Electron microscopic study of the myelinated nerve fibres and the perineurial cell basement membrane in the diabetic human peripheral nerves

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ABSTRACT

Objectives: To study the quantitative and ultrastructural changes in myelinated nerve fibers and the basement membranes of the perineurial cells in diabetic nerves.

Methods: The study was performed at the Department of Anatomy, Faculty of Medicine, King Abdul-Aziz University, Jeddah, Saudi Arabia from 2003 to 2005. Human sural nerves were obtained from 15 lower limbs and 5 diabetic nerve biopsies. The total mean and density of myelinated nerve fibers per fascicle were calculated, with density of microtubules and mitochondria in the axoplasm. The number of the perineurial cell basement membrane layers was counted, and thickness of the basement membrane was measured.

Results: Among the 15 diabetic and 5 normal human sural nerves, the average diameters, number and surface area of myelinated nerve fibers and axonal microtubules density were found to be less in diabetic nerves. Mitochondrial density was higher in diabetic axons. Thickness of the perineurial cell basement membrane had a greater mean, but the number of perineurial cell layers was less than that of the diabetic group. The inner cellular layer of the perineurium of the diabetic nerves contained large vacuoles containing electron-dense degenerated myelin. A few specimens showed degenerated myelinated nerve fibers, while others showed recovering ones. Retracted axoplasms were encountered with albumin extravasation.

Conclusion: Diabetes caused an increase in perineurial permeability. The diabetic sural nerve showed marked decrease in the myelinated nerve fibres, increase degenerated mitochondria, and decreased microtubules.
Diabetic neuropathy is one of the most frequent complications of diabetes. However, its pathogenesis remains unclear.\textsuperscript{1,2} Diabetic neuropathy was attributed mostly to the concomitant microangiopathy, which is a common finding in the diabetic nerves.\textsuperscript{3-7} Thickening of the wall of small blood vessels is a characteristic feature of diabetic microangiopathy, associated with atrophy, and loss of nerve fibers in human diabetic nerves.\textsuperscript{7,9} The blood-nerve barrier consists of the endoneurial blood vessels with the inner cellular layer of the perineurium protecting and regulating the endoneurial environment.\textsuperscript{10,11} The perineurium is a lamellated structure made up of concentric cell layers bordered on each side by basement membrane. The number of its concentric layers differs in normal fascicles and in different types of neuropathies. Structural changes in the perineurium impede its functional abilities both as a diffusion barrier and as a nutrient delivery system, thus preventing effective regulation of the endoneurial environment.\textsuperscript{12} Consequently, the quantitative and qualitative changes of the perineurium in diabetes appear important and need extensive study to complete the understanding of the pathogenesis of diabetic neuropathy. Diabetic neuropathy is also accompanied by loss and physiological dysfunction of the unmyelinated and myelinated nerve fibers.\textsuperscript{13} This could be secondary to hyperglycemia that increases the oxidative stress,\textsuperscript{14} inhibits Na\textsuperscript{+}/K\textsuperscript{+} adenosine triphosphatase and Ca\textsuperscript{2+} adenosine triphosphatase activities\textsuperscript{15} together with elevation of the cellular and membrane lipid peroxidation.\textsuperscript{16} These biochemical changes together with loss of vasa nervorum in diabetic neuropathy reduce perfusion of peripheral nerves and contribute to the affection of axonal organelles and ultimately axonal loss.\textsuperscript{17-20} Accordingly, the aim of the present study was to study the quantitative and qualitative changes in the basement membranes of the perineurial cells and the myelinated nerve fibers in diabetic nerves.

**Methods.** The study was performed at the Department of Anatomy, Faculty of Medicine, King Abdul-Aziz University, Jeddah, Saudi Arabia through the years 2003-2005. The study was approved by the Ethical Committee of the Faculty of Medicine, King Abdul-Aziz University. Fresh segments of human sural nerves were obtained from 15 amputated lower limbs (10 of diabetic patients and 5 of normal control persons) and from 5 nerve biopsies from diabetic patients. In all cases, diabetes was classified as type II diabetes, with a mean duration of 15 years (range 10-18 years). Tissues from diabetic and control groups were obtained immediately following limb amputation or nerve biopsies. Lower limb amputation was performed due to the effects of diabetic neuropathy; the patients had a mean age of 58 years (range 50-75). The patients used in the control group were all free of both diabetes and peripheral vascular disease. They had a mean age of 65 years (range 50-70). All patients gave their informed consent following adequate information on mode of data acquisition, processing, analysis, interpretation, and publication according to the local law. Nerve tissues were obtained by exposure of the sural nerves through transverse incisions behind the lateral malleoli. All tissue samples of the sural nerves were immediately immersed in buffered 2.5% glutaraldehyde for a period of 24 hours at 10°C. Secondary fixation was performed using 2% osmium tetroxide prior to dehydration in an ascending alcohol series with eventual embedding in epoxy resin. Transverse ultrathin sections of 0.06-micron thickness were cut and stained with uranyl acetate and lead citrate for electron microscopic examination.

**Quantitative analysis.** Five randomly selected areas of the perineurium of each fascicle were used to measure the thickness of the basement membrane of the perineurial cells using Swiss Vernier caliper (with 0.05 mm accuracy). Photographs were made at a final magnification of X 12,500 that showed much of the perineurium. Measurements were taken for the outer and inner basement membranes of the inner perineurial cells with the aid of an X7 hand-held magnifier. Measurements were made at 2-mm intervals along the length of the inner perineurial cell within each photograph field. The number of the perineurial cell layers and the perimeter of the nerve fascicle (endoneurium) was also counted in nanometers. One-micron sections of the entire nerve were obtained and stained with 1% toluidine blue in borax. With the aid of a magnifying lens, myelinated nerve fibres of all the nerve fascicles were counted from the obtained light photomicrographs. Diameters and surface areas of the axons of the myelinated nerve fibres (measured from the outer margin of the myelin sheaths) and the surface area of the endoneurium were calculated using the computerized image analyzing system. The mean surface area of the myelinated nerve fibres was divided by the surface area of the endoneurium to calculate the myelinated nerve fibres density per square millimeter in each fascicle. A total mean of myelinated nerve fibers densities were then calculated for each nerve, and then for each group. The number of the microtubules and mitochondria (total and degenerated) in the axoplasm of the studied axons was counted in the electron photomicrographs with final magnification of 40,000 using the image analyzing system. The microtubules density and mitochondria densities (total and degenerated) were then calculated for each axon and for the axons of the whole nerve. The mean density was then calculated for each group of nerves.

**Results.** The quantitative study. The mean perineurial cell basement membrane thickness (PCBM) of the control group was 405.86 ± 4.2 nm (range 290 - 564 nm) while that of the diabetic group was 524.55
The mean number of the perineurial cell layers in the control group was 6.4 ± 0.31 layers (range 3 - 7) while it was 7.4 ± 0.33 layers (range 4 - 8 layers). The mean fascicle perimeter in the control group was 118 ± 0.11 mm (range 59 - 143 mm), while that of the diabetic group was 138 ± 0.07 mm (ranging from 115 to 166 mm). The mean diameter of the myelinated nerve fibres in the control group was 4.18 ± 0.07 micron (range 4.17 - 4.26 micron), while it was 3.62 ± 0.01 micron in the diabetic nerves (range 3.52 - 3.62 micron). The average diameter was found to be less in the diabetic nerves as compared to the control group. The mean number of the myelinated nerve fibres in the control group was 5174 ± 32.3 (range 4865 - 5706) while it was 2022 ± 12.40 (range 1479 - 3198) in the diabetic group. The mean surface area of the endoneurium of the examined nerve fibres in the control group was 4407 ± 12.4 mm² (range 3931 - 5111) while it was 4667 ± 30.3 mm² (range 4432 - 5149) in the diabetic group. Consequently, the mean myelinated nerve fibres density in the control group was 1.174 ± 0.1 myelinated nerve fibres per mm² while it was 0.433 ± 0.04 myelinated nerve fibres per mm² in the diabetic group. The mean surface area of the myelinated nerve axon in the control group was 0.53 ± 0.01 micron² (range 0.30 - 0.72) while it was 0.432 ± 0.02 micron² (range 0.38 - 0.61) in the diabetic group. The mean number of microtubules in the control group was 16.3 ± 0.4 microtubules per axon (range 13 - 24), while that of the diabetic group was 12.7 ± 0.1 microtubules per axon (range 11 - 13). The mean density of microtubules in the control myelinated nerve axons was 26.4 ± 0.4 microtubules/micron (range 25.0 - 28.0) while that of the diabetic group was of 25.3 ± 0.2 (range 24.6 - 27.0 microtubules/micron²). The mean number of the total mitochondria per axon (healthy and degenerated) was 4.09 ± 0.09 (range 1 - 5) in the control group while it was 4.59 ± 0.10 mitochondria per axon (range 2 - 6) in the diabetic group. The mean number of the degenerated mitochondria per axon was 0.36 ± 0.004 (range 0 - 1) in the control group while it was 2.18 ± 0.01 degenerated mitochondria per axon (range 1 - 4) in the diabetic group. The mean density of the total mitochondria was less in the control group (7.71 ± 0.2 mitochondria per micron²) than that of the diabetic group (10.68 ± 0.3 mitochondria per micron²). The mean density of the degenerated mitochondria in the diabetic group (4.88 ± 0.02 mitochondria per micron²) was higher than that of the control group (0.68 ± 0.02 mitochondria per micron²).

The qualitative study. The perineurium of the control group showed compaction of its layers. The basal laminae covering the perineurial cells were sharply delineated, continuous, and well defined. The perineurial cells showed many pinocytotic vesicles. There were collagen fibrils between the layers of the perineurium. The sub perineurial space contained compacted collagen fibrils together with normal myelinated and unmyelinated nerve fibres (Figure 1a). The diabetic group showed apparent thick and hazy perineurial basement membranes compared to those of the control group. Such thickness was not uniform in the same membrane. In some of the studied specimens, the membrane appeared doubled with splitting of the doubled layers by intervening collagen fibrils. The inner cellular layer of the perineurium contained large vacuoles, which contained electron-dense membranes suggesting degenerated myelin. They also contained electron-lucent areas suggesting fat globules (Figure 1b). Other specimens showed disruption of some areas of the basement membrane indicated by the presence of collagen fibrils between the disrupted edges of the membrane and the underlying cell. The cellular layers of the perineurium contained an increased number of the pinocytotic vesicles that were prominent near their outer and inner limiting membranes. Electron dense patches suggesting extravasated albumin were present among the collagen fibrils lying between the cellular layers of the perineurium. Such patches were also demonstrated inside the perineurial cells and the pinocytotic vesicles (Figures 2a & 2b). The myelinated nerve fibres were distributed throughout the endoneurium. Each nerve fibre consisted of myelinated nerve fibres wrapped by Schwann cells. The myelin was arranged regularly in layers surrounding the axolemma. The axoplasm contained microtubules, neurofilaments, and mitochondria. The axoplasm was adherent to the inner layer of the myelin sheath. Each Schwann cell wrapped only one myelinated axon and was covered externally by its basal lamina. Its cytoplasm contained normal cytoplasmic content. The myelinated nerve fibres were surrounded by aggregates of collagen fibrils (Figure 3a). The axoplasm of the recovering myelinated nerve fibers contained electron-lucent membrane-bounded cytoplasmic vacuoles suggesting fat vacuoles. Most of the neurofilaments and microtubules appeared elongated probably due to the obliquity of the section or the presence of intra-axonal edema. The neurofilaments and microtubules were widely separated by electron-lucent areas suggesting the possibility of intra-axonal edema. The surrounding Schwann cell contained cytoplasmic electron-lucent vacuoles probably fat globules. Some of these globules contained electron-dense membranes, suggested to be remnants of the degenerated myelin. The myelin sheath showed marked thinning in some of the myelinated nerve fibres indicating that they were regenerating fibres. The nerve fibres were surrounded by collagen fibrils that were widely separated from each other by the intervening electron dense patches suggesting extravasated albumin (Figure 3b). In some specimens, degenerated myelinated nerve fibres were noticed. The axons of these fibres contained degenerated cytoplasm.
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The surrounding myelin appeared thin and disrupted. The surrounding Schwann cells showed degeneration of their cytoplasm and disruption and fragmentation of their basal laminae (Figure 4a). Other axons of the studied specimens showed normal myelin and axoplasm, while others showed only fissuring or partial disruption of the myelin sheaths. Some axons showed periaxonal edema containing degenerated myelin that resulted in axonal retraction. The cytoplasmic content of the retracted axons appeared normal. The axoplasmic organelles of the diabetic nerves also showed abnormal separation with the presence of electron-lucent areas in-between suggesting the presence of intra-axonal edema. Some of the mitochondria showed degenerative changes; this was in the form of mitochondrial swelling with ill-defined internal crista and disruption of their outer limiting membranes (Figures 4b & 5a). There were few recovered and regenerated myelinated nerve fibres.
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axons, indicated by their thin myelin sheaths and the healthy axoplasm. They had smaller diameters and their wrapping Schwann cells had many cytoplasmic processes with redundant basement membranes. The microtubules in the axoplasm were apparently increased and were aggregated. The axoplasm and cytoplasm of the wrapping Schwann cells showed electron-lucent vacuoles (Figure 5b).

Discussion. The current study shows that there was thickening of the basement membrane of the perineurial cells of the diabetic group. This thickening was not uniform throughout the membrane. Moreover, the present study shows that the absorbed degenerated fat globules split this membrane. In other specimens, collagen fibrils separated the basement membrane from the perineurial cells. In some specimens, disruption...
of this membrane at some sites leads to its either partial or complete discontinuity. Thickening of the basement membrane of the perineurial cells may be due to intervening absorbed substances or due to its duplication, as shown in the current study. These reasons may be the possible causes of its non-uniform thickening since the duplication and the intervening absorbed substances were not uniform throughout the whole length of the membrane. Hyperglycemia, which is the major metabolic abnormality of diabetes, has been shown to produce an up-regulation of several major basement membrane components, including collagen IV. 21,22

The present study showed several ultrastructural changes in the basement membrane of the perineurial cells of the diabetic nerves such as thickening. It also showed increased vacuoles and pinocytotic vesicles in the cellular layers of the perineurium. Also, there was albumin extravasation in the sub perineurial and between the layers of the epineurium. Consequently, these changes may suggest that permeability of the blood-nerve barrier was increased in the diabetic group. The increased extravasation of albumin and the degenerative changes in the endoneurium can increase the intra-neurial pressure in the endoneurium. 5 Such an increase will cause degeneration of the myelinated nerve fibers, which were significant in the diabetic group. 5,5

The present study also showed accumulation of intraneurial edema in some specimens that might also affect the viability of the axonal organelles. 23

The current study showed that there was a decrease in the number of the myelinated nerve fibres in the endoneurium of the diabetic nerves (their mean density was 0.433 fibers per mm² compared to the normal mean density 1.174 fibers per mm²). The present study also showed that there were several ultrastructural changes of the myelinated nerve fibres in the diabetic nerves. There was degeneration of the myelin sheath in the form of fissuring and separation of its lamellae. Also, there was degeneration of the myelin with the increased fat vacuoles in the cytoplasm of Schwann cells and in-between the cellular layers of the perineurium. In some cases, there was degeneration of the myelinated axons with disruption of its myelin and increased cytoplasmic vacuolation. The axoplasm appeared homogenous without identification of any of the cell organelles. The surrounding Schwann cells showed degenerative changes and fragmentation of its basal membrane. The loss of the myelinated nerve fibres due to degenerative changes may account for the decrease in their number and density as shown in the current study. It was also recorded during the current study that the mean surface area of the myelinated axons in the diabetic nerves was lower than those of the control. This may be due to the decreased mean diameter of these axons (3.62 micron in diabetic nerves compared to 4.18 micron in the control) that had been found during the study. It may be also due to counting the small-diameter regenerated or recovered axons that had been encountered during this study. It could be also due to the decrease in the number of the microtubules in the diabetic myelinated axons.

It has been shown that neuroblastoma underwent programmed cell death under high glucose or hyperosmolar conditions designed to mimic a diabetic state. 24,25 Hyperglycemia is believed to induce increased oxidative stress, 26 which in turn, may activate death effector proteins that disrupt the mitochondria.
membrane potential and trigger mitochondria to swell and become permeable. This can explain the ultrastructural changes in the mitochondria of the myelinated axons of the diabetic nerve in the current study. The present study showed swollen mitochondria with ill-defined internal cristae. Other mitochondria showed degeneration with amorphous degenerated content while others showed partial disruption of its surrounding membranes.

The current study showed that the average total number of mitochondria (healthy and degenerated) per axon was higher in the diabetic axons than in the control. It also showed that the average number of the degenerated mitochondria per axon was higher in the diabetic group than in the control. The current study also showed that the average total mitochondrial density was lower in the control group than in the diabetic. The average density of the degenerated mitochondria in the diabetic group was higher than in the control. Consequently, the increased total mitochondria per axon were largely due to the increased degenerated mitochondria and not due to actual increase in their number. The increased number and density of the degenerated mitochondria in the diabetic group can be explained by the increased permeability of the mitochondrial membrane secondary to hyperglycemia. Also the cyclic adenosine-monophosphate (cAMP) content is decreased in the dorsal root ganglia and sciatic nerves in diabetic rats. Such decreased cAMP may also occur in the human diabetic, and may contribute to the apoptotic changes in the nerve fibres including the axonal mitochondria. It was found that apoptotic changes occurred in parallel with activation of caspase-3, dependant on the concentration of glucose.

The current study also showed regenerating nerve fibres in the diabetic group. The regenerated neurons contained healthy thin myelinated axons wrapped by healthy Schwann cells. However, the regenerating nerve fibres were few in number and scattered throughout the endoneurium. Researchers suggested that impaired insulin signaling in type 1 diabetic nerves might be of greater significance in the regulation of neurotrophic and neuro cytoskeletal protein synthesis than hyperglycemia in explaining the differences in nerve fiber regeneration between type 2 and type 1 diabetes. Delayed nerve regeneration in diabetic animal models was attributed to abnormalities in proliferation/differentiation of Schwann cells. It has been recently reported that endothelins (ETs) regulate proliferation and phenotype in primary and immortalized Schwann cells. An extravascular role of ETs in peripheral nerves and Schwann cells has been reported. The increased sensitivity to ET-1 in nerves and immortalized Schwann cells exposed to high glucose may contribute to abnormal Schwann cells proliferation characterizing diabetic neuropathy.

The above-mentioned degenerative changes and the loss of nerve fibres were not constant in all the studied specimens. Some of the specimens showed normal axons with even apparent increase of the microtubules or neurofilaments. This might be explained by the presence of compensatory mechanisms or the presence of some regenerative axons. Consequently, it could be concluded that the affection of the myelinated axons in the diabetic patients was not universe but focal affection. The current study showed also the presence of a few regenerating and recovering myelinated nerve fibres. This may explain the recorded smaller magnitude of the effect of the therapeutic drugs used in clinical trials. The therapeutic drugs will induce regeneration to a certain extent since the capability of the myelinated nerve fibres of the diabetic nerve to regenerate is limited. Such limited capacity to regenerate can account also for the great loss of the myelinated nerve fibres in diabetic nerves.

The present study showed that the average number of the microtubules per micron of the cross-sectional area of the diabetic myelinated nerve fibres was 25.3 ± 0.2 microtubules/micron, while that of the normal nerves was 26.4 ± 0.4 microtubules/micron. Consequently, the density of the microtubules was nearly the same without any significant difference between the 2 groups. The average axonal area in the diabetic group was approximately 20% less in the normal control. It is suggested that the diabetic nerves show reduced content of the microtubules in a nerve trunk, as the relation between the axonal size and microtubules density remained near to the normal. However, some of the axons in the diabetic nerves showed uneven distribution of the microtubules inside the axoplasm.

The present study showed the increase of the mean values of the perimeters and the surface areas of the endoneurium of the examined nerve fascicles of the diabetic than the control group. The increased perimeter and surface areas of the endoneurium, as reported in the present study, could be explained by the increased endoneurial edema secondary to the increased permeability of the perineurium. Also, the perineurium appeared edematous, which may affect its resilience to stretch. The increased surface area can account partially together with the previously mentioned loss, for the decreased density of the myelinated nerve fibres in the diabetic nerves compared to the normal.

Pancræatic transplantation has not produced the expected beneficial effects on nerve dysfunction. Complete normalization of the blood glucose level has resulted in a notable but still mild degree of improvement in nerve function even 10 years after transplantation. This might indicate that diabetic neuropathy is a progressive disease even with reducing the blood glucose level to normal, and the degenerative changes in the nerve fibres are permanent although some regenerative
changes can occur. There was one limitation in our study, which was the reduced number of the obtained sural nerve specimens due to insufficient number of volunteers who agreed to join the study, and give their consent. It is recommended to repeat the study on other different nerves on larger numbers of diabetic patients.

It may be concluded from the current study, that diabetes produced ultrastructural changes in the perineurium that lead to an increase in its permeability. Also, the diabetic sural nerve showed marked decrease in the myelinated nerve fibres and several intra-axonal changes such as increased degenerated mitochondria, and decreased microtubules.

References