

Capillary number and percentage closed in human diabetic sural nerve

(morphometry/diabetes/polyneuropathy/pathology)

PETER JAMES DYCK, SEONA HANSEN, JEANNINE KARNES, PETER O'BRIEN, HITOSHI YASUDA, ANTHONY WINDEBANK, AND BRUCE ZIMMERMAN

Peripheral Nerve Center, Mayo Clinic and Mayo Foundation, Rochester, MN 55905

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ABSTRACT The number of capillaries per mm², minimum intercapillary distance, number of endothelial nuclei per capillary section, and percentage of capillaries closed were evaluated in transverse sections of fascicles of 45 control and 36 diabetic sural nerves. All controls and patients were prospectively studied to ascertain their diabetic and neuropathic status. An index of pathology was introduced and it was found to provide a sensitive and reliable measurement of the presence and severity of neuropathy. The number of capillaries and minimum intercapillary distance of diabetic nerves were not significantly different from those of controls ($P > 0.05$). Diabetic nerves exhibited a small but statistically significant increase in the number of endothelial nuclei per capillary that was positively correlated with the severity of neuropathy. The most striking abnormality was the statistically significant increase in the percentage of capillaries closed in patients with neuropathy as compared to those without neuropathy and controls. Among diabetics, this percentage increased with the severity of neuropathy ($P = 0.008$). The two capillary abnormalities that have been demonstrated may play a role in the development of diabetic polyneuropathy.

Diabetic polyneuropathy, a common complication of diabetes mellitus, is typically expressed as sensory loss, pain, and autonomic dysfunction in the feet and legs (1). Although the mechanisms underlying diabetic neuropathy remain unknown, chronic hyperglycemia may lead to metabolic derangement that directly affects neurons (axons) or Schwann cells or indirectly affects them by first altering another tissue—e.g., vessels. Since a higher rate of atherosclerotic heart disease and peripheral vascular disease occurs among diabetics (2, 3) and since arteriosclerosis with vessel occlusion is reported for vasa nervorum of nerve (4–6), arteriosclerosis has been postulated as a cause of diabetic neuropathy. This hypothesis, however, may not explain the common occurrence of an abnormality of nerve conduction and development of a diffuse neuropathy among diabetic patients who do not manifest peripheral vascular disease (7–10). Attention, therefore, has been focused on metabolic derangements that might affect neurons (or their peripheral axons) or Schwann cells. Among the metabolic mechanisms that have been considered are the following: (i) lipid alterations (11–13); (ii) accumulation of sorbitol and fructose (14, 15); (iii) decreased nerve *myo*-inositol (16); (iv) increased nonenzymatic glycosylation of protein (17, 18); (v) decrease of Na⁺,K⁺-ATPase (19); (vi) increased intraaxonal sodium (20); (vii) alterations in axonal flow (21) and axonal attenuation (22); and (viii) tissue dehydration (23). Since the nerve conduction abnormality is not readily reversed after near normalization of blood glucose for periods of up to 8 months, an irreversible nerve alteration or an intervening pathologic

alteration interposed between metabolic derangement and neuropathic dysfunction is inferred (24).

A functional and structural alteration of diabetic capillaries should be considered for this intervening pathologic alteration because (i) a generalized basement membrane abnormality is characteristic of diabetes (25–27); (ii) such an abnormality has also been described for diabetic nerve (28–31); (iii) increased permeability has been described for capillaries of several tissues (32–34); (iv) the complications of retinopathy, nephropathy, and neuropathy are correlated in the same patient (5, 35) and, since capillary dysfunction is involved in retinopathy and nephropathy, it may also be involved in neuropathy; (v) reduced nerve blood flow and oxygen tension have recently been described in streptozotocin diabetes (36); and (vi) the nerve conduction abnormality of streptozotocin diabetes can be partially prevented by oxygen supplementation (37). For nerve, however, capillaries have not been systematically studied and severity of capillary derangement has not yet been critically related to neuropathic deficit.

MATERIALS AND METHODS

Patient Selection. An ankle-level fascicular sural nerve biopsy was performed on 45 healthy subjects, recruited by advertisement from medical personnel and a recreational club for elderly people. Persons with a family history of neuropathy or diseases known to be associated with predisposition to neuropathy were excluded. Controls underwent the same evaluation for neuropathy as did diabetics.

A similar biopsy was performed on 36 patients with diabetes mellitus, as diagnosed by the National Diabetes Data Group criteria (38). Ten of these had insulin-dependent (ID) and 26 had non-insulin dependent (NID) diabetes by the criteria of the American Diabetes Association and by an algorithm developed by us. A neuropathy symptom score, a neurologic disability score (39), measured attributes of nerve conduction of peroneal and sural (and other) nerves, and detection thresholds of vibratory and cooling sensation on the great toe and dorsal foot (40), respectively, were obtained for each patient.

The minimal criteria used for the diagnosis of neuropathy was that two or more of the following evaluations gave abnormal results: (i) neuropathy symptom score ≥ 1 ; (ii) neurologic disability score ≥ 6 ; (iii) computer-assisted sensory examination (CASE) of toe or foot; and (iv) nerve conduction of limb nerves (two abnormal attributes in at least two nerves and in at least two limbs). By these criteria, 32 of the 36 diabetic patients had neuropathy (8 ID and 24 NID) and 4 did not (2 ID and 2 NID). Among neuropathy patients, the diagnosis was based on abnormalities in four evaluations in 14 patients, on three evaluations in 14 patients (but in 9 of

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Abbreviations: ID, insulin-dependent diabetes; NID, non-insulin-dependent diabetes; I_p , index of pathology; C/mm^2 , number of capillaries per mm²; N/C , number of endothelial nuclei per capillary section; %CC, percentage of capillaries closed.

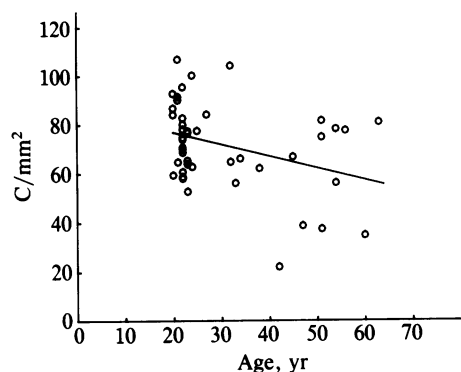


FIG. 1. C/mm^2 from transverse sections of fascicles of ankle sural nerves plotted against age for 45 healthy subjects. The regression line, fitted by the method of least squares, has a negative slope ($P = 0.008$), indicating that, on average, there are fewer capillaries with increasing age.

these only 3 of those evaluations had been obtained), and on two evaluations in 3 patients (but in one of these only three evaluations were done). One patient, with only one abnormality of three evaluations performed, was added to the neuropathy group because of an unequivocally abnormal index of pathology (I_p). Among the diabetics without neuropathy, two had one abnormal evaluation but, in all cases, the I_p was normal. The polyneuropathy among diabetic patients was of mild or moderate severity. All of the patients were ambulatory and most held jobs. None of these patients had proliferative retinopathy, renal failure, an elevated plasma creatinine value, or symptomatic peripheral vascular disease. Five patients had a history of coronary artery disease. By the World Health Organization criteria (41), definite hypertension was found in 10 patients and borderline hypertension in 7 patients.

Histologic Studies. To provide a measure of the severity of the pathologic abnormalities affecting nerve fibers, we used I_p . I_p is the product of the ratios of the observed-to-expected number of myelinated fibers per mm^2 and the observed-to-expected percentage of graded normal teased fibers. The use of I_p combines two components of pathologic abnormality (fiber loss and pathologic abnormality of remaining fibers) and normalizes results for age and sex. Such normalization for age and sex was important since the number of myelinated fibers per mm^2 and the percentage of teased fibers without abnormality were found to decrease with age, and sex differences were also found. In a separate study utilizing the same nerve biopsy specimens studied here, we have evaluated which morphometric parameters (I_p ; number of myelinated fibers per mm^2 ; number of large myelinated fibers per mm^2 ; percentage of abnormal teased fibers; percentage of

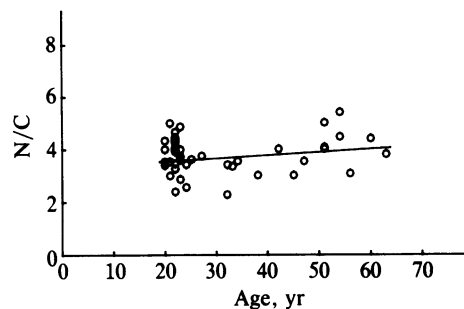


FIG. 2. Mean N/C from transverse sections of fascicles of ankle sural nerves plotted against age for 45 healthy subjects. The regression line, fitted by the method of least squares, does not show a significant slope ($P > 0.05$). A significantly positive slope was found for males only ($n = 27$; $0.025 < P < 0.05$).

teased fibers with de- and remyelination; and percentage of teased fibers with axonal de- and regeneration) provided the best predictor of neuropathy using clinical, nerve conduction and computer-assisted sensory examination criteria. The I_p provided the best measure, accurately predicting all cases with neuropathy followed in succession by number of myelinated fibers per mm^2 , number of large myelinated fibers per mm^2 , and then all teased fiber abnormalities.

The procedure of fascicular nerve biopsy, size of nerve sample, fixation, and histologic processing were the same for diabetic patients and controls and as previously described (42). Fixation was in isotonic glutaraldehyde and then the sample was immersed in isotonic osmium tetroxide at $10^\circ C$. The number of capillaries per mm^2 (C/mm^2), minimum intercapillary distance (MICD), number of endothelial nuclei per capillary section (N/C), and the percentage of capillaries closed (%CC) were evaluated by using our imaging system for nerve morphometry (ISNM) (43) in methylene blue-stained, semithin ($3/4-\mu m$), transverse, epoxy sections of fascicles of sural nerve. All evaluations were performed on coded slides, from controls and diabetic patients with and without neuropathy, randomly assigned and with identification marks covered. All vessels without muscle in their walls were evaluated. Usually, these microvessels (referred to as capillaries henceforth) were composed of a single layer of endothelial cells with or without pericytes. Generally, the capillaries were orientated in the plane of the fascicle and therefore were sectioned transversely. For technical reasons, we did not correct for split cell error in determining N/C . Capillaries were designated as closed when no lumen could be identified at a final high-dry magnification of $\times 1500$. Electron micrographs of capillaries of all nerves had been prepared to confirm whether vessels were open or closed.

Table 1. Regression analysis of associations of capillary parameters with age and sex

Subjects	Parameter	Males			Females			Males plus females		
		<i>a</i>	<i>b</i>	<i>r</i>	<i>a</i>	<i>b</i>	<i>r</i>	<i>a</i>	<i>b</i>	<i>r</i>
Control (18 females and 27 males)	C/mm^2	76.79	-0.280	-0.280	102.31	-0.860	-0.493*	86.06	-0.482	-0.359*
	MICD	0.08	0.000	0.099	0.07	0.001	0.352	0.08	0.001	0.194
	N/C	3.20	0.018	0.378†	3.61	0.000	0.008	3.34	0.012	0.233
	%CC	-3.08	0.290	0.388*	-2.39	0.250	0.229	-2.85	0.270	0.311*
Diabetic (15 females and 21 males)	C/mm^2	29.56	0.730	0.321	68.67	-0.052	-0.055	53.76	0.260	0.161
	MICD	0.15	-0.001	-0.266	0.12	-0.000	-0.423	0.13	-0.001	-0.261
	N/C	5.23	-0.011	-0.105	3.27	0.022	0.333	4.01	0.010	0.122
	%CC	16.88	0.285	0.124	-5.45	0.720	0.409	3.09	0.546	0.273

Regression lines are expressed in the following form: parameter value = $b(\text{age in years}) + a$; r is the correlation coefficient.

* $P < 0.025$.

† $P < 0.05$.

Associations with age were evaluated by using least squares regression analyses. Comparisons between regression lines were based on *F* tests with 2 degrees of freedom in the numerator.

RESULTS

C/mm². Among controls, the association of *C/mm²* with age was not significantly different between males and females (*P* > 0.05). *C/mm²* decreased with age (*P* = 0.008, Fig. 1). Since this decrease could be due to a decrease in the density of capillaries or an increase in the fascicular area of nerve and since only fascicular biopsies were utilized for this study, it was necessary to determine whether fascicular area increases with age. Evaluation of 14 other whole control sural nerves, taken post mortem, revealed no significant change in fascicular area. The decrease in *C/mm²* must, therefore, be authentic.

Among diabetics, the density of capillaries generally fell within the range of normal values and the slopes of regression lines relating *C/mm²* with age were not significantly different from those of controls (*P* > 0.05; Table 1).

MICD. No significant associations with age, sex, or disease states (diabetic vs. control) were observed (Table 1).

N/C. Among controls, *N/C* showed a small increase with age for men (*P* = 0.026), women (*P* > 0.05), and men and women combined (*P* > 0.05) (Fig. 2). The difference between sexes was not statistically significant.

To relate *N/C* in diabetics to expectations in health, the differences between the observed and expected values were computed. The expected values were obtained from linear regression in the control group. On average, these values were greater than 0 ($\Delta = 0.581$, *P* = 0.005), indicating that *N/C* values were elevated among diabetics. These differences were then plotted as a function of *I_p* (Fig. 3). The negative slope (*P* = 0.023) indicated that, among diabetics, the

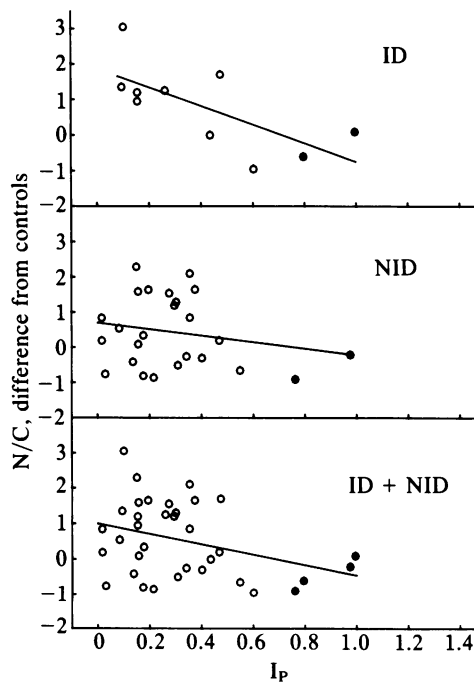


FIG. 3. Difference from mean values, considering age and sex, of *N/C* of transverse sections of fascicles of ankle sural nerves from diabetic patients plotted against *I_p*. ●, Patients without neuropathy; ○, patients with neuropathy. (Note that a higher *I_p* indicates less severe pathology.) A negative slope was found for patients with ID (*P* = 0.014), for patients with NID (*P* > 0.05), and for ID and NID combined (*P* = 0.023). These results indicate that a slight increase in number of nuclei is associated with more severe neuropathy.

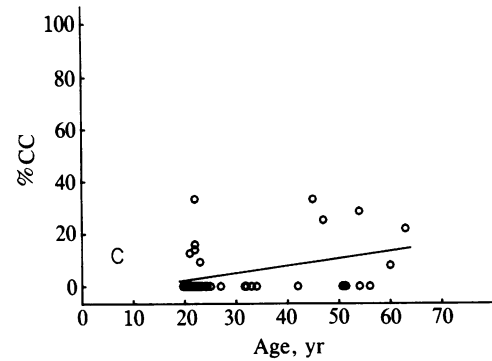


FIG. 4. %CC in transverse sections of fascicles of ankle sural nerves plotted against age for 45 healthy subjects. The regression line, fitted by the method of least squares, gave a positive slope for males (0.01 < *P* < 0.025), for females alone (*P* > 0.05), and for males and females combined (0.025 < *P* < 0.05, shown here).

increase in *N/C* is positively associated with the severity of nerve pathology.

%CC. Among controls, the %CC was significantly associated with age among males (*P* = 0.023) but not females (*P* > 0.05) (Fig. 4). However, the difference between sexes was not statistically significant and, when the groups were combined, the association with age remained (*P* = 0.021). Among diabetics, the %CC increased significantly with age for ID (*P* = 0.043), NID (*P* = 0.046), and combined ID and NID groups (*P* = 0.006).

To test whether the increase in the number of nuclei was a reflection of the higher percentage of hypertensives among these diabetics, *N/C* was plotted as a function of *I_p* for hypertensives and nonhypertensives. No significant differences were observed, although the regression line for hypertensives was slightly below that for nonhypertensives. Likewise, no effect of smoking could be demonstrated.

%CC in the nerves of diabetics was quite variable. On average, %CC was 5.6% for controls (*n* = 45), 23.7% for ID

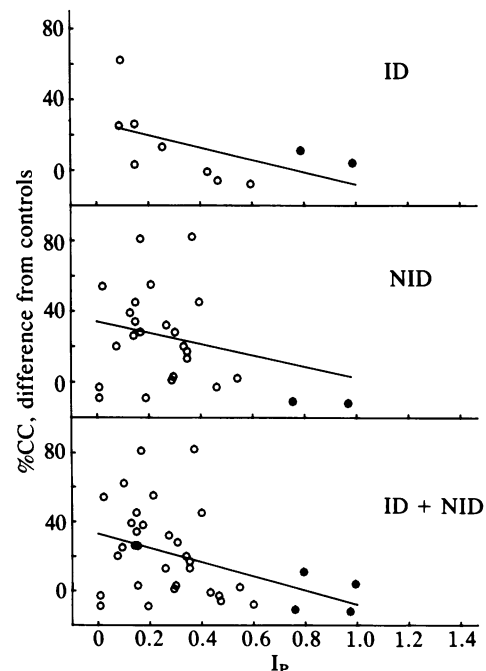


FIG. 5. Difference from mean values, considering age and sex, of %CC in diabetic nerves plotted against *I_p*. ●, Values from diabetic patients without neuropathy.

with neuropathy ($n = 8$, $P = 0.002$ as compared to controls), 38.6% for NID with neuropathy ($n = 24$, $P < 0.001$), 34.8% ID and NID with neuropathy ($n = 32$, $P < 0.001$), and 6.1% for ID and NID without neuropathy ($n = 4$, $P > 0.05$).

To test whether %CC related to the severity of neuropathy, we plotted the difference from control values (considering age and sex) as a function of I_p for ID, NID, and combined ID and NID nerves. An association was found for ID ($P > 0.05$), NID ($P = 0.045$), and ID and NID combined ($P = 0.008$) (Fig. 5).

Confirmation that these capillaries were closed came from a study of electron micrographs (Fig. 6).

DISCUSSION

Although sural nerves removed at biopsy (28, 29, 31, 39, 44–48) and post mortem (see ref. 1 for review) have been extensively studied, capillary closure has never been related to

neuropathologic abnormality. In the present quantitative study, rigorous design criteria were met: (i) control subjects and diabetic patients were prospectively evaluated to ascertain the presence and severity of neuropathy; (ii) in addition to scored evaluation of neurologic symptoms and signs, extensive electrophysiologic and computer-assisted sensory examination of detection threshold of vibratory and cooling sensations were used to detect and grade the severity of neuropathy; (iii) the type of diabetes was prospectively evaluated by utilizing laboratory tests and clinical characteristics and an algorithm; (iv) an index of pathology was used to quantitate sural nerve pathology; (v) the same nerve was used to make morphological measurements of capillaries and to judge severity of neuropathy; and (vi) sufficient numbers of nerves were evaluated to allow statistical comparisons.

Our results provide evidence of a structural abnormality of nerve capillaries in diabetes and of an association between pathologic abnormality of vessels and of nerve fibers. For

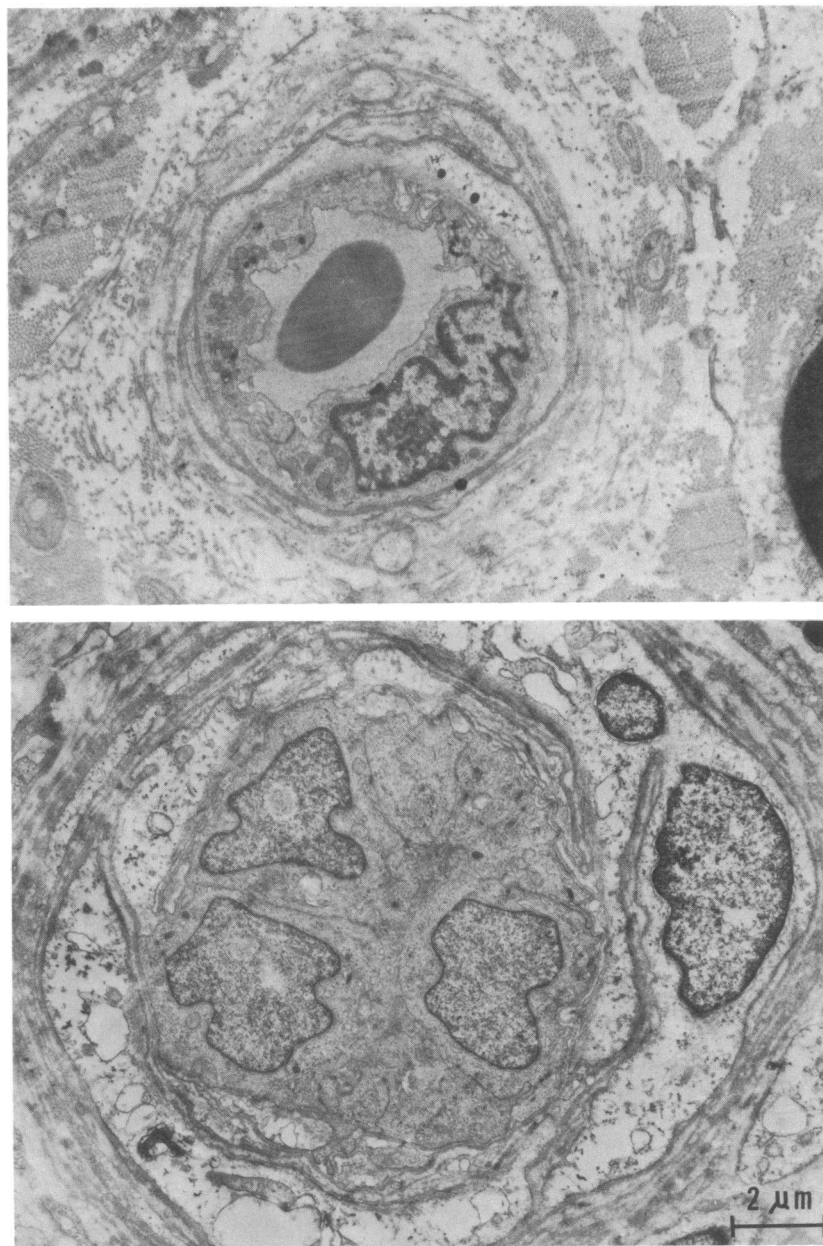


FIG. 6. Electron micrographs of capillaries from transverse sections of fascicles of ankle sural nerve of a control (*Upper*) and a diabetic with neuropathy (*Lower*). The diabetic capillary shows a closed lumen, hypertrophy and hyperplasia of endothelial cells, and thickening of the basement membrane.

diabetic nerves, including those with neuropathy, C/mm² was not significantly different from that of controls. The C/mm² values at various levels of the sciatic, tibial, and peroneal nerves in rat have been measured previously and provided values similar to those presented here (49). MICD was also not statistically different between diabetic nerves with neuropathy and controls. Because the capillaries are more disseminated in diabetes than in controls, decreased diffusion of blood gases or nutrients to nerve fibers because of separation of capillaries cannot be postulated.

The higher %CC in human diabetic nerves takes on added significance because not only were these percentages higher in patients with neuropathy than in those without neuropathy and controls but also they were related to the severity of the neuropathy. The alterations cannot simply be due to a different response to the surgical or histologic procedures, because these were alike for disease and control groups and structural changes (including increased N/C and thickened basement membranes) have been found. Because some of the severely affected nerves had normal percentages of capillaries closed, it is possible to argue that capillary closure is not the cause of fiber degeneration. Variability of results, however, may relate to the small number of capillaries per nerve specimen evaluated, fiber pathology at ankle sural level arising from more proximal sites of damage and closure representing only the most severe capillary lesions. The relative contribution of endothelial cell hypertrophy (or swelling), hyperplasia, exudation with capillary compression, intravascular occlusion, or another mechanism needs further systematic study. Results of such further studies may permit one to decide whether the increased percentage of capillaries that were closed are a factor in fiber degeneration or a response to such degeneration. The demonstrated increase in the number of nuclei per capillary indicates that endothelial nuclei undergo hyperplasia and not simply physiologic closure. We therefore suspect that capillary closure may relate to fiber degeneration. Nonphysiologic capillary closure, irrespective of how produced, may interfere with the exchange of gases and essential nutrients between the blood, endoneurial fluid, and neural tissue. Our findings suggest that capillary abnormalities play a role in the development of diabetic neuropathy, possibly as an intermediate step between metabolic derangement and pathologic abnormality of nerve fibers. Capillary abnormalities and closure have previously been shown to be involved in diabetic retinopathy (50, 51) and nephropathy (52).

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