Insulin-Ameliorated Peripheral Motor Neuropathy in Spontaneously Diabetic WBN/Kob Rats

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ABSTRACT. Rodent models of diabetes develop a slowing of nerve conduction velocity and mild axonal atrophy, but generally lack overt degenerative neuropathy. Spontaneously diabetic Wistar Bonn Kobori (WBN/Kob) rats develop severe diabetic peripheral motor neuropathy with a slowing of nerve conduction velocity. We examined the effect of glycemic control, using insulin implant, on neuropathic changes in these rats. Animals were divided into 2 groups: WBN group (spontaneously occurring diabetes rats) and WBN + insulin group (spontaneously occurring diabetes rats treated with insulin implants until 90 weeks of age). Conduction velocity was measured in sciatic–tibial motor nerves. These nerves also underwent qualitative and quantitative histomorphologic analysis. Mild to severe hyperglycemia (>200 mg/dl) and glycosuria (>100 mg/dl) were observed in the WBN group. In contrast, the blood glucose level of the WBN + insulin group fluctuated between normoglycemia (<200 mg/dl) and hyperglycemia. Conduction velocity significantly decreased in WBN group compared with WBN + insulin group. Morphologic analysis of the sciatic and tibial nerves of WBN group showed severe changes, including axonal degeneration, myelin distention, endoneurial fibrosis and microangiopathy. Insulin treatment corrected these changes without microangiopathy. These results suggest that insulin could decrease axonal atrophy and myelin distention of peripheral nerve in diabetic WBN/Kob rats. Observation of WBN/Kob rats revealed changes of axon, myelin and capillary caused by diabetes, thus indicating that this animal is a suitable model for investigating diabetic peripheral neuropathy.
KEY WORDS: diabetic neuropathy, insulin, peripheral nerve, rat.

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Peripheral neuropathy is one of the major complications of diabetes mellitus. Its pathogenesis remains to be clarified, but the duration and level of hyperglycemia have a serious impact on the development of neuropathy [8]. Thus, optimum glycemic control is needed to prevent peripheral neuropathy in human patients [1, 10]. In rodent models, insulin treatment prevents and improves the slowing nerve conduction velocity and morphologic changes of the peripheral sensory and motor nerves [5, 7, 24]. Impaired signaling through insulin receptors is emerging as a possible primary pathogenic mechanism that contributes to diabetes-induced damage to the nervous system [17]. These data suggest that insulin acts as a support factor for peripheral nerves.

Human diabetic peripheral neuropathy is characterized by nerve fiber loss, axonal degeneration and segmental demyelination with a slowing of nerve conduction velocity [8]. Rodent models of diabetes develop a slowing of nerve conduction velocity and mild axonal atrophy, but generally lack overt degenerative neuropathy, demyelination and fiber loss in the peripheral nerves. However, diabetic Wistar Bonn Kobori (WBN/KobSlc) rats, which occurred endocrine insufficiency by chronic pancreatitis, spontaneously develop long-lasting diabetes and severe diabetic peripheral motor neuropathy characterized by segmental demyelination and axonal atrophy with slowing of nerve conduction velocity [14, 16, 25]. In addition, endoneurial microangiopathic change is seen. Therefore, WBN/Kob rats may be useful for detecting morphological changes of peripheral nerve with glycemic control.

In the present study, we investigated the effect of glycemic control using insulin implants to determine whether or not it would improve the peripheral neuropathic changes induced in diabetic WBN/Kob rats.

MATERIALS AND METHODS

Animals and housing conditions: Male WBN/KobSlc rats were supplied by Japan SLC, Inc. (Hamamatsu, Japan). The animals were housed in stainless steel cages at a temperature of 20 to 26°C and a relative humidity of 40 to 70% under a 12/12-hr light/dark cycle; they were ventilated with filtrated fresh air and allowed free access to tap water and to a widely used standard pelletized diet for experimental rats (Charles River Formula 1, Oriental Yeast, Tokyo, Japan). The animals were handled according to the principles for all experimental procedures outlined in the Guide for the Care and Use of Laboratory Animals prepared by our institution (Setsunan University) and the Japanese Association for Laboratory Animal Science.

Experimental design: Male rats of the WBN/KobSlc strain developed spontaneous diabetes after 40 weeks of age [13, 20]. A total of 14 male WBN/KobSlc rats were randomly divided into 2 groups, and seven intact rats showing sponta-

neously occurring diabetes were used as the diabetes (WBN group). Seven rats were treated with sustained-release insulin implants (LinShin Canada Inc., Toronto, Canada; release rate: 2 U/24 hr/implant for >40 days) 2–3 times by subcutaneous injection 3 weeks, after they showed a spontaneously occurring severe diabetic state (WBN + insulin group). The moribund animals were necropsied, as were the dead animals (a total of 4 rats), during the examination period. The causes of death or the moribund condition in these rats were malignant lymphoma, hypoglycemia caused by the insulin treatment, urinary tract infection and ketoacidosis resulting from severe diabetes. The remaining 10 rats (a total of 5 rats in each group) were sacrificed at 90 weeks of age for morphological examinations.

Glycosuria and glycemia monitoring: Urinary glucose levels in fresh urine were measured semiquantitatively with a urine test paper (Wako Pure Chemical Industries, Osaka, Japan), and blood glucose levels in the tail vein samples were also measured semiquantitatively by using the glucose oxidase method (Glutest E, Sanwakagaku, Nagoya, Japan). The urinary and blood glucose levels were measured monthly after the rats reached 40 weeks of age. Blood samples from the tail vein, and fresh urine, were collected between 1:00 pm and 4:00 pm for measuring the fasting blood glucose level. The severity of hyperglycemia was defined as follows: normal, <200 mg/dl; mild, >200 mg/dl; moderate, >300 mg/dl; or severe, >400 mg/dl. The severity of glycosuria was defined as follows: normal, <100 mg/dl; mild, >100 mg/dl; moderate, >250 mg/dl; or severe, >500 mg/dl.

Motor nerve conduction velocity (MNCV): At the end of the experiment, rats were deeply anesthetized with ketamine (40 mg/kg IM; Ketalar, Sankyo, Tokyo, Japan) and xylazine (2.0 mg/kg IM; Seractal, Bayer, Tokyo, Japan). The right sciatic nerve was exposed by incisions in the regions of the great trochanter and ankle, and the distance between incisions was measured. Bipolar stimulating electrodes were placed on the nerves through the incisions, and bipolar recording electrodes were inserted percutaneously into either the interossei or lumbricalis muscle. The muscle potentials and conduction velocity were recorded using an electromyography system (Polygraph 360 System, Nippon-denki-sanei, Tokyo, Japan, and BioSignal Processing Program, Nihonsanteku, Osaka, Japan). The hind limb skin temperature was maintained at 37°C.

Histological and ultrastructural analysis: The animals were euthanized by exsanguination from the abdominal aorta under deep anesthesia with ketamine (40 mg/kg IM; Ketalar, Sankyo) and xylazine (2.0 mg/kg IM; Seractal, Bayer). The right sciatic and tibial nerves were removed and fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Samples were trimmed, dehydrated in an automated processor and embedded in paraffin. The left sciatic and tibial nerves were removed and fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. After fixation, tissue samples were postfixed in 1.5% osmium tetroxide solution (pH 7.4) for 2 hr and processed into epoxy resin. Semithin (1 μm) sections were cut and stained with toluidine blue. Ultrathin sections were cut, stained with uranyl acetate and lead

citrate and examined under an electron microscope (JEM 1200EX, JEOL, Tokyo, Japan).

Morphometrical analysis: For morphometric analysis, semithin cross-sections of a distal portion of tibial nerve were used. Digital images (20 × objective lens, 3,900 × 3,090 pixels) were captured using a digital camera (DC500, Leica Microsystems, Wetzlar, Germany) attached to a light microscope (DM5500, Leica Microsystems). The sections were analyzed morphometrically by image processing and analysis software (Ultimage Pro version 2.6.1, Graftek, Austin, France). Morphometric parameters analyzed were of the total fascicular area; the number and size (cross-sectional area) of myelinated nerve fibers, myelin and axons; and the mean fiber, axon and myelin size (cross-sectional area). Fiber occupancy (nerve fiber area/fascicular area) was calculated by dividing the total area of myelinated fibers into the total fascicular area. Fiber density (number of fibers/mm^2) was calculated by dividing the total number of myelinated fibers into the total fascicular area.

Statistical analysis: Data are presented as mean ± SD. One-way ANOVA was used with the Tukey multiple comparisons test to determine whether values differed. A *P* value of less than 0.05 was considered statistically significant. Statistical analyses were performed by using the StatMate III program (ATMS, Tokyo, Japan).

RESULTS

Blood and urine glucose levels: Mild to severe hyperglycemia (>200 mg/dl) and glycosuria (>100 mg/dl) were observed in the WBN group at approximately 40 weeks of age. In contrast, the blood glucose level of the WBN + insulin group fluctuated between normoglycemia (<200 mg/dl) and hyperglycemia. Although blood glucose levels were kept under 200 mg/dl immediately after treatment with insulin implants, they gradually increased before the next treatment (Fig. 1 and Table 1).

Motor nerve conduction velocity: The conduction velocity significantly decreased in the WBN group (35.0 ± 3.6 m/sec) compared with the WBN + insulin group (41.0 ± 1.7 m/sec) (Table 1).

Morphological analysis: Histopathologic analysis of the sciatic and tibial nerves of WBN group showed severe changes in myelinated nerve fibers (Fig. 2). The affected fibers were characterized by axonal atrophy and myelin distention (Fig. 2). Loss of myelinated fibers was observed around endoneurial vessels (Fig. 2). These changes in myelinated nerve fibers were corrected by insulin treatment; in particular, the axons showed an almost normal shape (Fig. 3). Endoneurial fibrosis was constantly detected in the WBN group, and endoneurial vessels displayed thickening of the wall and perivascular fibrosis (Fig. 2). Electron microscopically, endoneurial vessels had reduplication of the basal lamina (Fig. 4). Insulin treatment inhibited endoneurial fibrosis, but the vascular lesions between the two groups were not different (Figs. 3 and 4).

Morphometric analysis revealed marked reduction of fiber occupancy in the WBN group compared with the WBN +
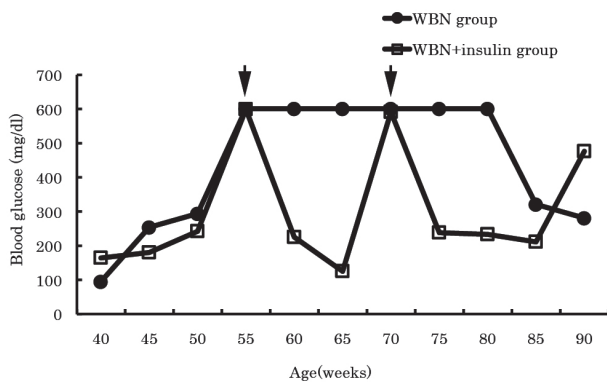
Fig. 1. Change of representative blood glucose levels in WBN and WBN + insulin groups. Insulin treatment (arrows).

Table 1. Body weight, blood glucose and motor nerve conduction velocity

		Body weight (g)	Blood glucose (mg/dl)	MNCV (m/sec)
WBN	mean	375.3	431.3	35.0
	S.D.	16.6	62.9	3.6
WBN + insulin	mean	429.8	212.8	41.0[##]
	S.D.	34.0	147.7	1.7

MNCV: motor nerve conduction velocity. ## $P<0.01$ vs WBN.

insulin group ($P<0.01$) (Table 2). The axon/fiber ratio, mean axon size and mean fiber size were significantly decreased in WBN group, but mean myelin size was similar in both groups. The total number of nerve fibers in WBN group was decreased, but no significant difference was found between
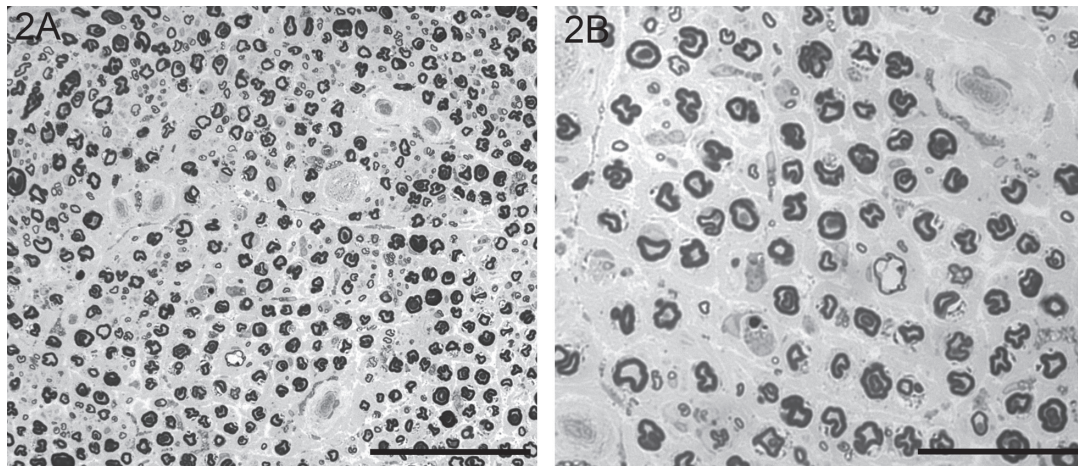

Fig. 2. Representative sections of tibial nerve in WBN group. The affected myelinated fibers are characterized by axonal atrophy and myelin distention. Loss of myelinated fibers is observed around endoneurial vessels. Endoneurial fibrosis is constantly detected in WBN group, and endoneurial vessels display thickening of the wall and perivascular fibrosis. Toluidine blue stain; bar, 100 μm (A), 50 μm (B).
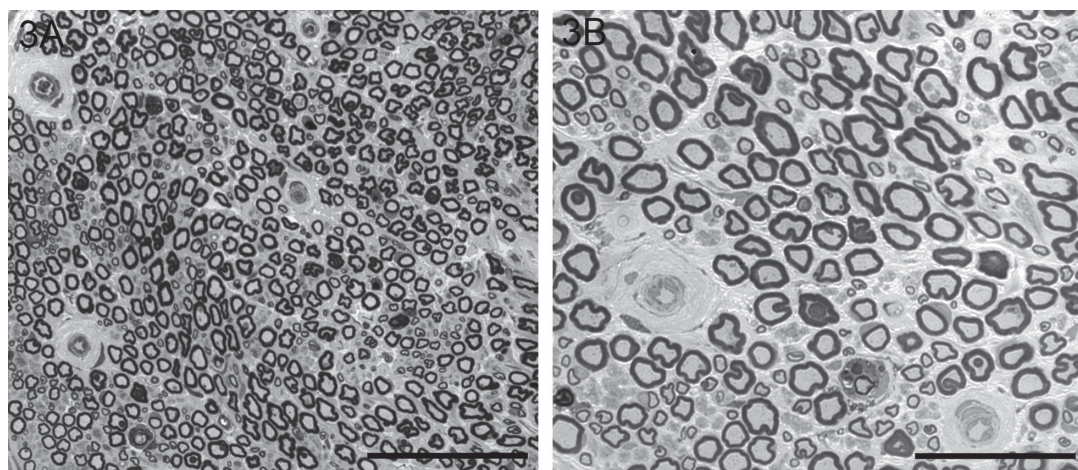

Fig. 3. Representative sections of tibial nerve in WBN + insulin group. Myelinated nerve fibers are corrected by insulin treatment; axons especially display an almost normal shape. Insulin treatment inhibits endoneurial fibrosis, but endoneurial vessels display thickening of the wall and perivascular fibrosis. Toluidine blue stain; bar, 100 μm (A), 50 μm (B).
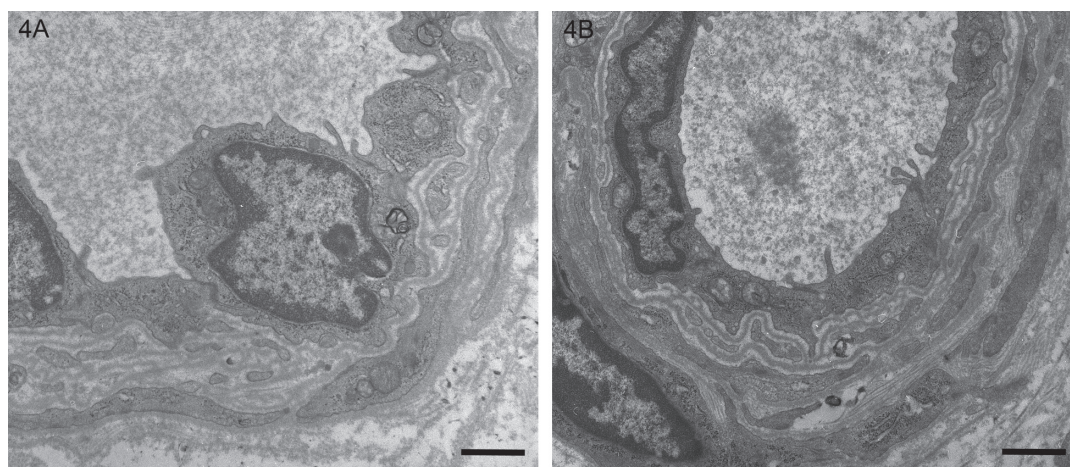
Fig. 4. Representative ultrastructural sections of endoneurial vessel in WBN (A) and WBN + insulin (B) groups. Endoneurial vessel has reduplication of basal lamina in both groups. Uranyl acetate and lead citrate; bar 1 μm.

Table 2. Morphometric data of Tibial nerve

		Total fascicular area (μm^2)	Fiber occupancy (%)	Fiber number	Fiber density (fiber number/ mm^2)	Axon/ fiber ratio	Mean fiber size (μm^2)	Mean axon size (μm^2)	Mean myelin size (μm^2)
WBN	mean	231917.4	25.7	1554.4	7021.5	0.18	38.1	6.9	31.2
	S.D.	48352.5	2.5	167.5	2032.2	0.02	7.5	1.8	5.8
WBN + Insulin	mean	243757.1	35.0##	1721.4	7072.5	0.34##	48.8#	16.5##	33.0
	S.D.	7324.6	5.6	167.9	790.0	0.04	5.9	2.6	5.5

$P<0.01$ vs WBN. # $P<0.05$ vs WBN.

WBN and WBN + insulin groups. The fiber and axon-size frequency histogram showed a shift to smaller fiber size in WBN group compared with WBN + insulin group (Fig. 5).

WBN + insulin rats showed individual difference in the severity of nerve fiber's lesion and endoneurial fibrosis, but were apparently different from WBN rats. There was no correlation between MNCV and morphological changes in WBN + insulin groups.

DISCUSSION

The present study showed that glycemic control with insulin could relieve peripheral neuropathic changes in diabetic WBN/Kob rats including axonal atrophy and myelin distension. Human diabetic peripheral neuropathy is characterized by nerve fiber loss, axonal degeneration and segmental demyelination with endoneurial microangiopathy [8]. Multifactorial pathogenesis was suggested including accelerated polyol pathway, enhanced advanced glycation product formation and increased oxidative stress [8]. These factors involve directly and indirectly the development of diabetic neuropathy affecting nerve tissues or through vascular tissues. Insulin treatment reduced neuropathic lesions in the peripheral nerves of experimental diabetic animals [3, 11, 23]. Indeed, insulin receptors exist in the axons of peripheral neurons [5, 19], and insulin may stimulate them to maintain axonal structure. Thus, insulin is considered

to normalize axonal changes caused by diabetic peripheral neuropathy. These experimental studies used a streptozotocin (STZ)-induced diabetic model which was characterized by reduction of myelinated fiber size due to reduced axonal caliber and axonal atrophy [23]. It was difficult to elucidate the relationship between myelin and insulin with the STZ model. WBN/Kob rats spontaneously develop diabetic peripheral motor neuropathy characterized by segmental demyelination and axonal degeneration. The prevention to collapse the myelin sheath suggested that insulin is related to maintaining the structure of myelin.

Electrophysiological analysis revealed a significant amelioration of MNCV by insulin treatment, but its effect was very mild. Clinical and experimental studies indicated that insulin treatment could improve conduction velocity deficits [1, 2, 10, 21]. In particular, insulin intensive treatment was effective for inhibiting the progression of neuropathy, compared to conventional insulin therapy [1, 10]. In experimental animals, amelioration of biochemical and morphological abnormalities of the peripheral nerve can be achieved by the application of insulin infusion therapy rather than daily injections of insulin [3, 12, 22]. Also, fluctuating blood glucose levels may influence the degree of amelioration by insulin [12, 22]. In the present study, the average blood glucose level of insulin-treated rats was half that of non-treated diabetic rats, but fluctuated with insulin treatment. These findings suggest that fluctuating blood glucose
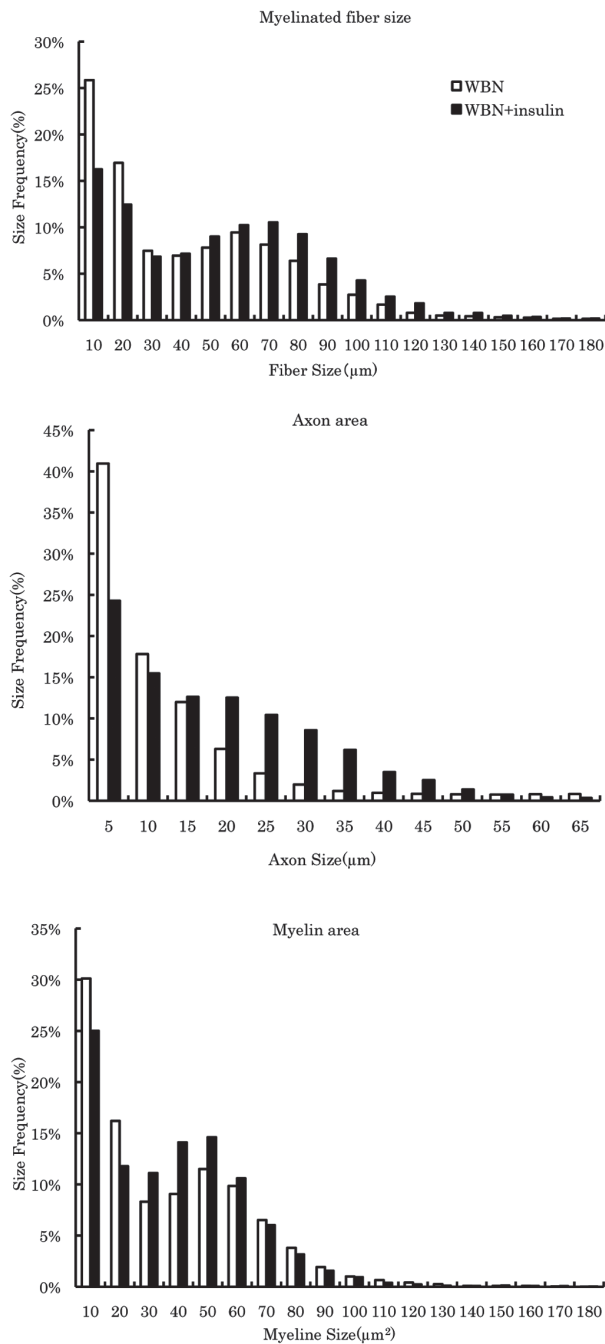
Myelinated fiber size

Axon area

Myelin area

Fig. 5. The myelinated fiber, axon and myelin size frequency histogram in WBN and WBN + insulin groups. The fiber and axon-size frequency histogram show a shift to smaller fiber size in WBN group compared with WBN + insulin group.

levels affect nerve conduction of the peripheral nerve.

Endoneurial capillary basement membrane thickening with endothelial cell hypertrophy was the characteristic structural changes of human diabetic neuropathy [6]. There was a correlation between the degree of basement membrane

thickening and the severity of myelinated fiber abnormality [4, 9]. The duration of diabetes mellitus was significantly associated with the area occupied by reduplicated basement membrane [6]. Furthermore, peripheral nerve functions may deteriorate along with the progression of microangiopathy in diabetic patients [15]. However, our present data did not reveal a significant amelioration of microangiopathy by insulin treatment, in spite of the decrease of peripheral neuropathic changes and a significant amelioration in MNCV. Similar microangiopathic changes of endoneurial microvessels occurred in rats with hyperinsulinemia and insulinoma [18]. Thus, it is suggested that inadequate glycemic control and hyperinsulinemia together brought about microangiopathic change in our rats.

In conclusion, our results suggest that insulin could decrease axonal atrophy and myelin distension of peripheral nerves in diabetic WBN/Kob rats. In WBN/Kob rats, changes in axon, myelin and capillary caused by diabetes could be observed, thus indicating that this animal was a suitable model for investigating diabetic peripheral neuropathy.

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