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Original Article

Effect of deoxycorticosterone acetate-salt-induced hypertension on diabetic peripheral neuropathy in alloxan-induced diabetic WBN/Kob rats

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Abstract: The relationship between hypertension and diabetic peripheral neuropathy (DPN) has recently been reported in clinical research, but it remains unclear whether hypertension is a risk factor for DPN. To investigate the effects of hypertension on DPN, we analyzed morphological features of peripheral nerves in diabetic rats with hypertension. Male WBN/Kob rats were divided into 2 groups: alloxan-induced diabetic rats with deoxycorticosterone acetate-salt (DOCA-salt) treatment (ADN group) and nondiabetic rats with DOCA-salt treatment (DN group). Sciatic, tibial (motor) and sural (sensory) nerves were subjected to qualitative and quantitative histomorphological analysis. Systolic blood pressure in the two groups exhibited a higher value (>140 mmHg), but there was no significant difference between the two groups. Endoneurial blood vessels in both groups presented endothelial hypertrophy and narrowing of the vascular lumen. Electron microscopically, duplication of basal lamina surrounding the endothelium and pericyte of the endoneurial vessels was observed, and this lesion appeared to be more frequent and severe in the ADN group than the DN group. Many nerve fibers of the ADN and DN groups showed an almost normal appearance, whereas morphometrical analysis of the tibial nerve showed a significant shift to smaller fiber and myelin sizes in the ADN group compared with DN group. In sural nerve, the fiber and axon-size significantly shifted to a smaller size in ADN group compared with the DN group. These results suggest that combined diabetes and hypertension could induce mild peripheral nerve lesions with vascular changes. (DOI: 10.1293/tox.2015-0033; J Toxicol Pathol 2016; 29: 1–6)

Key words: diabetes, neuropathy, hypertension, rat

Introduction

Data on the effects of peripheral nerves in hypertension were scarce in both clinical and experimental studies. Patients with essential hypertension showed a reduced number of active sensory nerve fibers without affecting myelination[1]. Evidence gathered from spontaneously hypertensive (SHR) rats revealed that the vascular supply to the peripheral nerves was impaired by thickening of the arterial wall and luminal narrowing of interfascicular arteries[2] and that high blood pressure affected the sural nerve, especially the small myelinated fibers[3]. However, the influence of hypertension on nerve function has yet to be elucidated.

Peripheral neuropathy is one of the major complications of diabetes mellitus. Although its exact pathogenesis is not fully understood, the duration of hyperglycemia and poor glycemic control have a serious impact on the development of neuropathy[4] . Other risk factors including hypertriglycemia, body mass index, smoking and hypertension play an important role in the incidence of neuropathy[5]. Also, the relationship between hypertension and the incidence of neuropathy was found to be significantly strong in type 1 and 2 diabetes patients[6–8]. However, in some clinical studies, a negative association between hypertension and neuropathy was found in diabetic subjects[9]. In experimental animals, only two studies showed that hypertension had a mild additive contribution to diabetic neuropathy, but these studies were conducted only with SHR and SHR hybrid rats[10, 11]. Since the data from animal experiments are very poor, there is a need to analyze whether hypertension and hyperglycemia affect the peripheral nerve using other animal models of diabetes and hypertension.

Human diabetic peripheral neuropathy is characterized by nerve fiber loss, axonal degeneration and segmental demyelination with a slowing of nerve conduction velocity[4]. Many diabetic animal models including the alloxan-induced diabetes model have been studied to clarify the pathogenesis of neuropathy. An alloxan-induced model develops rapid and overt hyperglycemia, a slowing of nerve conduction velocity and mild axonal atrophy, but generally lacks overt degenerative neuropathy, demyelination and fiber loss in the

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peripheral nerves. Diabetic Wistar Bonn Kobori (WBN/KobSlc) rats, which evidenced endocrine insufficiency due to chronic pancreatitis, spontaneously develop long-lasting diabetes and severe diabetic peripheral motor neuropathy characterized by segmental demyelination and axonal atrophy, with slowing of the nerve conduction velocity[12–14]. In addition, an endoneurial microangiopathic change is seen. WBN/Kob rats may be useful for detecting morphological changes of the peripheral nerve occurring in conjunction with hyperglycemia. However, WBN/kob rat show overt hyperglycemia and glucosuria from about 40–60 weeks of age. Thus, WBN/Kob rats in this study were given alloxan in order to accelerate the diabetic condition from an early age.

The aim of the present study was to investigate the effect of superimposed hyperglycemia on peripheral nerve morphology under acute severe hypertension using deoxycorticosterone acetate (DOCA) salt, in alloxan-induced diabetic WBN/Kob rats and non-induced 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, which are 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. The study was approved by the Committee for Animal Experiments of Setsunan University.

Experimental design

A total of 20 male WBN/Kob rats were divided into two groups at 10 weeks of age: the ADN and DN group. The 10 rats in the ADN group, aged 10 weeks, were given a single dose of alloxan (Sigma-Aldrich Japan, Tokyo, Japan) via the tail vein at a dosage level of 40 mg/kg body weight. The concentrations were set up as a given dose to see which a rat can survive for a long period of time after developing signs of diabetes and which dose induces continuous glycosuria. From 13-weeks-old, rats were injected subcutaneously once a week with DOCA (Sigma-Aldrich Japan, Tokyo, Japan) at a dose of 50 mg/kg body weight suspended in corn oil (50 mg DOCA/mL corn oil), and received 1% NaCl (Wako) added to tap water. The 10 nondiabetic rats in the DN group received DOCA and NaCl from 13 weeks of age, as did the ADN group.

The moribund and dead animals (4 each per group) were necropsied during the examination period. The causes of death or moribund condition in these rats resulted from severe hypertension and renal failure. The original plan was to perform necropsy at 37 weeks of age. However, because the number of remaining rats was about half of the original number, the 6 remaining rats in each group were sacrificed at 27 weeks of age (ADN group) and 31 weeks of age (DN group) for morphological examinations.

Glycosuria, glycemia and blood pressure monitoring

Urinary glucose levels in fresh urine were measured semiquantitatively with urine test paper (Wako Pure Chemical Industries, Osaka, Japan), and blood glucose levels in the tail vein blood samples were also measured semiquantitatively by using the glucose oxidase method (Glutest E, Sanwa Kagaku, Nagoya, Japan). Urinary and blood glucose levels were measured monthly from 10 weeks of age. Blood samples from the tail vein, and fresh urine, were collected between 1:00 pm and 4:00 pm to measure 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. Blood pressure was measured monthly from 10 weeks of age by the tail-cuff method using the Non-Invasive Blood Pressure Monitor for Mice and Rats (MK-2000, Muromachi Kikai Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions. Five consecutive measurements were averaged, and the mean value was calculated.

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, tibial and sural nerves were removed and fixed by immersion 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. Sections (4 μm thick) were stained with HE, Luxol fast blue and Masson's trichrome, and analyzed morphologically. The left sciatic, tibial and sural nerves were removed and fixed by immersion 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).

Morphometric analysis

For morphometric analysis, semithin cross sections of a distal portion of the tibial and sural nerves were used, with one section each of the nerves being used per animal. For tibial nerve samples, a terminal portion of the tibial nerve of about 5 mm long from just proximal to the branching of the lateral and medial planter nerve was used. For sural nerve samples, a terminal portion of sural nerve of about 5 mm long from just proximal to the terminal branching was used.

Digital images (20× objective lens, 3900 × 3090 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 (IP Lab version 4.0, BD Biosciences, Rockville, MD, USA). The morphometric parameters analyzed were of the total fascicular area; the numbers and sizes (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 by the total fascicular area. Fiber density (number of fibers/mm^2) was calculated by dividing the total number of myelinated fibers by the total fascicular area. Histograms for the size frequency of nerve fiber, axon and myelin, separated into class intervals increasing by 10 μm^2, were constructed.

Statistical analysis

Data are presented as the mean ± SD. The Student's *t*-test was used to compare the significance of differences in the mean values. Comparisons between histograms were made by the Mann-Whitney *U* test. A *P* value of less than 0.05 was considered statistically significant. Statistical analyses were performed by using the StatMate III software (ATMS, Tokyo, Japan).

Results

Glycosuria, glycemia and blood pressure monitoring

The average body weight of the ADN group was significantly decreased compared with that of the DN group from 13 weeks of age to the time of necropsy (at the time of necropsy, ADN group, 261.7 ± 29.9 g: DN group, 370.8 ± 51.2 g: $P<0.001$). Severe hyperglycemia (>300 mg/dL) and glycosuria (>500 mg/dL) continued from the day of alloxan injection to the time of necropsy in the ADN group, but all rats of the DN group showed normal glycemia and no glycosuria. The systolic blood pressures of the ADN and DN groups exhibited a higher values in 23 weeks of age (ADN group, 145.3 ± 11.9 mmHg: DN group, 161.6 ± 25.3 mmHg), but there was no significant difference between the two groups.

Morphological analysis

Many nerve fibers of the ADN and DN groups had an almost normal appearance without nerve fiber loss in the three nerves, but some nerve fibers in the sural nerve of the ADN group showed slight axonal atrophy (Fig. 1). In endoneurial vessels, narrowing of the lumen with endothelial hypertrophy was observed in tibial and sciatic nerves of the ADN and DN groups (Fig. 2A, B, Table 1). Some animals in both groups showed edema and fibrosis in endoneurium (Fig. 2A, B, Table 1). In fact, these vascular and endoneurial lesions were detected equally in the ADN and DN groups (Table 1). Electron microscopically, duplication of the basal lamina surrounding the endothelium and pericytes of the endoneurial vessels was presented in the ADN group (Fig. 2D). Many collagen fibers were also present around the vessels of both groups (Fig. 2C, D). A duplicated basal lamina was also seen between collagen fibers in the ADN

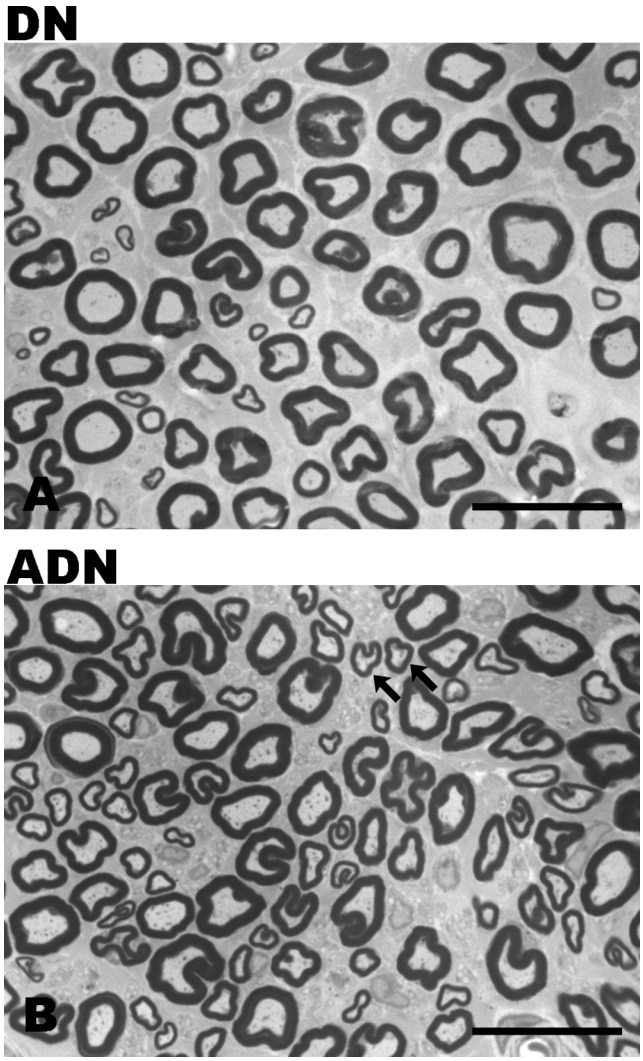

Fig. 1. Representative sections of sural nerve in the DN (A) and ADN (B) groups. The small-sized myelinated fibers are increased in the ADN group (arrows). Endoneurial fibrosis is observed in both groups. Toluidine blue stain. Bar = 20 μm.

Table 1. Histopathological Findings of Vascular and Endoneurial Regions of Tibial, Sural and Sciatic Nerves

	n	Narrowing of vascular lumen	Endoneurial fibrosis/edema
Tibial nerve			
ADN group	6	4	3
DN group	6	2	4
Sural nerve			
ADN group	6	0	3
DN group	6	0	3
Sciatic nerve			
ADN group	6	4	2
DN group	6	3	2

group, but in the DN group, edema was observed among collagen fibers (Fig. 2E, F).

Morphometrical analysis

In the tibial nerve, the fiber occupancy and fiber density of the DN group were slightly decreased compared with those of the ADN group, and the mean myelin size of the ADN group was slightly decreased compared with that of the DN group (Table 2). However, no morphometric parameters, including the aforementioned parameters, showed significant differences between the ADN and DN groups. In the sural nerve, there were also no significant differences in these parameters between the ADN and DN groups (Table 2). The fiber and myelin size frequency histogram for the tibial nerve showed a significant shift to a smaller size in the ADN group compared with the DN group (Fig. 3), and the fiber and axon size frequency histogram for the sural nerve showed a significant shift to a smaller size in the ADN group compared with the DN group (Fig. 3).

Discussion

The present study demonstrated that superimposed hyperglycemia under conditions of DOCA salt hypertension can induce peripheral nerve lesions, but the severity was mild. The sural nerve in the ADN group showed an increased number of small myelinated fibers with mild axonal atrophy, as was the cases with alloxan- and streptozotocin-induced diabetic peripheral nerve lesions[15, 16]. The hypertensive SHR sural nerve also presented an increased number of small myelinated fibers with axonal atrophy[3]. In this study, both groups showed the same degree of hypertension, but in the sural nerve, axonal lesions of the diabetic ADN group were more evident than in the nondiabetic DN group. Thus, it is probable that the axonal lesions of the sural nerves in the diabetic ADN group progressed by the addition of hyperglycemia to hypertension. In addition, a decreased myelin area was observed in the tibial nerve of the diabetic ADN group. This is in accordance with the myelin thinning in the sural nerve of Zucker Diabetic Fatty rats with hypertension and SHR rats with diabetes[10, 11]. However, our results were different from those of previous reports regarding the type of affected nerve. In this study, myelin lesions were detected in the motor tibial nerve but not the sensory sural nerve. Demyelination of diabetic WBN/Kob rat was predominantly observed in the motor nerve. [12, 14] Thus, the motor nerve of WBN/Kob rat may be vulnerable compared with the sensory nerve.

Endoneurial vascular lesions were induced in both groups. Morphological findings such as narrowing of the vascular lumen, endothelial hypertrophy, and duplication of the basal lamina surrounding the endothelium were consistent with previous reports of diabetic patients and animals[13–15, 17–19]. Similar vascular lesions were demonstrated in hypertensive animal model rats (SHR rats) [2, 3]. The present study found the more severe endoneurial vascular lesions, especially duplication of the basal lamina surrounding the endothelium, in the ADN group rather than the DN group. These results suggest that both hypertension and diabetes deteriorated these lesions in the ADN group. The basal lamina of the endothelium may be the most affected area in the peripheral nerve by diabetes, because it showed the same levels of hypertension in both groups.

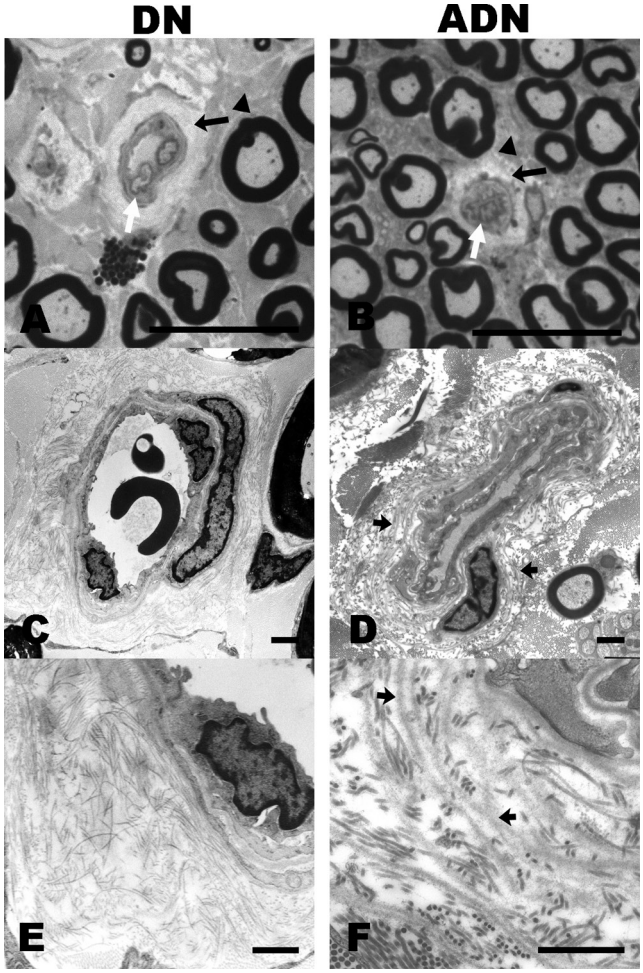

Fig. 2. Representative sections of sciatic nerve in the AND and DN groups. (A, B) In endoneurial vessels (white arrows), narrowing of the lumen with endothelial hypertrophy is observed in both groups. Both nerves show edema (black arrow) and fibrosis (arrowhead) in the endoneurium. (C, D) Electron microscopically, duplication of the basal lamina (arrows) surrounding the endothelium and pericytes of endoneurial vessels is seen in the ADN group. Many collagen fibers are also present around the vessels of both groups. (E, F) High magnification of Figs. 2C and 2D. Duplicated basal laminae (arrows) of the ADN group are also seen between collagen fibers, but in the DN group, edema is observed among collagen fibers. Toluidine blue stain. Bar=20 μm (A and B). Uranyl acetate and lead citrate. Bar = 1 μm (C–F).

The ADN group (27 weeks of age) and DN group (31 weeks of age) were autopsied at different weeks of age. As the animals of the two groups were treated with DOCA, the duration of hypertension in the ADN group was short compared with that in the DN group. However, vascular and nerve lesions of the ADN group showed more severe

Table 2. Morphometric Analysis of Tibial and Sural Nerves

		Fiber occupancy (%)	Fiber density (fiber number/ 100 μm^2)	Axon/fiber ratio	Mean fiber size (μm^2)	Mean axon size (μm^2)	Mean myelin size (μm^2)
Tibial nerve							
ADN group	Mean	45.77	10096.71	0.43	45.58	18.59	26.98
	SD	7.28	1891.30	0.06	3.83	2.43	3.61
DN group	Mean	40.54	8489.60	0.39	47.98	18.96	29.02
	SD	3.96	573.46	0.04	6.38	3.32	5.89
Sural nerve							
ADN group	Mean	44.03	11832.12	0.45	40.42	17.15	21.78
	SD	11.56	4454.98	0.05	6.95	4.02	4.54
DN group	Mean	45.14	11585.11	0.48	39.51	18.16	21.35
	SD	5.68	2120.77	0.08	5.41	2.40	5.41

No significant difference between the two groups.

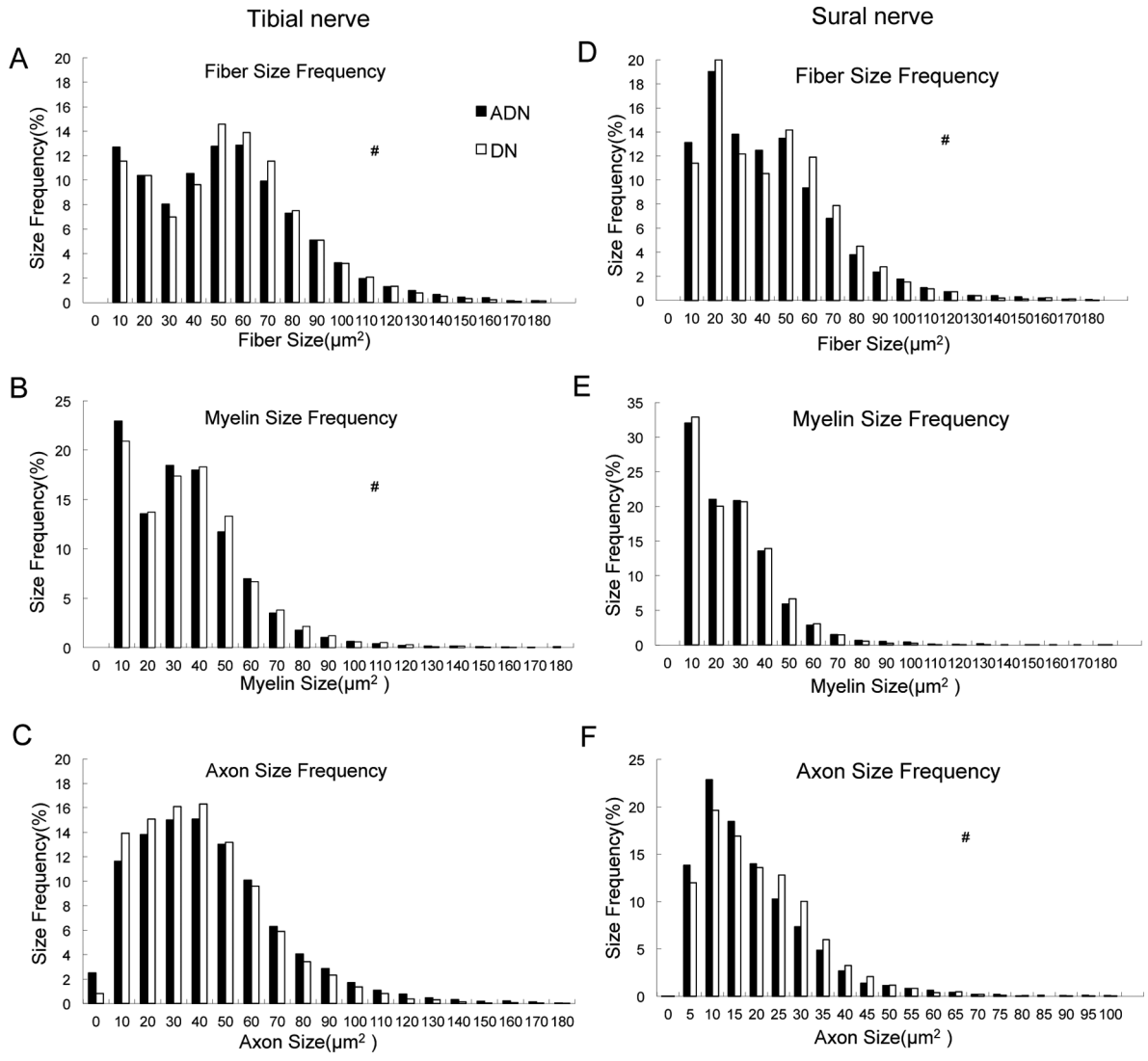

Fig. 3. Myelinated fiber, axon and myelin size frequency histograms for the tibial (A–C) and sural (D–F) nerves in the ADN and DN groups. The fiber and myelin size frequency histogram for the tibial nerve shows a shift to a smaller size in the ADN group compared with the DN group. In sural nerve, the fiber and axon size frequency histogram is shifted to a smaller size in the ADN group compared with the DN group.

changes than those of DN group. Thus, it was possible to compare the two groups without regard to autopsy age. Previous papers reported that alloxan treatment induced axonal lesions[15, 16]. In this study, axonal lesions in the ADN group were evident in only the sural nerve. Therefore, it was highly probable that nerve lesions in the ADN group were induced by diabetes and hypertension.

Diabetic peripheral neuropathy is closely related to the severity of endoneurial vascular lesions[17, 20, 21]. Indeed, vascular dysfunction is a key cause of diabetic peripheral neuropathy. In this study, nerve lesions of the hypertensive and diabetic ADN group with vascular lesions were more severe than those of the hypertensive DN group with vascular lesions. It was thus suggested that hypertension and diabetes deteriorated the nerve and vascular lesions. However, the severity of nerve lesions was very mild even in the hypertensive and diabetic ADN groups. Thus, in this experimental condition, hypertension and hyperglycemia induced by alloxan and DOCA have only a small effect on diabetic peripheral neuropathy in our diabetic model. The precise reason for this is unclear, but it is possible that the short duration of hypertension and diabetes could not induce apparent nerve lesions.

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Disclosure of potential conflicts of interest: The authors declare that they have no competing interests.

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