The procedure used is necessarily invasive and we feel it would have been unethical to include more patients in the non-neuropathic diabetic group, particularly as we had demonstrated significant differences in the fluorescein appearance time and intensity of fluorescence between the two diabetic groups. As we excluded patients with peripheral vascular disease, the main complication resulting from the procedure, apart from the discomfort, was minor inflammation around the wound site. This was present in four subjects and resolved completely with oral antibiotic treatment. There were no long-term complications such as localized numbness and tingling encountered after nerve biopsy.

Could epineurial arterio-venous shunting contribute to the pathogenesis of diabetic distal symmetric sensory polyneuropathy or is it the result of nerve damage? It is well established that there is arterio-venous shunting in the diabetic neuropathic leg which leads to distended, "arterialised" veins [39–42] that have elevated oxygen tension [42]. The presence of epineurial arterio-venous shunts in the neuropathic subjects and the reduced intensity of fluorescence has led us to speculate that similar mechanisms may be taking place at the surface of the nerve leading to a "steal" effect rendering the endoneurium ischaemic. This is consistent with previous studies showing the multifocal nature of nerve fibre degeneration [8, 9] and a reduction in peripheral nerve oxygen tension in human diabetic neuropathy [11]. In rats, the creation of proximal limb arterio-venous shunts results in 50 to 75% reduction in endoneurial blood flow within the distal sciatic nerve and consequent functional and morphological abnormalities [43].

At what stage during the course of neuropathy these abnormalities of epineurial vessels develop has not yet been determined. Sural nerve photography and fluorescein angiography may be employed to study: a) the effect of drugs such as aldose reductase inhibitors or peripheral vasodilators on nerve blood flow; b) subjects with early distal symmetric neuropathy; c) diabetic subjects with acute painful neuropathy; d) unusual clinical syndromes of diabetic neuropathy such as that which follows the institution of insulin therapy [44]; and e) neuropathies of other causes than diabetes.

Recently we have demonstrated that exercise-induced nerve conduction increment is impaired in chronic sensory motor neuropathy [12], suggesting impaired nerve blood flow. Approximately 15% of the non-neuropathic diabetic subjects failed to increase their nerve conduction after exercise. We speculate that these may be the diabetic subjects who will eventually develop neuropathy. Clearly, such subjects need to be studied further by sural nerve photography and fluorescein angiography to see if they have very early microvascular abnormalities.

This study provides a new and direct measure of epineurial vessel structure coupled with an assessment of blood flow. While nerve biopsy material provides information regarding the structure of nerve fascicles and vessels, it does not give a dynamic view of function. If possible all available techniques for studying nerves should be applied in cross-sectional and prospective studies in diabetic neuropathy. The study reported here provides further evidence for the role of microvascular disease in the progressive damage so common in the diabetic nerve.

Acknowledgements. The technical assistance of the Department of Medical Illustration, in particular, Mr. N. Campbell, at the Royal Hallamshire Hospital, is gratefully acknowledged. We thank Ms. L. Parnell for the post-operative review of subjects. We also thank the neurosurgical theatre staff at Royal Hallamshire Hospital particularly Ms. E. Ramsden.

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Received: 8 February 1993
and in revised form: 29 June 1993

Dr. S. Tesfaye
Walton Hospital,
Rice Lane,
Walton,
Liverpool L9 1AE
UK