[Environmental Health and Preventive Medicine 5, 53-59, July 2000]

Original Article

The Effects of Ginkgo Biloba Extract (GBe) on Axonal Transport, Microvasculature and Morphology of Sciatic Nerve in Streptozotocin-induced Diabetic Rats

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Abstract

To evaluate the protective effects of *Ginkgo biloba* extract (GBe) which has antioxidant activity against peripheral neuropathy due to diabetes mellitus, slow axonal transport and morphology of sciatic nerve including endoneurial microvessels were examined in 12 rats with diabetes mellitus induced by streptozotocin (STZ, 60mg/kg, b.w., i.p.). Six of the diabetic rats were treated with 0.1 % of GBe for 6 weeks from one week after the STZ injection. Serum glucose and lipid peroxide levels in GBe-treated diabetic rats were significantly lower than those in untreated diabetic rats ($p<0.01$, respectively), though the serum glucose level was higher than that in the control rats. L-[^{35}S] methionine pulse radiolabeling with subsequent gel fluorography demonstrated that mean velocities (Vmean) of actin and β-tubulin, i.e. slow component b (SCb) transport in untreated diabetic rats were significantly lower than those in control rats ($p<0.05$, respectively); mean diameter of axons in the former rats was significantly smaller than that in the latter ($p<0.01$). Vmean of actin transport in GBe-treated diabetic rats was significantly faster than that in untreated diabetic rats ($p<0.05$). Vmean of slow axonal transport was significantly correlated with mean diameter of axons in the three groups of rats combined ($p<0.01$). On electron microscopy, severe altered endoneurial microvessels decreasing in luminal area together with endothelial cell degeneration or hypertrophy, pericyte debris and basement membrane thickening were observed in untreated diabetic rats; on the other hand these findings were less prominent in the diabetic rats treated with GBe. It is suggested that GBe treatment may protect disturbed slow axonal transport and pathological alterations of peripheral nerve with abnormal endoneurial microvasculature from diabetes mellitus by antioxidant activity.

Key words: *Ginkgo* alkaloides, neuropathy, tublin, actin, neurofilament

Introduction

Diabetic neuropathy is associated with the disturbance of slow axonal transport [1-4], which carries cytoskeletal and related proteins through the axoplasm at variable velocities for maintenance of neural function and morphology [5, 6], and with structural derangement of nerve fibers such as axonal degeneration, atrophy and myelinated nerve fiber decrease [7-10]. Disturbed slow axonal transport affects the delivery of slow components such as neurofilament (NF) triplets, α, β-tubulin, actin and probably retrograde axonal transport, which influence protein synthesis in nerve cell bodies [2, 4, 6]. These changes lead to the abnormal integrity of axons and to the alteration of nerve structure, and finally play an important role in the progression of the neuropathy in diabetic patients [3, 9-11].

Recently, it has been widely reviewed that one of the pathogeneses of diabetic neuropathy is closely related to the abnormalities of endoneurial microvasculature including endothelial cell, basement membrane and pericyte [12-15]. A number of studies demonstrated that the pathological changes of endoneurial microvessels in the peripheral nerves of patients with diabetic neuropathy induce the decreases in both nerve blood flow and endoneurial oxygen tension [16-18], resulting in endoneurial ischemia or hypoxia [19, 20]. These conditions can easily get into low antioxidant status and give a cause of oxidative stress such as lipid peroxidation to the neurovascular cells by generating free radicals [21-23]. Thus, abnormal endoneurial microvasculature in the diabetic process contributes to aggravate nerve structure [10, 13, 14, 20] which may be related to disturbed axonal transport [24-26].

Ginkgo biloba extract (GBe) has been known as a therapeutic

Received Oct. 15 1999/Accepted Feb. 3 2000
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agent for peripheral vascular disease and cerebral insufficiency[27]; it has strong pharmacological effects including antioxidant activity, which protects the vascular endothelial cell damage from oxidative injury by scavenging free radicals[28-30] and vasoregulating activity, which promotes the microvascular circulation by improving hemorheological factors[31, 32]. Because it has been suggested that antioxidants[18, 21-24, 33-35] or vasoregulating agents[36, 37] exert therapeutic effects on nerve damage and/or dysfunction caused by microvascular abnormalities and endoneurial hypoxia, GBe is expected to prevent diabetic neuropathy. The present study was, therefore, conducted to examine whether or not GBe can protect slow axonal transport and pathological alterations with abnormal endoneurial microvasculature of the sciatic nerve in rats with diabetes induced by streptozotocin (STZ).

Materials and Methods

Animals and Treatment

Eight week-old male Wistar rats (Charles River Co., Japan) weighing 300 - 330g were housed in plastic cages with a 12 : 12 hrs artificial light cycle at a room temperature of 21 ± 1.5 ℃ and a humidity of 56 ± 3 %. All rats had access to CRF-1 food (Charles River Co., Japan) and water *ad libitum*. The animals were divided into three weight matched groups (6 rats for each). Group 1 (untreated diabetic rats) and 2 (GBe-treated diabetic rats) were given a single intraperitoneal injection of STZ at a dose of 60 mg/kg body weight in 0.01M cold citrate buffer (pH 4) at a concentration of 12 mg/ml to induce diabetes mellitus at the start of the experiment. One week later, only the rats with a serum glucose of 250 mg/dl or higher were selected from these two groups and served as the study subjects. The GBe-treated diabetic rats were then given 0.1% of GBe (SK Chemicals Co. Ltd., Korea), which contains 24 % of gingkoflavon-glycoside and 6 % of terpenelactone, in drinking water for the following 6 weeks. The third group (control rats) were intraperitoneally injected with a citrate buffer solution as a vehicle instead of STZ at the start of the experiment.

Measurement of serum glucose, body weight and lipid peroxide

Blood samples (0.5 ml) were collected from the tail vein at 0, 2, 4 and 7 weeks; serum glucose was measured using a blood chemistry analyzer (Model 7020, Hitachi Co., Japan). Body weight was also measured just before blood collection.

At the end of the experiment, serum lipid peroxidation was accessed by measuring thiobarbituric acid-reactive substance (TBARS) as an index of lipid peroxide in the serum. TBARS level was expressed as nmol malondialdehyde (MDA)/ml serum and was determined by the method of Yagi[38]; 20 μl of serum was put into 4 ml of N/12 H_2SO_4 and phosphotungstic acid (10%, 0.5 ml) was added. After standing for 5 min at 18 ℃, the mixture was centrifuged at 3,000 g for 10 min. To the sediment suspended in 4 ml of distilled water, 1 ml of TBA reagent was added and heated for 60 min at 95 ℃ in an oil bath. After cooling and adding 5 ml of *n*-butanol, the mixture was shaken and centrifuged (3,000 g, 15 min), and *n*-butanol layer was taken for fluorometric measurement at 553 nm with 515 nm excitation, using luminescence spectrophotometer (Aminco-B2, SLM Instruments Inc., USA). The value of fluorescence was calculated by comparison with a standard prepared from tetramethoxypropane.

Axonal transport studies

Three weeks after STZ injection, 100 μCi/ml aliquots of L-[^{35}S] methionine (Du Pont Co., USA) in an isotonic PO_4 buffer solution (pH 7.4) was injected into the right ventral horn of the spinal cord (L_3 to L_5), after a partial laminectomy of the rats by a glass capillary microinjection method[39, 40] under sodium pentobarbital anesthesia. The sectioned sites were sutured soon after the operation and the animals were allowed to recover. All rats were killed at 3 weeks after intraspinal injection of L-[^{35}S] methionine; right sciatic nerves were rapidly dissected from the ventral root to the division at the knee and kept frozen at -80 ℃ until the next step. The nerves including the ventral root were then cut into 6 mm consecutive segments on ice cold plastic plates and homogenized in 250 μl aliquots of a medium containing 2.3 % (w/v) sodium dodecyl sulfate (SDS), 5 % (v/v) 2-mercaptoethanol, 10 % (w/v) glycerol and 6.25 mM Tris-HCl, pH 6.8. The homogenates were boiled in a water bath at 100 ℃ for 3 min and centrifuged (15,000 g, 15 min). Aliquots (40 μl) of clear supernant were used to detect radioactivities by scintillation counting and the same aliquots were then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% gradient slab gels for the separation of cytoskeletal and related proteins. The obtained gels were stained with Coomassie Brilliant Blue for subsequent fluorography.

The distribution of radioactivities of slow axonal components transported along the sciatic nerve was assessed from the densitometric reading of fluorograms with a bio-imaging analyzer (BAS 2000, Fuji Photo Film Co.Ltd., Japan). The radioactivity of each nerve segment (*Ri*) was expressed as the percentage of total radioactivity by applying the following formula: $Ri\ (\%) = (ri / \Sigma ri) \times 100$, where ri is the radioactivity in the ith-segment of the nerve. The cumulative radioactivity (*Cx*) was calculated by summing the averages of Ri at different distances, *x* (× 6, mm), transported from the spinal cord. A fitted curve for the distribution of *Cx* was then obtained according to the previous methods[4, 40]; the mean transport velocity (Vmean) was calculated by dividing the *x* value corresponding to the 50th percentile of total *Cx* value by the time (21 days) elapsed after the isotope injection.

Mophological studies

Segments which were 2 mm in length, transversely cut from the middle portion (80 mm from the spinal cord) of the left sciatic nerves of animals from each group were fixed in 5 % glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 20 min. The tissue was then postfixed in 2 % osmium tetroxide for 24 hrs, dehydrated through a series of graded alcohol solutions, and embedded in Epoxy resin. Transverse sections of 0.75 μm thick were prepared with an LKB-Ultratome and stained with 1 % toluidine blue.

The stained sections were examined by light microscopy and morphometrical analysis was performed with a computer assisted image analyzer (IPAP, Sumitomo Co., Japan). The parameters analyzed were fiber density (number of nerve fiber/mm^2) and axon diameter; the former was for more than 20 % of fascicles in each nerve, and the latter was for 200 fibers per nerve. For the observation of endoneurial microvessels, prepared ultrathin sections were stained with methanolic uranyl acetate and lead citrate, and examined by electron microscopy (JEM-1200EX, JEOL Ltd., Japan).

Table 1 Diffrences in slow axonal transport, morphometry of the sciatic nerve and serum lipid peroxidation among GBe-treated diabetic, untreated diabetic, and control rats (Averages with ranges in parentheses)

	GBe-treated diabetic rats (n=5)	Untreated diabetic rats (n=5)	Control rats (n=6)
Vmean (mm/day)			
Actin	1.53 (1.28-1.79)[a]	1.26 (1.06-1.61)[c]	1.74 (1.49-2.02)
β-Tubulin	1.47 (1.22-1.75)	1.24 (1.04-1.59)[c]	1.67 (1.42-1.94)
68kDa NF subunit	1.06 (0.76-1.34)	0.89 (0.59-1.17)	1.12 (0. 82-1.36)
Morphometry			
Mean diameter of axons (μm)	3.68 (3.56-3.92)	3.48 (3.24-3.73)[d]	3.81 (3.62-4.02)
Fiber density (x10^2 fibers/mm^2)	116.4 (100.2-129.7)	110. 3 (94.6-123.5)	120.2 (106.3-132.4)
Serum lipid peroxide			
TBARS (nmol MDA/ml)	1.04 (0.82-1.20)[b]	1.53 (1.32-1.72)[d]	0.97 (0.84-1.11)

a, b Significantly different from untreated diabetic rats at $p < 0.05$ and $p < 0.01$, respectively (Student's t-test).
c, d Significantly different from control rats at $p < 0.05$ and $p < 0.01$, respectively (Student's t-test).
Abbreviations as in text.

Statistical analysis

Differences in serum glucose, body weight, lipid peroxide, mean velocity of slow axonal transport, and morphometric parameters of nerve fibers between the three groups of rats were analyzed by Student's t-test. A correlation between the mean velocity of slow axonal transport of total protein and the mean diameter of axons in the three groups of rats combined was assessed by Spearman's correlation test.

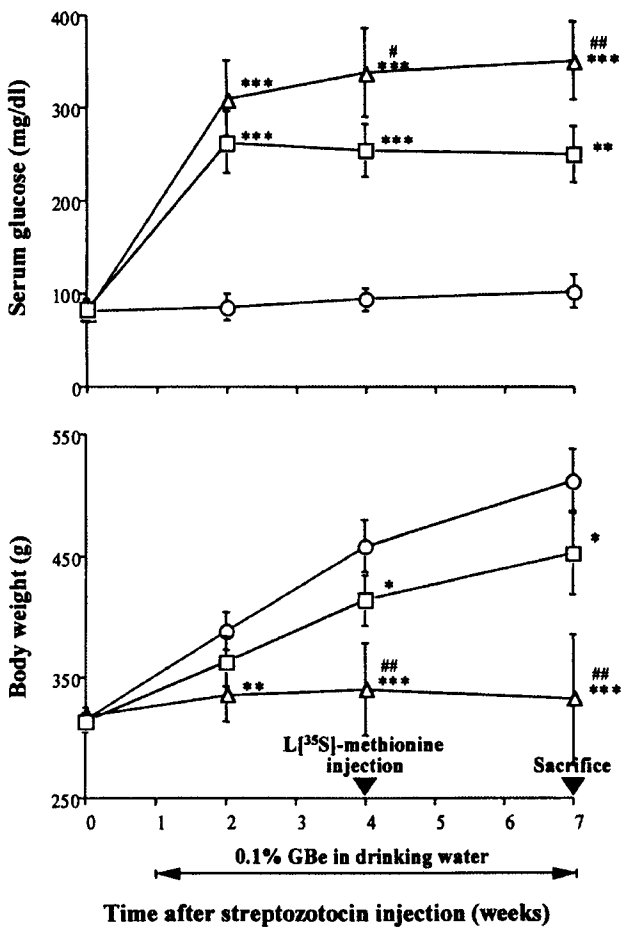

Fig. 1 Changes in serum glucose and body weight in untreated diabetic (△), GBe-treated diabetic (□), and control (○) rats (6 rats for each). The values are mean ± standard deviation.
*, **, *** Significantly different from control rats at p<0.01, p<0.001 and p<0.0001, respectively.
#, ## Significantly different from GBe-treated diabetic rats at p<0.05 and p<0.01, respectively.

Results

Untreated diabetic rats showed a significant increase in serum glucose and a decrease in body weight as compared with the controls (Fig. 1); In GBe-treated diabetic rats, serum glucose was significantly lower and body weight was higher than those in the untreated diabetic rats (p<0.01, respectively), though being significantly different from the controls (Fig. 1).

Cx in fitted curve for total slow axonal transport along the sciatic nerve at the proximal sites from spinal cord in untreated diabetic rats was significantly higher than that in the GBe-treated diabetic rats up to 12 mm and than that in the control rats up to 24 mm (Fig. 2). Visual inspection of the fluorographs showed that axonal transport of slow components in untreated diabetic rats was retarded in distal sites as compared with the GBe-treated diabetic rats and the control rats (Fig. 3).

Vmean of slow axonal transport, nerve morphometrical and serum lipid peroxide values in the three groups of rats are shown in Table 1. Vmean of actin and β-tubulin, i.e. slow component b (SCb) and mean diameter of axons were significantly lower and

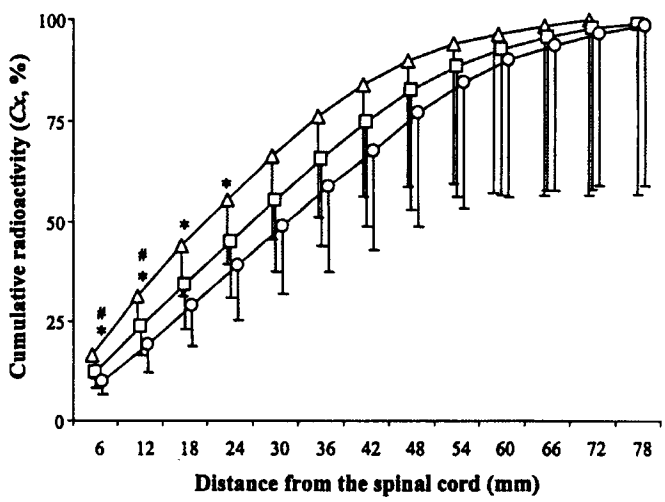

Fig. 2 Cumulative radioactivity for total slow components transported along the axons in sciatic nerves 21 days after intraspinal injection of L [^{35}S]-methionine in untreated diabetic (△), GBe-treated diabetic (□), and control (○) rats (5 rats for each). The values are expressed as relative proportion to the estimated total radioactivity transported (mean ± standard deviation).
*, # Significantly different from control rats and GBe-treated diabetic rats, respectively, at p<0.01.

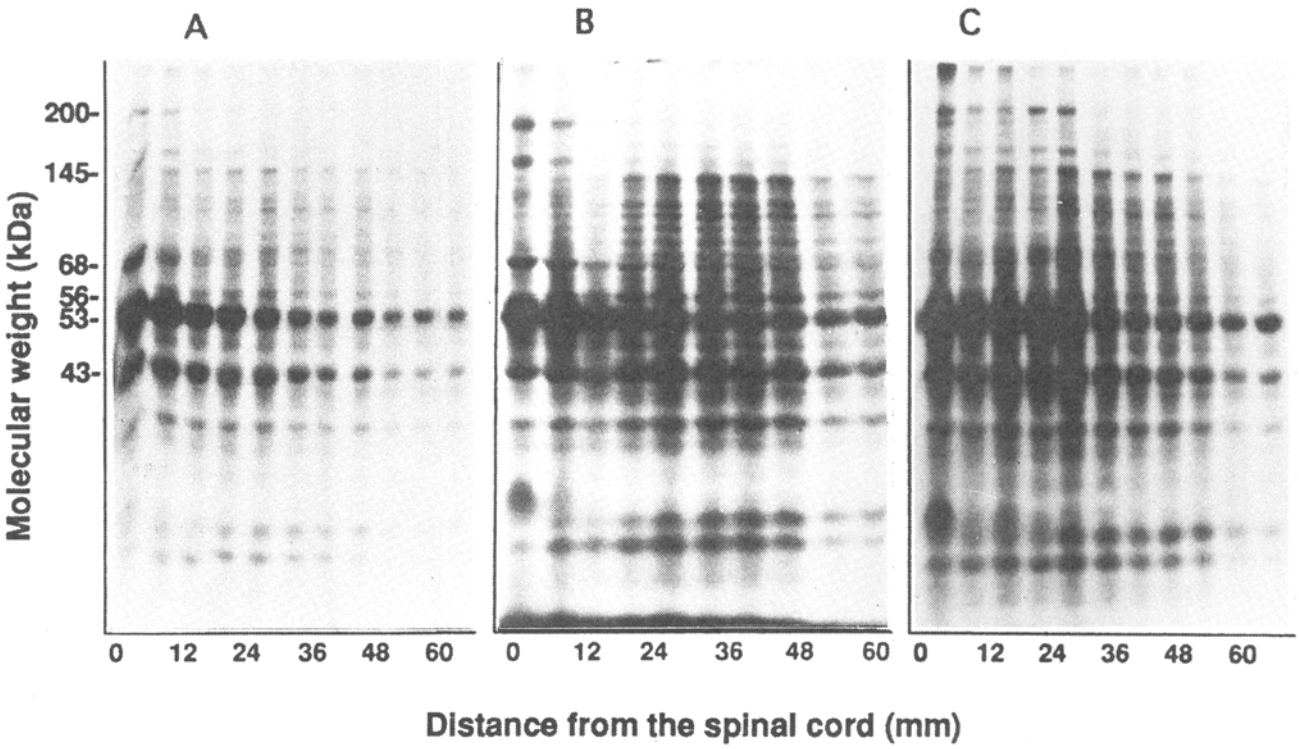

Fig. 3 Fluorograph of radiolabeled slow axonal transport in the sciatic nerves 21 days after intraspinal injection of L [^{35}S]-methionine in untreated diabetic rats (A), GBe-treated diabetic rats (B), and control rats (C).

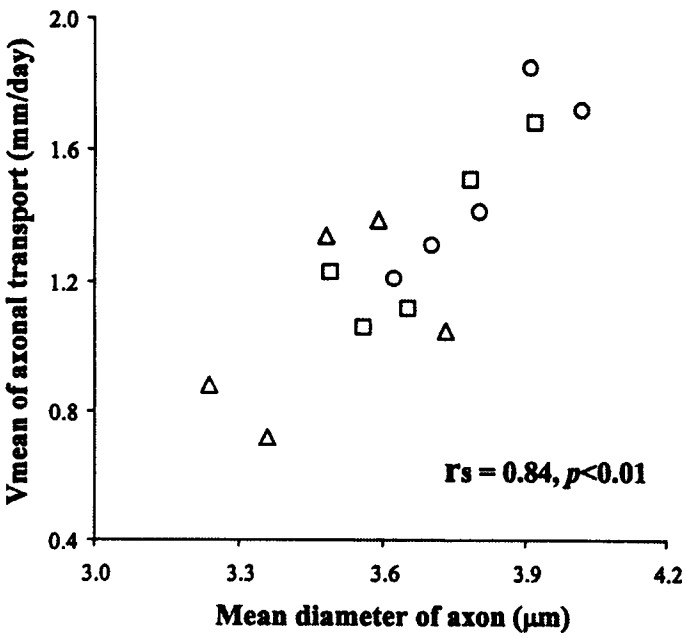

Fig. 4 The relationship between Vmean of slow axonal transport and mean diameter of axons in the sciatic nerves in untreated diabetic (△), GBe-treated diabetic (□), and control (○) rats combined (5 rats for each). rs = Spearman's rank correlation coefficient.

serum lipid peroxide was higher in untreated diabetic rats than those in the control rats. In GBe-treated diabetic rats, Vmean of actin was significantly faster and serum lipid peroxide was lower than those in the untreated diabetic rats. There were no significant differences in Vmean, morphometrical and serum lipid peroxide values between the GBe-treated diabetic rats and the control rats. Vmean of slow axonal transport was significantly correlated with mean diameter of axons in the three groups of rats (Gbe-treated diabetes, untreated diabetes and control) combined (Fig. 4).

Visual inspection of light microphotographs revealed nerve fiber loss and small nerve fibers in the sciatic nerve of the untreated diabetic rats, compared with those of the control rats; these changes were less prominent in those of the GBe-treated diabetic rats (Fig. 5-I). Additionally, under electron microphotographs, the untreated diabetic rats demonstrated severe alterations of endoneurial microvessels, i.e. closure or decrease in luminal area due to the endothelial cell degeneration or hypertrophy, pericyte aggregation and basement membrane thickening, in the sciatic nerves as compared with the GBe-treated diabetic rats (Fig. 5-II).

Discussion

In the present study, STZ-induced diabetic rats demonstrated a significant retardation of slow axonal transport, axon size reduction and impairment of endoneurial microvessels in the sciatic nerve, together with hyperglycemia and high serum lipid peroxidation. On the other hand, in STZ-diabetic rats treated with GBe for 6 weeks, slow axonal transport and axon size were not significantly affected as compared with the untreated STZ-diabetic rats and endoneurial microvessel alterations with serum lipid peroxidation were also less severe, although those levels were not completely recovered to the normal ranges. Therefore, GBe might have the potential for prevention of adverse changes in both function and morphology in the peripheral nerve of the diabetic patients.

The axonal transport of the STZ-diabetic rats in this study affected the flow of cytoplasmic matrix of the cytoskeletal and

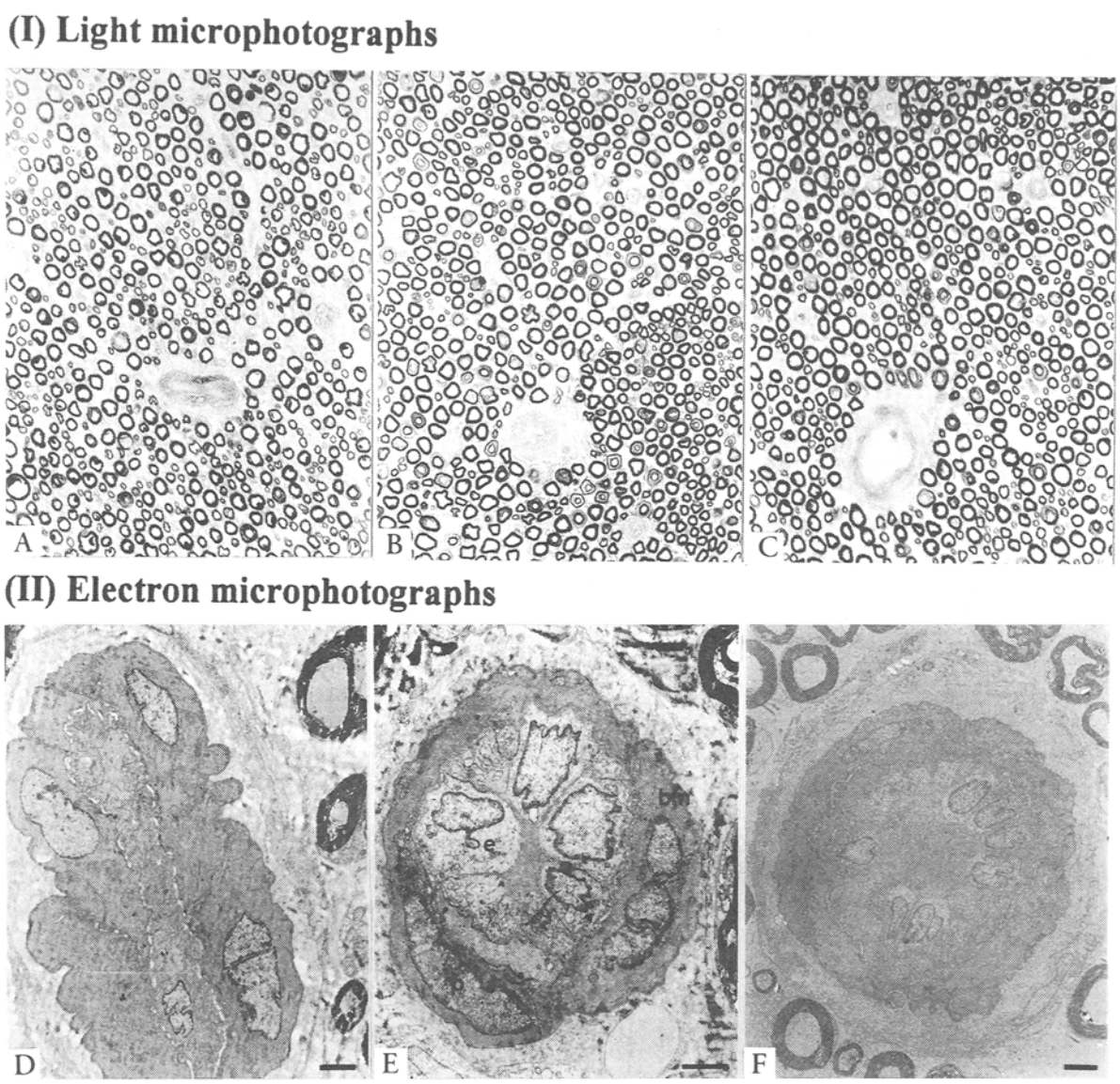

Fig. 5 Light microphotographs (I) for the sciatic nerves and electron microphotographs (II) for the endoneurial microvessels in untreated diabetic rats (A, D, and E), GBe-treated diabetic rats (B and F), and control rats (C). Nerve fiber loss and axon diameter decrease are observed in untreated diabetic rats with alterations of endoneurial microvessels due to endothelial cell (e) degeneration or hypertrophy, pericyte (p) aggregation and basement membrane (bm) thickening as compared with the control rats. Such regions are less prominent in those of the GBe-treated diabetic rats. Bars = 2 μm.

related proteins, particularly lower molecular proteins, i.e. β-tubulin, actin rather than NF subunits. The cause for the delay in axonal transport of slow components was likely due to a decrease in their coherent rates of movement 5). This disturbance of SCb transport with the reduced axon size in the STZ-diabetic rats is supported by the previous studies [1-3, 6]. As there was a significant correlation between velocities of slow axonal transport and axon diameters in the three groups of rats combined, it is suggested that the disturbance of slow axonal transport affects the integrity of axoplasm, which leads to axonal degeneration, atrophy and finally nerve structural alterations.

The pathological finding under electronic microscopic examination of endoneurial microvessels in the sciatic nerve of the STZ-diabetic rats in this study was shown as a closure or decrease in their luminal size due to the endothelial cell degeneration or hypertrophy, pericyte debris and basement membrane thickening like in the previous studies [10, 14, 15, 41-44]. Thus, it is possible to infer that the peripheral nerve of diabetic patients with neuropathy can fall into the endoneurial ischemia or hypoxia by reducing endoneurial blood flow due to the impaired endoneurial microvasculatures. This condition may aggravate the microvascular cell exhibiting edema, luminal blebs, and mitochondrial cristae degeneration by its oxidative injury. Also, it was observed that severities between impairment of endoneurial microvessels and nerve fiber loss and axon size reduction were related in the sciatic nerve, suggesting that microvascular abnormalities are closely associated with the alteration of nerve structure or axonal transport.

In the diabetic rats treated with GBe, endoneurial microvessels with both of axonal transport and nerve fibers were getting better. This is probably because the component of GBe, flavonoid fraction acted as a potent antioxidant, reinforcing protection of the neuro-vascular system from oxidative stress by inhibiting intracellular lactate dehydrogenase leakage, lipid peroxide, phospholipid increase and superoxide dismutase, ATPase decrease in mitochondria related to free radical-induced

pathology [28-30, 45-47]. In the present study, serum lipid peroxidation in the GBe-treated diabetic rats was significantly lower than that in the untreated diabetic rats which may provide evidence that GBe protects microvascular cells from oxidative injury by antioxidant activity. GBe also plays a role of vasoregulation by acting on neuromediator release and hemorheological factors such as platelet activating factor, endothelium derived releasing factor and prostacyclin [31, 32, 48, 49].

Thus, it appears that GBe prevents structural derangement of nerves including axons from disturbed axonal transport in the diabetic process by protecting microvascular abnormalities through its antioxidant activity. Alternatively, as hyperglycemia and body weight decrease were less severe in the GBe-treated diabetic rats than in the untreated diabetic rats, the progression of diabetes itself may be suppressed by GBe, resulting in mild changes in axonal transport and nerve morphology. A further study is necessary to clarify the mechanisms of GBe on diabetic neuropathy.

Acknowledgements

The authors wish to thank Dr. S.H. Park, Department of Public Health and Occupational Medicine, Graduate School of Medicine, and Dr. H.Y. Yoon and Dr. K.W. Cho, Department of Veterinary Internal Medicine, Dr. S.H. Hong, Department of Veterinary Surgery, Dr. K.I. Chung, Department of Veterinary Pathology, Dr. S.Y. Nam, Department of Veterinary Anatomy, Faculty of Applied Agriculture, The University of Tokyo, for their technical assistance. Generous gift of *Ginkgo biloba* extract (GBe) from SK Chemicals Ltd. is also greatly appreciated.

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