AN EARLY DIAGNOSTIC TOOL FOR DIABETIC NEUROPATHY: CONDUCTION VELOCITY DISTRIBUTION

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Accepted 9 July 2010

ABSTRACT: Diabetes is a metabolic disorder that affects much of the human population. As a secondary complication, diabetic neuropathy causes time-dependent damage to peripheral nerves. In this study, experimental diabetes was induced by streptozotocin (STZ; 50 mg/kg intraperitoneally) in rats. Diabetic animals were grouped into those with 2 or 4 weeks of diabetes, whereas a control group received only the STZ vehicle (0.1 M citrate). Sciatic nerves were dissected, and compound action potentials (CAPs) were recorded. Results deduced by conventional calculation carried less information when compared with conduction velocity distribution (CVD) obtained by a computer-based mathematical model. Using the conventional approach, statistically significant changes were first seen in the fourth week of diabetes, whereas results deduced by CVD measurement could be seen in the second week. Consequently, the CVD calculation provides more information for the early diagnosis of neuropathies compared with classical conduction velocity measurements.

Muscle Nerve **43:** 237–244, 2011

Diabetes involves secondary complications such as neuropathies. Diabetic neuropathy is the common name for nerve damage caused by diabetes mellitus. Diabetic neuropathic conditions affect the entire human nervous system and cause pain and weakness in the hands, arms, feet, and legs.[1] Diabetic patients also experience a loss of sensation in the feet and legs and/or asymptomatic progressive loss of peripheral nerve function.[2] These conditions may result in the development of foot ulcers and infections, followed by amputation.[3] Early diagnosis and management are the best precautions for diminishing such progression.

Measurement of nerve conduction velocity (NCV) is among the most important diagnostic techniques in peripheral neuropathies.[4,5] This measurement can be made easily by dividing the distance between stimulating and recording electrodes by the time for the compound action potential (CAP) to travel this distance (Δt, latency).[6] Latency is measured by obtaining the time to the onset of CAP.[7] The NCV calculated by using latency carries information only for the nerve fibers having the largest axon diameters and the fastest conduction velocities. Accordingly, we can say that classical NCV measurements do not give information about alterations in smaller diameter axons caused by neuropathic conditions.[8,9] It is commonly known that diabetic neuropathy causes damage to the myelin sheath,[10] resulting in slowing of the conduction velocity in myelinated fibers. As with all other neuropathies, the effect of diabetes is dissimilar in nerve fibers with different axon diameters.[11] In diabetic neuropathy, the level of damage to the nerve fibers is directly associated with exposure time of diabetes. Furthermore, diabetes also affects both large- and small-diameter nerve fibers over the same period of time.[8,12,13]

The conduction velocity distribution (CVD) histogram calculation is an accurate way to gather information about the contribution of each nerve fiber group of different conduction velocities that make up the CAP.[8,14–16] Therefore, with CVD histogram calculations, damage to different groups of nerve fibers at early stages of neuropathology, such as diabetic neuropathy, can be shown.

The aim of this study is to determine the alterations in CVD of the rat sciatic nerve at early stages of diabetic neuropathy and to investigate the efficacy of CVD as an early diagnostic tool in peripheral neuropathies.

METHODS

Organization of Experimental Group of Animals and Diabetes Induction. This study was approved by the ethics committee of the Selcuk University Experimental Medicine Research and Application Center (Approval No. 2006/56). Due to gender-dependent differences in the rat sciatic nerve fiber CVDs, only male Sprague-Dawley rats weighing 250–300 g (12–14 weeks old) were used for study. After birth, 5 rats were housed per cage at ambient temperature and humidity on a 12/12-h light/dark cycle. All animals received food and water ad libitum. For this study, two diabetes

Abbreviations: $\dot{V}_{max}$, maximum time derivative of compound action potential; $\Delta t_{latency}$, time delay between the moment the stimulus is delivered and the onset of compound action potential; Δt_{peak}, time delay between the moment the stimulus is delivered and the compound action potential amplitude reaches its maximum value; ANOVA, analysis of variance; BG, blood glucose; CAP, compound action potential; c_m, membrane capacitance; CON, control group; CVD, conduction velocity distribution; DM2, 2-week diabetic group; DM4, 4-week diabetic group; DPN, diabetic peripheral neuropathy; IDDM, insulin-dependent diabetes mellitus; MD, maximum depolarization; NCV, nerve conduction velocity; r_i, intracellular axial resistance along axons and dendrites; r_m, resting membrane resistance; STZ, streptozotocin; λ, space constant; τ, time constant

Key words: compound action potential, diabetic peripheral neuropathy, nerve conduction velocity distribution, rat, sciatic nerve

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Published online 15 January 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/mus.21837

(DM2 and DM4) groups were used. Diabetes was induced by a single intraperitoneal (IP) injection of 50 mg/kg STZ (dissolved in 0.1 M sodium citrate, pH 4.5). The control group was given a comparable volume of the citrate vehicle. One week after the STZ injection, animals with at least threefold higher blood glucose levels ($\geq$300 mg/dl) were accepted as diabetic. For purposes of this study, animals were studied 2 weeks after injection (DM2) and 4 weeks after injection (DM4). For electrophysiological recordings, 10 rats were used for the control group (CON), 8 rats were used in the DM2 group, and 10 rats were used in the DM4 group. All chemicals used were purchased from Sigma-Aldrich Chemie (Steinheim, Germany).

Preparation of Sciatic Nerves and Experimental Setup. Under light anesthesia (30 mg/kg sodium pentobarbital), sciatic nerves were dissected from the hind paw of the rats killed by cervical dislocation. Nerves were then rapidly transferred to the recording chamber, which was superfused with fresh modified Locke solution (in millimoles per liter): 140 NaCl, 5.6 KCl, 2.2 $CaCl_2$, 1.2 $MgCl_2$, 10 glucose, and 10 Tris-[hydroxymethylaminomethane] (pH 7.4), at a constant rate to maintain the temperature at 33.2°C (which was monitored online by BiosigW software). Stimulations were given from proximal ends of the nerve trunk through a stimulus isolation unit (Model SIU5; Grass Instruments Co., Quincy, Massachusetts) using a stimulator (Model S88K; Grass Instruments Co.). Square-wave pulses of supramaximal amplitude, 100-μs duration, and 1-Hz frequency were used for nerve stimulation. CAP recordings were performed by using a suction electrode from the tibial branch of the isolated nerve trunk. Amplified (Model CP511 AC amplifier; Grass Instruments Co.) CAP signals were digitized by an A/D converter (Model PCL 1710; Advantech) at a 50-kHz sampling rate and acquired with the BiosigW data acquisition software and stored on a hard disk for further analysis.

Analysis. To investigate the functional alterations caused by diabetes, strength–duration curves were plotted to calculate the rheobase and chronaxie values, and mathematical procedures were conducted on all CAP recordings. Because the rheobase is the minimal intensity to produce a response, the amplitude of the CAP over 0.1 mV was accepted as a minimal electrical response.

In this study, two different conduction velocity calculations were obtained. For this purpose, two time differences ($\Delta t_{\text{latency}}$ and Δt_{peak}) were measured; $\Delta t_{\text{latency}}$ is the time delay between the moment the stimulus is delivered and the onset of CAP, and Δt_{peak} is the time delay between the moment the stimulus is delivered and the CAP amplitude reached its maximum value. When Δx is determined as the distance between stimulating and recording electrodes:

$$\text{CV}_{\text{latency}} = \Delta x / \Delta t_{\text{latency}} \quad (1)$$

$$\text{CV}_{\text{peak}} = \Delta x / \Delta t_{\text{peak}} \quad (2)$$

Conduction velocities for each experimental group were estimated by using eqs. (1) and (2), where Δx was taken as 40 mm.

The maximum time derivatives of CAPs ($\dot{V}_{\text{max}}$) and the areas under the CAPs were also computed. Maximum time derivatives, which correspond to the maximum rate of change in the rising phase of CAPs, can also be used as an index of the conduction activity of nerve fibers in a bundle. The area under the CAP is proportional to the number of excited nerve fibers, so areas under the CAPs were calculated.

To obtain information about the individual activities of nerve fiber groups having different conduction velocities, CVD histograms were developed using a mathematical model that was enhanced[14] using the model proposed by Cummins et al.[15] The basic principle of the model based on the statements of CAP can be expressed as:

$$CAP(t) = \sum_{i=1}^{N} w_i f_i(t - \tau_i)$$

where CAP(t): the observed compound action potential as a function of time, N is the number of fiber classes, w_i is the amplitude weighting coefficients for class i, and $f_i(t)$ is the single-fiber action potential in class i.[14–16] The weighting coefficients (w_i) are general parameters to account for all influence on the contribution of each fiber class to the observed CAP. To estimate the individual activities of nerve fiber groups from CAPs, the CVDs for all nerves of the CON, DM2, and DM4 groups were calculated.

The CVD histogram is divided into three subgroups, *slow*, *medium*, and *fast*, for the reason that the visually augmented effect of diabetes on NCV can be helpful for ease of interpretation.

Pathology. Formaldehyde-fixed peripheral nervous tissue samples were processed with autothecnicon, embedded in paraffin, sectioned by using a microtome and prepared on slides. The slides were stained with hematoxylin–eosin. Stained specimens were examined using a light microscope (Eclipse E400; Nikon, Japan). For each specimen, cross-sections were imaged using a photograph attachment

Table 1. General parameters of the experimental animal groups.

	CON ($N = 24$)	DM2 ($N = 8$)	DM4 ($N = 10$)
Body weight (g)	282.07 ± 2.47	233.27 ± 4.21*	223.35 ± 2.19*
Blood glucose (mg/dl)	105.90 ± 1.52	353.86 ± 6.97*	674.26 ± 27.71*

Values are given as mean ± SEM. N represents number of animals.
**$P < 0.05$ indicates significance level vs. the control (CON) group.*

(Coolpix 5000; Nikon). Photographs of the micrometer microscope slide (Stage Micrometer MBM11100; Nikon) were also taken during the procedure and then transferred into a PC environment and viewed using image analysis software (Clemex Vision Lite v3.5). The length was calibrated by comparing the photograph of the specimen with the photograph of the Nikon micrometer microscope slide, which was taken under the same magnification. Ten nerve fiber diameters were measured for each animal in the experimental group, and mean values were calculated with the Clemex Vision Lite v3.5 program. The reader was blinded to the origin of the specimen.

Statistics. Unless otherwise specified, comparisons between groups were done using one-way analysis of variance (ANOVA), followed by the Duncan post hoc test for multiple comparisons when analysis of variance indicated significant results. $P < 0.05$ was considered significant. Data are presented as mean ± SEM.

RESULTS

STZ injection produced a greater than threefold increase in blood glucose (BG) concentrations after 1 week. BG concentrations of animals were checked every week (data not shown) and just before the experimental sessions (Table 1) to confirm the continuation of induced diabetes. After the initial confirmation of diabetes, a time-dependent increment was seen in BG concentrations until the experimental sessions. In addition, there was significant body weight loss in all diabetic groups of animals ($P < 0.05$) (Table 1).

The parameters of neuronal excitability, rheobase, and chronaxie were calculated from the strength–duration curves. Rheobase, which is the minimal stimulus strength required to trigger the production of an electrical response, was found to be significantly increased for both diabetic groups when compared with controls ($P < 0.05$) (CON = 2.34 ± 0.02 V, DM2 = 2.49 ± 0.03 V, DM4 = 2.44 ± 0.02 V) (Fig. 1A). The chronaxie value, which is the minimal duration of a stimulus that produces a response when a stimulus two times the rheobase is applied to the nerve bundle, was found to be significantly increased 4 weeks after diabetes induction when compared with controls ($P < 0.05$) (CON = 18.82 ± 0.17 μs, DM2 = 19.08 ± 0.18 μs, DM4 = 20.27 ± 0.24 μs) (Fig. 1B).

Sample CAP traces are given in Figure 2 for each group in the same time axis. The figure further shows that diabetes dramatically affected the shape of the rat sciatic nerve CAP for both DM2 and DM4. This finding resulted from a change in the maximum depolarization (MD) value of the CAP and in the number of active fibers making up the CAP. The MD values and CAP areas, calculated for each group (Table 2), were found to be significantly decreased for both diabetic groups ($P < 0.05$). The maximum time derivative of CAP, which is also called upstroke velocity ($\dot{V}_{max}$), can also be

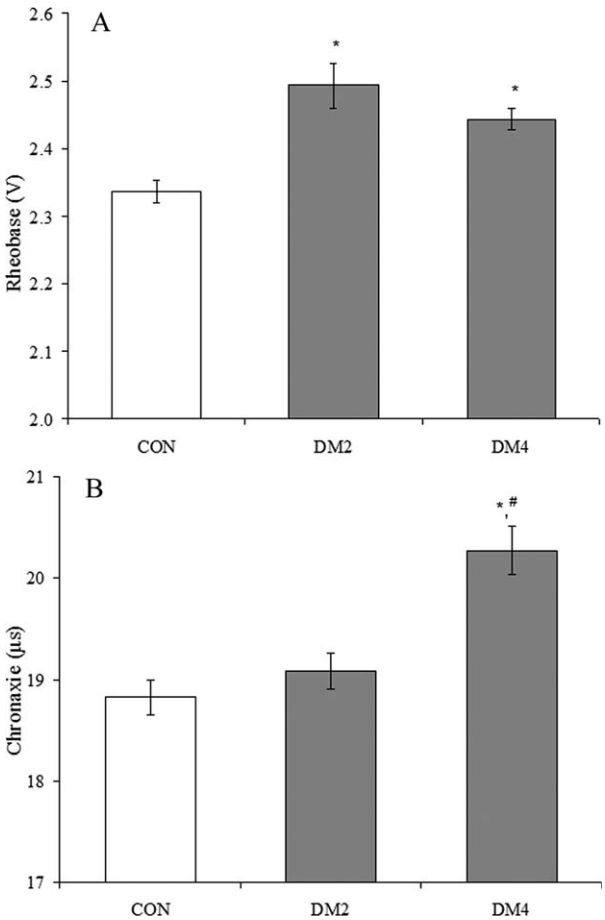

FIGURE 1. Rheobase **(A)** and chronaxie **(B)** values obtained from the experimental groups of animals. *$P < 0.05$ vs. control (CON); #$P < 0.05$ vs. the 2-week diabetic (DM2) group. Values are given as mean ± SEM.

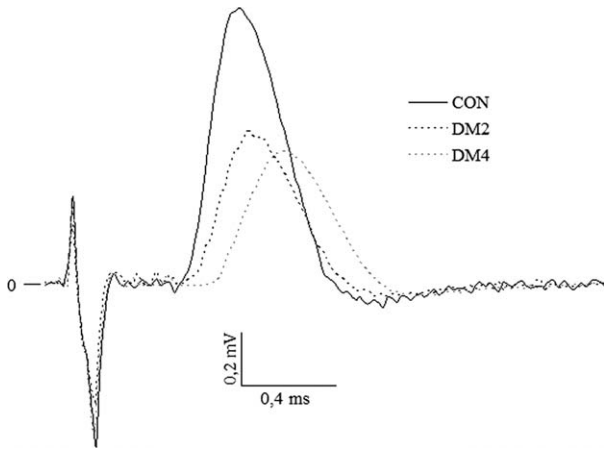

FIGURE 2. Sample CAP traces from a single nerve for each group 40 mm away from the stimulating electrodes. The solid black line represents the control group (CON), the dashed black line represents the 2-week diabetic group (DM2), and the dashed gray line represents the 4-week diabetic group (DM4).

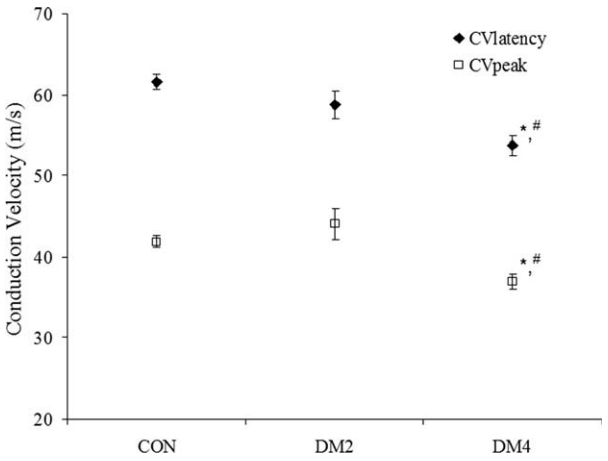

FIGURE 3. Measured conduction velocity values ($CV_{latency}$, CV_{peak}) of each group of animals (CON, DM2, and DM4). $^{*}P < 0.05$ vs. control (CON) group; $^{\#}P < 0.05$ vs. 2-week diabetic (DM2) group. Values are given as mean ± SEM.

used as an index of the conduction activity of nerve fibers that constitute the nerve bundle.[17] The upstroke velocities ($\dot{V}_{max}$) of the CAPs were calculated for each group and are also shown in Table 2. Although the $\dot{V}_{max}$ decreased for both diabetic groups, a significant decrease was seen only in the DM4 group ($P < 0.05$).

In this study, two different conduction velocity calculations ($CV_{latency}$ and CV_{peak}) were measured, as detailed in the Methods section, and the results are plotted in Figure 3. A statistically significant decrease in NCV was found only in the DM4 group for both $CV_{latency}$ and CV_{peak} ($P < 0.05$).

CVD histograms are shown in Figure 4 and were developed using an inverse mathematical model for each group. As in our previous studies,[18,19] to provide a better assessment of the effect of diabetes on fiber groups with different conduction velocities, three conduction velocity subgroups were defined according to the place in which notable changes in NCVs take place. The borders in these subgroups are shown on the CVD histograms as dashed lines in Figure 4. The designations and ranges of these subgroups are as follows: slow, 8–29 m/s; medium, 31–50 m/s; and fast, 51–70 m/s. For each of these subgroups, relative contributions were recalculated, and the changes in relative contribution of these subgroups for CON, DM2, and DM4 are given in Figure 5. There were significant changes in all conduction velocity subgroups of both DM2 and DM4 ($P < 0.05$). In addition, in the DM4 group the contribution of fast fibers was found to be significantly decreased compared with the DM2 group ($P < 0.05$). Therefore, the contribution of the slow-conducting group was significantly increased in the DM4 group compared with the DM2 group ($P < 0.05$) (Fig. 5).

In pathological studies, axon diameters were measured and were found to be increased significantly for both the DM2 and DM4 groups when compared with controls ($P < 0.05$) (Fig. 6). Although there was an increment in diameter with the time of diabetes, no significant difference emerged in the comparison between the DM4 and DM2 groups ($P < 0.05$). The average axon diameter value for the CON group was 9.44 ± 0.50 μm, whereas it was 11.55 ± 0.29 μm for the DM2 group and 12.53 ± 0.45 μm for the DM4 group. Demyelination, axonal swelling, and intraaxonal degeneration with neurofilament depletion were apparent in light photomicrographs of cross-sections of sciatic nerves. These pathological conditions became more prominent with the time from onset of diabetes (Fig. 7).

Table 2. Calculated parameters from CAP recordings of experimental animal groups.

	CON	DM2	DM4
MD (mV)	0.94 ± 0.06	0.50 ± 0.04*	0.37 ± 0.02*
Area (mV/ms)	0.60 ± 0.03	0.42 ± 0.03*	0.36 ± 0.02*
$\dot{V}_{max}$ (mV/ms)	3.38 ± 0.25	2.97 ± 0.41	1.33 ± 0.09*,†

Values are expressed as mean ± SEM.
$^{*}P < 0.05$ vs. control group (CON).
$^{\dagger}P < 0.05$ vs. 2-week diabetic group (DM2).

DISCUSSION

In this study we have investigated the time-dependent effects of experimental diabetes on nerve fibers with different conduction velocities in the sciatic nerve. The STZ diabetic rat is a well-studied model of diabetic neuropathy, and atrophy of the myelinated fibers is seen as a reproducible and predominant lesion of the peripheral nerve in this model. Studies of nerve fiber preparations have shown that nodal abnormalities, such as paranodal

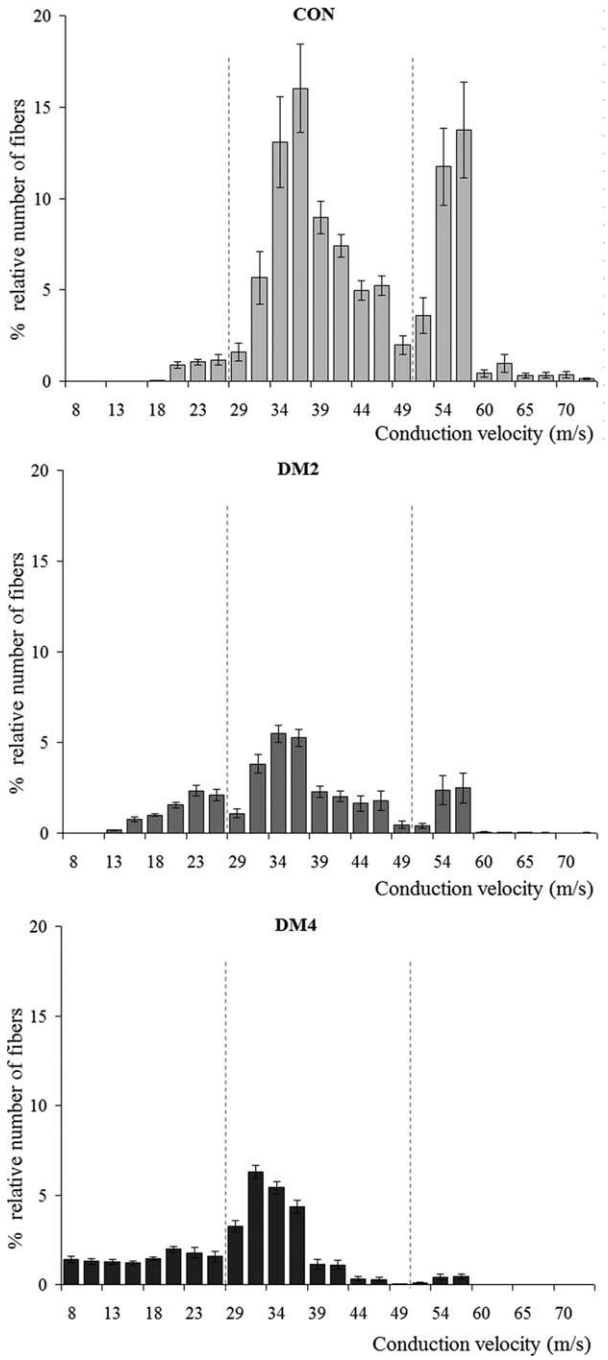

FIGURE 4. Calculated conduction velocity distribution (CVD) histograms of each of the groups (CON, DM2, and DM4). Values are given as mean ± SEM. Percent relative contributions in the DM2 and DM4 group are computed relative to CON values.

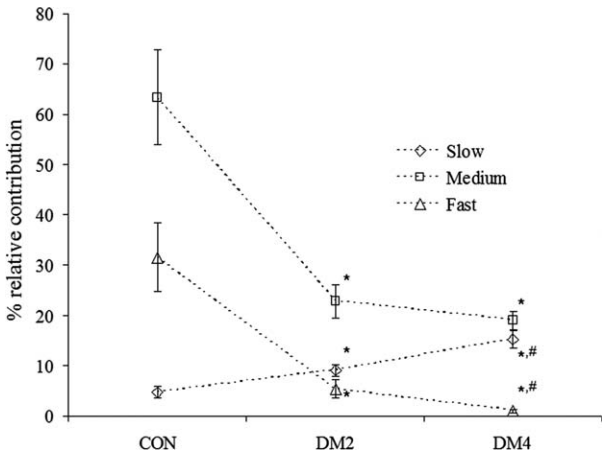

FIGURE 5. Percent relative contributions of constituted conduction velocity subgroups as described in the Results (slow: 8–29 m/s; medium: 31–50 m/s; fast: 51–70 m/s). Values are given as mean ± SEM.

and segmental demyelination and axonal degeneration, are produced in this model after 4 weeks of diabetes.[12,20,21] These findings are similar to those of neuropathy in human insulin-dependent diabetes mellitus (IDDM) as a secondary complication. Within a few days after injection of STZ, hyperglycemia develops, and the blood glucose level increases gradually. It is well known that the level of alterations in peripheral nerve conduction in diabetic peripheral neuropathy (DPN) depends on the time that the nerve is exposed to high blood glucose. Accordingly, impairment in peripheral nerves starts with the development of hyperglycemic conditions. In our study, the sciatic nerves of 2- and 4-week diabetic animals were used to observe the time-dependent alterations caused by diabetes. Our data show that there are deleterious effects of diabetes on nerve conduction, which is in agreement with previous investigations.[22,23]

Generally, rheobase and chronaxie values are used as indices of nerve excitability. An increase in rheobase and chronaxie values is an indicator of decreased excitability. Although the increase in the chronaxie value of the DM2 group was not significant (but was significant in rheobase, at $P < 0.05$), when these two parameters are considered together, one can easily conclude that excitability began to decrease after 2 weeks of diabetes (Fig. 1). The decrement in membrane excitability can be attributed

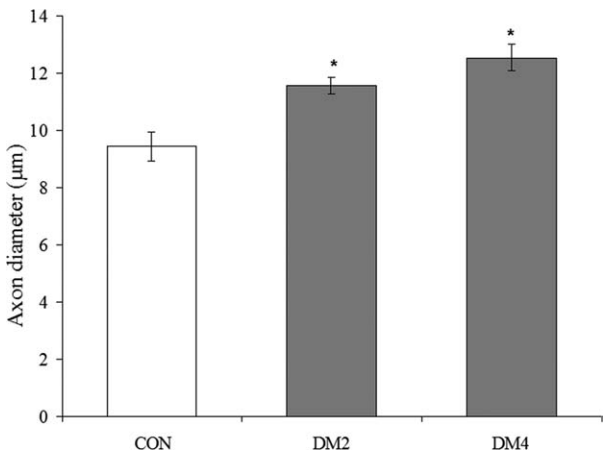

FIGURE 6. Average axon diameter values measured from sciatic nerves of each group of animals (CON, $N = 10$; DM2, $N = 8$; DM4, $N = 10$). $^{*}P < 0.05$ vs. the control (CON) group. Values are given as mean ± SEM.

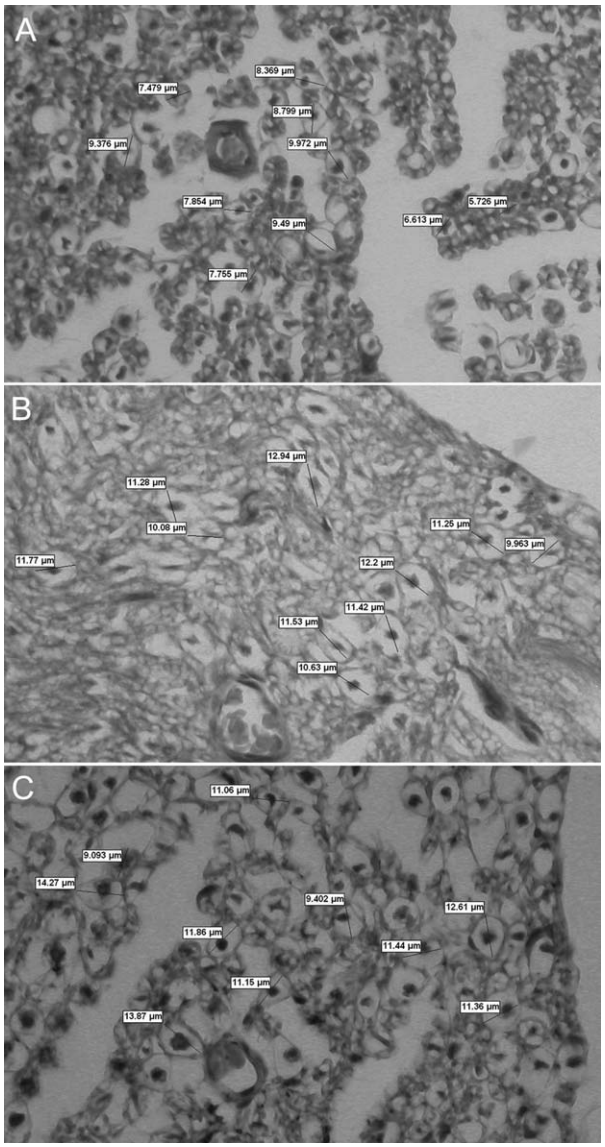

FIGURE 7. Light photomicrographs of cross-sections of sciatic nerves from control **(A)**, DM2 **(B)**, and DM4 **(C)** experimental animals. Axon diameters were measured using the Clemex Vision Lite v3.5 image analysis program.

to both channel activity and passive membrane properties (important for electrical signaling), such as time ($\tau = r_m c_m$, where r_m is resting membrane resistance and c_m is membrane capacitance) and space constants [$\lambda = \sqrt{(r_m/r_i)}$], where r_i is the intracellular axial resistance along axons and dendrites.[24]

The measured peak depolarization (MD) values of the CAP were significantly reduced in both the 2- and 4-week diabetic groups ($P < 0.05$), which can be attributed to an alteration in the CVD of the nerve due to the nature of the CAP. We also calculated the area of the CAP waveforms, which decreased in both diabetic groups ($P < 0.05$). The area was proportional to the number of activated nerve fibers, supporting its use as a measure of conduction block in the nerve.[25] From this point of view, there was a decrement in the number of nerve fibers that actively contribute to the CAP, and thus there are alterations in the CVDs (Table 2). Because the time derivative of the rising phase of the CAPs yields the activity of the fastest conducting fibers in the nerve bundle,[17] the upstroke velocity of CAP ($\dot{V}_{max}$) was calculated and was found to decrease in the diabetic groups, although significance was found only in the 4-week diabetic group ($P < 0.05$). If we simply used this finding as an indicator of the effects on the fastest conducting fiber group, we may conclude that diabetes affected this group later than the others (slow and medium) (Table 2).

To test this hypothesis, two different conduction velocities were calculated, using eqs. (1) and (2). $CV_{latency}$ reflects the fastest conducting fibers, whereas CV_{peak} gives information about most of the fiber groups that make up the nerve. Although there seemed to be a decrease in the $CV_{latency}$ value for the 2-week diabetic group, a significant change was found in both $CV_{latency}$ and CV_{peak} values in the 4-week diabetic group ($P < 0.05$) (Fig. 3). These findings are consistent with morphometric studies conducted at 4 weeks on STZ diabetic rats, and they are explained by fiber diminution and decreased axon/myelin ratio.[12] These conventional velocity measurements, used mostly in clinical studies, show the effects of diabetes solely on the nerve conduction of the fastest and medium groups. However, to understand the velocity changes in relatively slow fibers of the nerve caused by diabetes, determination of the CVD histograms may be a useful tool. This method requires forward and backward calculation but gives detailed information about the relative number of active fibers for discrete conduction velocity values in a nerve bundle.[8,14–16,26] The CVD histograms given in Figure 4 show that, although NCV ranged from 18.1 to 77.8 m/s for the CON group, it ranged from 13.0 to 64.8 m/s and 7.8 to 59.6 m/s in the DM2 and DM4 groups, respectively. These results indicate that, as the duration of diabetes increases, a proportion of fibers shift to slower conduction velocities.

The percentage contribution of the subgroups (slow, medium, and fast) obtained from CVD histograms for CON, DM2, and DM4 are given in Figure 5. We see that, although relative contributions of the fast and medium subgroups decreased, the contribution of the slow subgroup increased significantly in both diabetic groups ($P < 0.05$). Although the relative contribution (%) of the fast subgroup was 31.48 for the CON group, it almost vanished for the DM2 and DM4 groups. This means that the fast subgroup was already affected at 2 weeks. This finding may appear to be in

contrast to the literature at first sight. In histological and morphometric studies, small fibers with slow conduction velocities are found to be much more susceptible to diabetes than larger ones with medium and fast conduction velocities.[12] We also know that fibers with relatively slow conduction velocity are the first affected nerve fibers by pathological conditions such as diabetes. If the CVD findings are evaluated with this knowledge, the increase in the contribution of slow-conducting fibers can be attributed to slowed conduction of the medium and fast subgroups that leads to an increase in the slow subgroup. This interpretation was supported by the significant decrease in the contribution of the medium and fast subgroups starting from the second week of experimentally induced diabetes.

The slowing in conduction velocity with diabetes may have multiple explanations. Because axonal degeneration may change the passive properties of the membrane (τ and λ), there may be a decrease in the conduction velocity. Reduction in the axon/myelin ratio can cause an increment in membrane capacitance (c_m) and produce a decrement in conduction velocity.

In sensory fibers (fastest conducting fibers), enlargement or swelling of the nodal and paranodal axon is one of the earliest structural changes seen in diabetes. It correlates with an early Na/K–ATPase defect and increased intraaxonal Na^+ concentration.[27] The firing rate of ion channels (opening and closing) can also affect nerve conduction velocity. The decrement in channel activity caused by diabetes may lead to a decrement in conduction velocity. Slightly widened nodes of Ranvier may also retard conduction velocity.[12,21,28–31] Some of these findings, such as axonal swelling and degeneration, were also evident in our pathological study results. In the photomicrographs of cross-sections of sciatic nerves from the diabetic groups, progressive demyelination was clearly seen. Axon diameter changes and structural abnormalities were distinct in both diabetic groups. The short-term effects of diabetes on our CVD findings were supported by the pathological findings.

This study has shown that a decrement in nerve excitability with diabetes was seen as early as 2 weeks of diabetes. Although conventional NCV measurements (CV_{peak} and $CV_{latency}$) show a significant change in the fourth week of diabetes, this significant change can be seen by CVD histograms deduced by mathematical modeling in 2 weeks ($P < 0.05$).

Bertora et al.[32] used the collision method for calculating CVD and found similar results within motor and sensory nerve fibers of subclinical neuropathy in diabetic patients. Although the collision method for determining CVD is more practical in terms of clinical applicability when compared with the mathematical methods, results determined by collision methods have limited sensitivity.[33–36] So, for determining CVDs from nerves, the two methods complement one another, and knowledge obtained from this study may be a guide for the physician.

This study was presented at the 20th National Biophysics Congress, Mersin, Turkey, and at the 9th International Congress of the Polish Neuroscience Society, Warsaw, Poland. This research was partially supported by the Scientific Research and Project Coordination Center of Selcuk University (Fund No. BAP 07202012), Meram, Konya, Turkey, and includes part of S.T.'s master's of science thesis.

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