ELSEVIER

Journal of Biomechanics 37 (2004) 879–888

JOURNAL OF BIOMECHANICS

www.elsevier.com/locate/jbiomech
www.JBiomech.com

Differences between collagen morphologies, properties and distribution in diabetic and normal biobreeding and Sprague–Dawley rat sciatic nerves

B.E. Layton[a], A.M. Sastry[a,b,*], H. Wang[b], K.A. Sullivan[c], E.L. Feldman[c], T.E. Komorowski[d], M.A. Philbert[d]

[a] *Department of Biomedical Engineering, University of Michigan, Ann Arbor, MI, USA*
[b] *Department of Mechanical Engineering and Applied Mechanics, University of Michigan, 2350 Hayward Str., 2250 G.G. Brown, Ann Arbor, MI 48109-2125, USA*
[c] *Neurology Department, Medical School, University of Michigan, Ann Arbor, MI, USA*
[d] *Environmental Health Sciences, University of Michigan, Ann Arbor, MI, USA*

Accepted 4 November 2003

Abstract

Both structural and functional differences between normal and diabetic nerve have been observed, in human patients and animal models. We hypothesize that these structural differences are quantifiable, morphologically and mechanically, with the ultimate aim of understanding the contribution of these differences to permanent nerve damage. The outer collagenous epineurial and perineurial tissues of mammalian peripheral nerves mechanically and chemically shield the conducting axons. We have quantified differences in these collagens, using whole-nerve uniaxial testing, and immunohistochemistry of collagens type I, III, and IV in diabetic and normal nerves. We present results of two studies, on normal and diabetic BioBreeding (BB), and normal, diabetic and weight-controlled Sprague–Dawley (SD) rats, respectively. Overall, we measured slightly higher uniaxial moduli (e.g. 5.9 MPa vs. 3.5 MPa, BB; 10.7 MPa vs. 10.0 MPa, SD at 40% strain) in whole nerves as well as higher peak stresses (e.g. 0.99 MPa vs. 0.74 MPa, BB; 2.16 MPa vs. 1.94 MPa, SD at 40% strain) in the diabetics of both animal models. We measured increased concentrations of types III and IV collagens in the diabetics of both models and mixed upregulation results were observed in type I protein levels. We detected small differences in mechanical properties at the tissue scale, though we found significant structural and morphometric differences at the fibril scale. These findings suggest that whole-tissue mechanical testing is not a sufficient assay for collagen glycation, and that fibrillar and molecular scale assays are needed to detect the earliest stages of diabetic protein glycation.

Keywords: BioBreeding; Sprague-Dawley; Collagen; Diabetes; ECM; AFM

1. Introduction

Diabetic neuropathy may affect as many as 50% (Feldman et al., 2001; Gavin et al., 1998; DCCT, 1995) of the over 15 million American diabetics (Thomas and Tomlinson, 1993; O'Connor et al., 1998). This number is likely to increase, with the increasing number of diabetic patients (Kopelman and Hitman, 1998). The clinical symptoms of diabetic neuropathy include, but are not limited to, paresthesia, dysesthesia, neuropathic pain, and diminished reflexes (Greene et al., 1997). The disease accounts for at least half of all nontraumatic amputations in the United States (American Diabetes Association, 1984). The primary cause of diabetic neuropathy, namely, prolonged hyperglycemia is well known, but despite over 30 years of active research dedicated to understanding this disease, its full etiology remains elusive.

Elevated blood glucose is used to diagnose both Type I and Type II diabetes. Metabolic (e.g. Winegrad and Greene, 1976; Stevens et al., 1996), neurochemical, vascular (e.g. Myers et al., 1986; Cameron and Cotter, 1994), morphological, and mechanical hypotheses (Myers et al., 1986) have all been advanced to explain

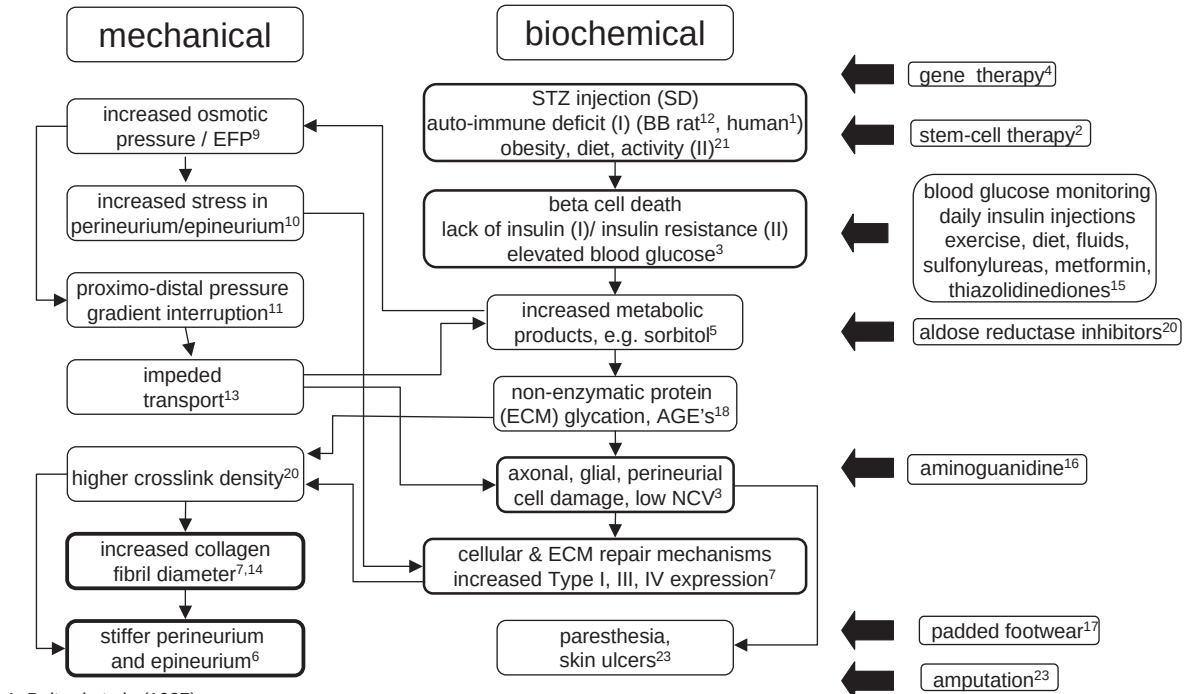
*Corresponding author. Tel.: +1-734-764-3061; fax: +1-734-647-3170.
E-mail address: amsastry@umich.edu (A.M. Sastry).

0021-9290/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jbiomech.2003.11.008

the pathology of diabetic neuropathy. Relationships between metabolic (neurochemical) and transport (vascular) factors has also been explored. Attempts to link metabolic factors to morphological changes include those of Sima et al. (1986) and Wright and Nukada (1994). The galactose-induced diabetes model has been used to demonstrate the relationship between biochemistry and mechanics with the presence of increased endoneurial osmotic pressure in peripheral nerves (Low et al., 1980; Myers and Powell, 1984). This biochemical–mechanical link has also been explored in lead neuropathy (Low and Dyck, 1977).

In diabetic neuropathy, it has also been postulated that increases in endoneurial fluid pressure (EFP) contribute to permanent nerve damage (Myers and Powell, 1984). One possible explanation for this effect is that locally enhanced interfibrillar loads result in upregulation of collagens (Kessler et al., 2001), creating a positive feedback. Others, (e.g. Sanders and Goldstein, 2001) studying in vivo cyclic loading of pig skin, have reported quantifiable changes in collagen fibril architecture, but not in gross tissue morphology. Reihsner and Menzel (1998) found that long-term in vitro incubation with glucose-6-phosphate (a more highly reactive sugar than native glucose) brought about significant changes in skin stiffness at all loads tested. They also found that viscous losses were present at high loads, and that the differences were directionally dependent. They further correlated these changes with the degree of glycoslyation, as measured by fluorescence, showing that increases in stiffnesses and viscosities were lessened by the addition of aminoguanadine to their incubations. Reddy et al. (2001) found the femurs and tibias of STZ-diabetic (streptozotocin) rats to be weaker than those of controls. Pavlin et al. (2001) found an upregulation of osteocalcin (OC), alkaline phosphatase (ALP), and type I collagen (collagen I) in their in vivo mouse tooth model where mechanical loading played the crucial role.

Some relationships among the possible mechanical and biochemical mechanisms involved in diabetic neuropathy have been investigated; a schematic of these factors and their possible relationships, and clinical interventions is shown in Fig. 1. Previously, both Type I and Type II diabetes have been linked to quantitative, structural changes in the extracellular matrix proteins of nerve, such as (1) nonenzymatic glycation, (Sensi et al., 1995; Sternberg et al., 1985); (2) increased expression of extracellular matrix proteins such as collagen at the transcriptional (Muona et al., 1991, 1993a, b) and translational (Benazzoug et al., 1998) levels; and (3)



1. Boitard et al., (1997)
2. Faustman (2002)
3. Feldman et al., (2001)
4. Froguel (1997)
5. Greene et al., (1988)
6. Low et al., (1980)
7. Muona et al., (1989)
8. Muona et al., (1994)
9. Myers et al., (1979)
10 Myers et al., (1986)
11. Myers et al., (1988)
12. Nakhooda et al., (1977)
13. Nukada (1993)
14. Odetti et al., (2000)
15. Patel (1999)
16. Reihsner R., Menzel E.J., (1998)
17. Roy (1988)
18. Sensi (1995)
19. Sima et al., (1990)
20. Sternberg et al., (1985)
21. Williams et al., (2002)
22. Wilson and Leiter (1990)
23. Windebank and Feldman (2001)

Fig. 1. Relationships between (some known and some proposed) mechanical and (known) biochemical pathways involved in progression of diabetic neuropathy. Traditional and novel treatments shown on the right are designed to treat the disease at various points in its progression.

increased collagen fibril diameters, in diabetic rat sciatic endoneuria (Muona et al., 1989), diabetic human sural endoneuria and perineuria (Bradley et al., 2000) and diabetic rat sciatic epineuria, endoneuria, and tail collagens (Wang et al., 2003). However, no clear linkages have yet been established among peripheral neural collagen upregulation, structure, and mechanical properties.

To examine possible connections among mechanical characteristics of nerve tissue, and the underlying biochemical mechanisms regulating production of collagenous extracellular matrix, we conducted a series of experiments ranging from the nanoscale to the tissue scale. Our specific hypotheses were that (1) greater presence of the major structural peripheral nerves collagens, types I, III and IV, arise in diabetic rats, (2) differences in healthy and diabetic tissues detected at the microscale, manifest as mechanical property differences detectable at the whole tissue scale in a diabetic rat model, (3) genetic and induced models of diabetic and nondiabetic animals exhibit similar differences, and (4) that it is glycation, rather than weight loss resulting from Type I diabetes, that produces differences in tissue, at all scales. We used rat models to examine collagen content and nerve tissue mechanical properties (hypotheses 1 and 2), Use of BioBreeding (BB) model (Nakhooda et al., 1977; Like et al., 1982; Butler et al., 1983; Sima et al., 2000) and STZ treated Sprague–Dawley (SD) models allowed investigation of hypothesis 3; we used one set of weight control animals in the SD study to investigate hypothesis 4. Our study was unique, to our knowledge, in comparing differences in both tissue-scale mechanical properties and collagen content; we additionally used both genetic and phenotypical animal models for Type I diabetes.

2. Methods

Healthy BB and SD rats were purchased from Biomedical Research Models (Worchester, MA, USA), and Charles River Labs (Cambridge, MA, USA), respectively. The 10 diabetic and eight control inbred BB age-matched male rats and twenty-seven age-matched male outbred Sprague–Dawley (SD) rats used, were cared for according to University, State, and Federal standards, in accordance with the National Research Council's "Guide for the Care and Use of Laboratory Animals". The BB rats required additional, intensive, care using the husbandry guidelines of Guberski (1994). Eight SD rats were used as weight controls and 12 were made diabetic with STZ injection (e.g. Pop-Busui et al., 2001). Body weights were taken (BB daily, SD weekly) with an Ohaus triple-beam balance (Fisher). Blood glucose measurements were taken as described elsewhere (Pop-Busui et al., 2001). Nerve conduction velocity (NCV) measurements were made (BB tail motor and sensory; SD sciatic motor) at the end of each study (BB 16 week (wks); SD 12 wks) with a NeuroMax 1002, XLTEK (Oakville, Ont., Canada).

Comparisons among three specific measures related to structural collagen remodeling in the tissues of rats were examined, including (1) diagnosis of diabetes (including blood glucose, body weight, and nerve conduction velocity), (2) mechanical properties of whole nerve (including stiffness, strength, and stress relaxation), (3) content analysis of collagens (including immunohistochemical staining for types I, II, and IV collagen).

Our experiments were performed on excised whole sciatic nerve. For reference, excellent reviews have been provided for both rat (Ushiki and Ide, 1986, 1990), and human (Thomas et al., 1993; Gamble and Eames, 1964) peripheral neuroanatomy. Uniaxial mechanical testing was performed on excised tissue from the animals' left sides; immunohistochemistry and imaging experiments were performed on tissue taken from the right sides. Briefly, 20 mm of the (left) sciatic nerve, consisting of the single-fascicle portion between the sciatic notch and sural–tibial–peroneal trifurcation, was gently excised from each side. Skin was carefully removed from the fascia, and the nerve was exposed with a small incision posterior to the femur. The nerve was then mobilized with microscissors before being transected at the proximal and distal ends. Nerves were stored in cold phosphate-buffered saline until testing.

Specimens used for mechanical testing were thawed at room temperature for approximately 5 min, then clamped in a pin-and-groove grip, affixed to an Ohaus digital scale (model TP2KS). Strain was applied using a Bodine Electric Company DC stepper motor controlled by a Parker Digiplan PK3 driver. An interclamp length of 10 mm and displacement rate of 0.1 mm/s (strain rate 0.01/s) were used. Zero strain was defined as the point at which a force of 2.0×10^{-5} N was detected. Nerves were held fixed, in successive cycles, at the strains and times shown in Table 3, with approximately 30 s of rest between cycles. Because the two studies were run consecutively (first SD, then BB) we altered strain levels in the BB study to reflect the need for very high levels of strain in order to detect any significant differences in mechanical response.

All specimens exhibited classical stress relaxation (Fig. 2). Data were collected at a rate of 1 Hz and stored on an Apollo Domain Series 4000 digital computer. Measurement times were consistent among all groups; measurements were taken when stress relaxation responses were flat. Four parameters were recorded/calculated for each strain cycle: (1) maximum force, F_ε, defined as the peak force within a strain cycle; (2) maximum stress, σ_ε, defined as the peak force within

a strain cycle; divided by original cross sectional area (3) elastic modulus, E_ε; defined as the slope of the linear region of a stress–strain plot, and (4) stress relaxation, r_ε, defined as relaxation force at the end of a given cycle divided by maximum force. After mechanical testing, five cross sections were measured and averaged for data reduction using NIH Image analyses of SPOT RT II digital camera section images. Nerves were not preconditioned. To achieve a steady state force reading, the peak of each strain cycle was held fixed for progressively longer times, with increasing strain.

Immunohistochemistry (Janssen, 1994) was performed on fresh-frozen, OCT-imbedded, 10 μm-thick transverse sections of whole sciatic nerve mounted on glass slides (Fisherbrand Superfrost/Plus). Specimens for immunohistochemistry were stored at −80°C, thawed (∼5 min), then rinsed (∼5 min) in Copeland jars on an agitator in 0.1 M PBS pH 7.2. Normal goat serum (NGS) (Gibco) was applied (∼20 min) in a humid container, 10 μl of primary collagen antibody was applied (∼60 min), then specimens were rinsed (3 cycles, of 5 min each) in 0.1 M PBS pH 7.2 in Copeland jars on an agitator. NGS was reapplied (∼20 min), then secondary goat–anti-rabbit antibody (Molecular Probes, Eugene, OR) labeled at 647 nm was applied (∼60 min) in dark drawer. Finally, specimens were then rinsed again (2 cycles, of 5 min each) in 0.1 M PBS pH 7.2, and (∼5 min) in tap water. Specimens were covered (Corning cover glass #1 22 × 50 mm), using mounting media (Biomedia™ Gel/Mount), stored at −20°C overnight and imaged under an Olympus inverted confocal microscope using FluoView v3.0 laser scanning microscope software at 100×. Initial focusing was performed manually for each section to maximize signals. A Kallman filter was applied twice, and a 1028 × 1028 pixel image was saved for each specimen. Nerve sections were approximately 1 mm in diameter, with each pixel (Fig. 3) representing approximately 1 μm^2. Three representative 10 × 10 pixel were used for analysis in epineurial/perineurial tissues; two 100 × 100 pixel portions were used for analysis of endoneurial tissues. Pixel intensities within each 10 × 10 or 100 × 100 region were summed then the average values taken within each group. Perineuria and epineuria were not differentiated in selection of subimages as was done in the high-magnification images of Lorimer et al. (1992). Only in the type IV images were epineuria and perineuria readily distinguishable. Vague outlines of interfascicular perineuria were visible; none of these were selected for analysis. Only portions of tissues adjacent to epineuria were analyzed.

To compare means in the three-group (control, weight control, diabetic) SD study, one-way ANOVA ($p<0.05$) with post-hoc Student–Newman–Keuls tests was implemented with the compare-means function in SPSS, SPSS Inc, Chicago IL. For the two-group (control, diabetic) BB study, means comparisons were performed in Microsoft® Excel's Student's two-tailed heteroscedastic t-test.

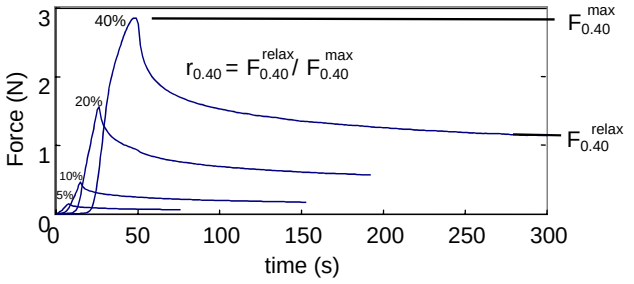


Fig. 2. Uniaxial testing result from a weight-control Sprague–Dawley rat whole sciatic nerve showing toe region, linear region, and stress relaxation region. The four traces represent successive strainings to 5%, 10%, 20% and 40%.

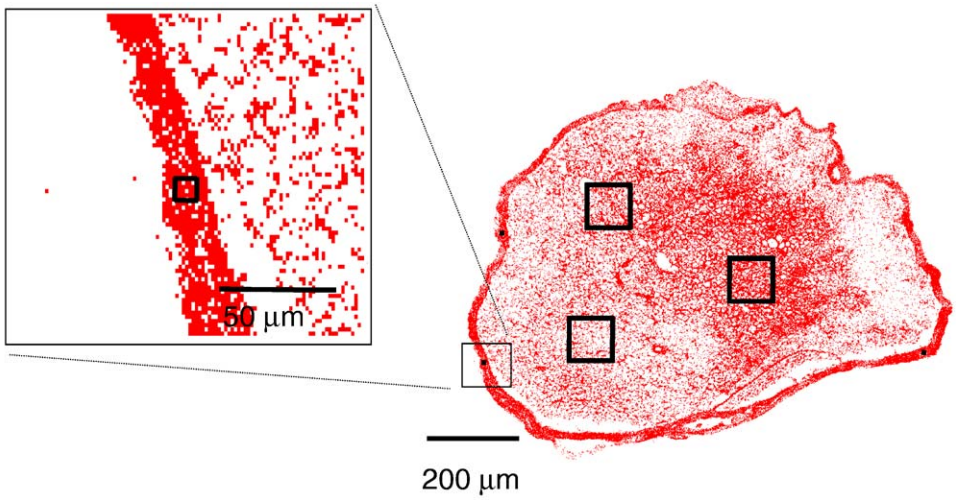


Fig. 3. Example of sampling a 1028 × 1028 pixel image for endoneurial (100 × 100 pixels) and epineurial (10 × 10 pixels) collagen types I, III and IV. The image shown is an example of diabetic Sprague–Dawley rat whole nerve stained for type III collagen.

3. Results

Values of $p<0.05$ (95% confidence of nonrandom differences in mean values) were judged significant in all cases; significant values are underlined for two-group BB comparisons reported here. For the three-group comparisons of the SD study, results are tabulated such that entries in the last three columns represent Student–Newman–Keuls post-tests, for d = diabetic, c = control, w = weight control groups, ranked by mean. Continuous connecting lines indicate no significant difference between or among linked groups.

Average blood glucoses of diabetic animals were maintained at or above 300 mg/dl. (BB—Table 1; SD—Table 2). Insulin overdose (<2 U/kg) resulted in hypoglycemia, causing death in two of the BB animals. Body masses fluctuated by as much as a 20% gain to a 10% drop over the previous day. Overdose of STZ resulted in the premature death of one of the SD animals. Nerve conduction velocities were, as expected, significantly diminished in diabetics in all measurements. Similar results were observed in the SD study (Table 3).

Uniaxial testing of whole nerve revealed only slight elevation in maximum stress and stiffening of tissues, and no significant differences in stress relaxation in either BB (Table 4) or SD (Table 5) rats. Diabetic whole nerves exhibited diminished cross sectional areas.

Overall, immunohistochemistry revealed larger amounts of types I, III and IV collagens in diabetic BB (Table 6) and SD (Table 7) nerves. Significantly larger amounts of types III ($p<0.001$) and IV ($p=0.010$) collagen were found in diabetic epineurial/perineurial BB tissues. Significant differences in type I collagen content were not detected between diabetic and control BB epineurial/perineurial or endoneurial nerve tissues.

Table 1
Summary of biobreeding rat clinical results at the conclusion of the 16-week study

Variable	Control (8)	Diabetics (10)	p
Age (days)	190±14	188±9	0.741
Duration (days)		113±1	
Body mass (g)	447±28	295±40	<u>≤0.001</u>
Blood glucose (mg/dl)	60±5	380±21	<u>≤0.001</u>
Tail motor NCV (m/s)	37.0±3.4	32.3±4.9	0.058
Mid tail sensory NCV (m/s)	46.6±5.5	41.8±2.9	0.055
Prox tail sensory NCV (m/s)	50.5±3.5	45.8±3.0	<u>0.016</u>

Results are average values±1σ. Results with $p<0.05$ are underlined.

Table 3
Whole nerve uniaxial straining protocol

Strain	Relaxation time (s)	Study
5	60	SD
10	100	BB, SD
20	180	BB, SD
40	300	BB, SD
80	300	BB
160	300	BB

Strain rate was 0.1 mm/s (~0.01/s).

Table 4
Summary of biobreeding rat whole sciatic nerve uniaxial testing results at the conclusion of the 16-week study

Variable	Controls (8)	Diabetics (10)	p
D (mm)	1.24±0.07	1.17±0.08	0.094
$F_{0.10}$ (N)	0.036±0.036	0.010±0.011	<u>0.021</u>
$F_{0.20}$ (N)	0.196±0.198	0.108±0.126	0.186
$F_{0.40}$ (N)	0.861±0.541	1.02±0.650	0.554
$F_{0.80}$ (N)	2.62±0.529	3.53±1.54	0.194
$\sigma_{0.10}$ (MPa)	0.028±0.020	0.013±0.014	0.119
$\sigma_{0.20}$ (MPa)	0.13±0.10	0.14±0.17	0.899
$\sigma_{0.40}$ (MPa)	0.74±0.57	0.99±0.71	0.462
$\sigma_{0.80}$ (MPa)	3.35±1.83	4.06±1.56	0.402
$E_{0.10}$ (MPa)	0.30±0.21	0.15±0.17	0.168
$E_{0.20}$ (MPa)	0.64±0.52	0.79±1.07	0.728
$E_{0.40}$ (MPa)	3.5±2.7	5.9±3.7	0.178
$E_{0.80}$ (MPa)	8.9±5.7	13.1±8.0	0.229
$r_{0.10}$ (%)	48±9	51±16	0.619
$r_{0.20}$ (%)	37±6	37±6	0.912
$r_{0.40}$ (%)	31±6	32±12	0.789
$r_{0.80}$ (%)	29±15	26±17	0.737
ε_{yield}	0.38±0.22	0.15±0.13	0.070
W_v (MJ/m^3)	1.51±0.63	1.12±0.76	0.333

Results are average values±1σ.
Results with $p<0.05$ are underlined.

Table 2
Summary of Sprague–Dawley rat clinical results at the conclusion of the 12-week study

Variable	Control (7)	Wt control (8)	Diabetic (11)	ANOVA	SNK low	SNK mid	SNK high
Age (days)	152	152	152				
Duration (days)	89	89	89				
Body mass (g)	507±31	342±8	337±32	<0.001	<u>*d*</u>	<u>*W*</u>	c
Blood glucose (mg/dl)	100±18	94±16	341±60	<0.001	<u>*w*</u>	<u>*C*</u>	d
NCV (m/s)	49.3±10.6	34.1±3.3	36.9±6.6	0.002	<u>*w*</u>	<u>*D*</u>	c

Results are average values ± 1σ.
Entries in the last three columns represent Student–Newman–Keuls tests, for d = diabetic, c = control, w = weight control groups, ranked by mean; continuous connecting lines indicate no significant difference between or among linked groups.

Table 5
Summary of Sprague–Dawley rat whole sciatic nerve uniaxial testing results at the conclusion of the 12-week study

Variable	Control (7)	Wt control (7)	Diabetic (7)	ANOVA	SNK low	SNK mid	SNK high
D(mm)	1.19±0.08	1.18±0.11	1.07±0.23	0.294	d	w	c
$F_{0.05}$(N)	0.01±0.00	0.04±0.05	0.04±0.05	0.331	c	w	d
$F_{0.10}$(N)	0.05±0.03	0.14±0.17	0.21±0.14	0.105	c	w	d
$F_{0.20}$(N)	0.46±0.21	0.65±0.48	0.52±0.20	0.628	c	d	w
$F_{0.40}$(N)	1.82±0.36	1.95±0.51	1.45±0.28	0.145	d	c	w
$\sigma_{0.05}$(MPa)	0.01±0.00	0.04±0.04	0.04±0.03	0.194	c	w	d
$\sigma_{0.10}$(MPa)	0.05±0.02	0.15±0.17	0.23±0.14	0.047	c	w	d
$\sigma_{0.20}$(MPa)	0.56±0.30	0.59±0.48	0.70±0.36	0.812	c	w	d
$\sigma_{0.40}$(MPa)	1.94±0.31	1.62±0.32	2.16±0.55	0.144	w	c	d
$E_{0.05}$(MPa)	0.30±0.17	0.73±0.81	0.72±0.58	0.343	c	d	w
$E_{0.10}$(MPa)	0.13±0.06	0.24±0.25	0.36±0.16	0.073	c	w	d
$E_{0.20}$(MPa)	3.04±1.33	3.34±2.53	2.59±1.13	0.761	d	c	w
$E_{0.40}$(MPa)	10.0±3.9	9.0±3.8	1.07±2.5	0.709	w	c	d
$r_{0.05}$(%)	46±10	38±13	43±15	0.472	w	d	c
$r_{0.10}$(%)	38±6	36±7	37±9	0.778	w	d	c
$r_{0.20}$(%)	41±6	32±4	34±5	0.013	w	d	c
$r_{0.40}$(%)	31±17	37±6	34±6	0.645	c	d	w

Results are average values $\pm 1\sigma$.
Entries in the last three columns represent Student–Newman–Keuls tests, for d = diabetic, c = control, w = weight control groups, ranked by mean; continuous connecting lines indicate no significant difference between or among linked groups.

The greatest abundance of fibrillar collagens assayed in the SD study were found in the weight control animals' tissues; both epineurial/perineurial types I ($p<0.001$), III ($p=0.013$) and IV ($p<0.001$) and endoneurial types III ($p=0.011$) and IV ($p<0.001$) collagens were found in greater abundance than in the control tissues. Diabetic tissues also showed significantly higher abundance of collagens in epineurial/perineurial types I ($p=0.021$), III ($p=0.031$) and IV ($p<0.001$) and endoneurial type IV ($p<0.001$) tissues than the control tissues. Weight control tissues had significantly more fibrillar endoneurial (types I and III) collagen than even diabetic tissues, though overall, the collagen contents were similar for the diabetic and weight control tissues—both were larger than for control tissues.

4. Discussion

We quantified differences in the relative amounts and morphology of the major fibrillar collagens in diabetic and normal rat peripheral nerve. The four factors examined were chosen in order to clarify the possible origin of differences in behavior of the structural collagens, as either in the types, arrangements, or locations of fibrils. We were also interested in the scale at which these changes manifested.

We found support for our first hypothesis; differences in collagen expression were detected in six of the 12 comparisons. Our finding was consistent with Muona et al. (1993a) who found collagen types I and IV to be upregulated in an in vitro preparation of Schwann cells, perineurial cells and fibroblasts in the presence of excess glucose; they also determined that type VI collagen was upregulated (Muona et al., 1993a). In a human in vivo study, Bradley et al. (2000) found that collagen types I, III, IV, V and VI were upregulated in the endoneurium and types IV and V were upregulated in the perineurium. The major inconsistency of our results with these, is that we found no upregulation of type I collagen in either of the BB compartments, or in SD diabetic endoneurium. The consistent findings in both animal models were for upregulations of collagen types III and IV in the endoneurium.

Apparent upregulations of collagens were also found in weight-control animal tissues. Various other workers have reported no change or downregulation of collagen in the presence of undernourishment. In most of these cases, however, generalized protein assays were performed, with the exception of Reif et al. (1993), who found diminished total protein content in malnourished rat liver, but normal collagen/protein ratios for types I, III and IV content with and immunohistochemical technique, and Spanheimer et al. (1991) who found diminished cartilaginous collagen (presumably type II) content in malnourished rats with a [^{3}H]proline assay. Two groups have reported results using hydroxyproline to assay generalized, assembled collagens. Blankenship et al. (1993) found no change in a generalized collagen content in malnourished rat lung with a, and Yue et al.

Table 6
Summary of biobreeding rat in situ immunohistochemistry results at the conclusion of the 16-week study

Variable	Controls	Diabetics	p
Epi/perineurial type I collagen	1.00 ± 0.39 (81)	0.94 ± 0.42 (86)	0.361
Epi/perineurial type III collagen	1.00 ± 0.37 (55)	1.62 ± 0.46 (90)	<0.001
Epi/perineurial type IV collagen	1.00 ± 0.29 (47)	1.23 ± 0.56 (55)	0.010
Endoneurial type I collagen	1.00 ± 0.66 (52)	1.08 ± 0.49 (56)	0.457
Endoneurial type III collagen	1.00 ± 0.54 (48)	1.25 ± 0.80 (60)	0.053
Endoneurial type IV collagen	1.00 ± 0.50 (32)	1.14 ± 0.75 (38)	0.348

Units are fluorescent units normalized to the control values. In brackets are number of regions viewed to obtain the averages.
Results are average values $\pm 1\sigma$.
Results with $p<0.05$ are underlined.

Table 7
Summary of Sprague–Dawley rat in situ immunohistochemistry results at the conclusion of the 12-week study

Variable	Control	Wt control	Diabetic	ANOVA	SNK low	SNK mid	SNK high
Epi/perineurial type I collagen	1.00 ± 0.54(45)	1.43 ± 0.67(63)	1.33 ± 1.05(81)	0.025	c	d	w
Epi/perineurial type III collagen	1.00 ± 0.40(57)	1.28 ± 0.75(61)	1.25 ± 0.91(79)	0.081	c	d	w
Epi/perineurial type IV collagen	1.00 ± 0.42 (36)	1.55 ± 0.47(40)	1.42 ± 0.53(54)	<0.001	c	d	w
Endoneurial type I collagen	1.00 ± 0.56(33)	1.32 ± 0.67(38)	0.96 ± 0.56(54)	0.012	d	c	w
Endoneurial type III collagen	1.00 ± 0.57(50)	1.47 ± 1.09(43)	1.06 ± 0.63(52)	0.010	c	d	w
Endoneurial type IV collagen	1.00 ± 0.67(29)	2.06 ± 0.70(27)	2.08 ± 1.30(36)	<0.001	c	w	d

Units are fluorescent units normalized to the control values. In brackets are number of regions viewed to obtain the averages.
Entries in the last three columns represent Student–Newman–Keuls tests, for d = diabetic, c = control, w = weight control groups, ranked by mean; continuous connecting lines indicate no significant difference between or among linked groups.

(1986) found diminished ability to make generalized collagens in diabetics, but not in malnourished rats with a subcutaneous induced wound preparation. The reduction in lysyl oxidase in malnourished rat lung tissue reported by Madia et al. (1979) may help explain this latter result, however, given the importance of this enzyme in assembly of fibrillar collagens.

We found no significant differences between weight-control and control nerve diameters, so that it seems unlikely that the higher density of collagens observed immunohistochemically in the weight-control tissues was simply due to possible reduction in cellular components of the nerve. Thus, the reasons behind the upregulations we observed are not yet clear. Future work will include Western and Northern blots of tissues, to determine more quantitatively the types of collagens in the tissues, and also whether the alterations were translational or transcriptional.

Two specific findings were that epi/perineurial type I collagens were apparently upregulated in both the weight controls ($p<0.001$) and diabetics ($p=0.021$) in the SD study. It is reasonable to conjecture that type I collagen, with its larger volume fraction and fibrillar structure, would have the greatest effect on global mechanical properties among the collagens present. However, we found this not to be the case; indeed, our second hypothesis was clearly not supported. Specifically, one might expect upregulation of type I collagens to be most strongly associated with higher stresses at high strains, in which wavy fibrils are aligned, if upregulation was causal to generalized tissue stiffening. However, SD weight controls showed 16% lower stresses ($p=0.096$); the result for SD diabetics, though statistically insignificant, was 11% greater ($p=0.535$) at 40% strain. Conversely, in the BB model, there was no appreciable upregulation of type I collagen in either the endoneurium ($p=0.457$) or in the epi/perineurium ($p=0.361$), and although insignificant, there was an average increase of 33% in maximum stress at 40% strain ($p=0.462$) and 21% in maximum stress at 80% strain ($p=0.402$). Thus, upregulations in the major peripheral nerve collagens apparently do not manifest as differences in tissue-scale mechanical differences in these rat models. As a side note, the overall reductions in diabetic whole nerve diameters reported here were consistent with previous results, which showed diminished endoneurial cellular components (Jakobsen, 1976a, b).

Our third hypothesis, that BB animals would exhibit similar differences as the SD animals (diabetic vs. control), was largely supported. Generalized collagen upregulations were found in both models, as were

insignificant differences in mechanical results. However, genetically diabetic animals may possibly be less susceptible to type I collagen upregulation due to diabetes in the peripheral nerve, per our findings.

Finally, our fourth hypothesis, that glycation rather than weight loss is responsible for tissue alterations, was only partially supported. While collagens were generally upregulated in both weight control and control SD animals, the morphologies of weight control versus diabetic collagens were markedly different. Specifically, we found (as reported in Wang et al., 2003) significantly larger collagen fibrils in diabetics, in both endoneurial (49, 43 and 42 nm, for diabetic, control and weight control) and epineurial collagen (78, 72 and 72 nm, for diabetic, control and weight control) for the SD model. Larger collagen fibrils were also found in BB diabetics, in endoneurial (49 vs. 42 nm), epineurial (82 vs. 76 nm) and tail collagens (160 vs. 125 nm). Thus, though food restriction and diabetes may both result in generalized collagen upregulation, only diabetes appears to produce significantly larger fibril diameters. This suggests glycation as the mechanism by which structural changes are induced in the fibrils themselves, and possibly a shared mechanism with mineral deficiency or food restriction in collagen upregulation.

Clearly, the way in which microstructure affects tissue behavior merits further study. Though tissue stiffening has been described as an outcome of long-term diabetes in several clinical studies, e.g. work on rat cardiac tissue (Woodiwiss et al., 1996) and human skin (Reihsner and Menzel, 1998), the significant alterations in collagens assayed in this study did not produce measurable tissue-scale stiffness changes. Either content or geometry of fibrillar collagens would be expected to affect both axial (fibril direction) and transverse mechanical properties, at least locally (but perhaps not at the tissue scale).

The remodeling found in by our studies may thus be either glucose (Benazzoug et al., 1998), or mechanically (Kessler et al., 2001) mediated. Determination of the relationship between glycation-induced geometry changes, and local load distribution (resulting in mechanically mediated changes) remains to be determined using detailed models. Though the slight increases measured in *axial* maximum stress and elastic modulus reported here are generally consistent with both the upregulation of the collagen types studied and with the increase in fibrillar collagen fibril (types I and III) diameter observed, correlation of these properties requires detailed modeling of fiber straightening and load transfer within the fibril bundles. The effect of larger fibril diameter on mechanical properties must also be determined in order to link structural to physiological alterations. Examples of similar investigations include work in single neurons growing on glycated laminin or type IV collagen in vitro (Luo et al., 2002) or in investigation of the role of ECM on regrowth of sensory receptors (Dubovy, 2000) and distal axons (Chamberlain et al., 2000), or artificial even ECM construction (Zhang et al., 2002). Work in fibril properties has been initiated elsewhere at the molecular (Ayad et al., 1994), fibrillar (Hulmes, 2002), and tissue (Fung, 1993) scales. Indeed, the differences between diabetic and control tissues in both the BB and SD models are possibly consistent with significantly elevated, local, *transverse* loads in the tissue. Such local loads have been implicated in collagen upregulation (Pavlin et al., 2001; Sanders and Goldstein, 2001) and will be studied as part of our future work. Detailed modeling would be required to determine whether significant increases could be expected in transverse loads.

Future work will include use our results as input to a bundle model for deformation of the various types, arrangements and sizes of collagens found. This will require a sufficiently detailed model of a single collagen fibril; ultimately, we aim to develop bounds for both transverse and axial tissue and local (fibril-scale) loads, in order to elucidate the relationship between collagen upregulation and glycation and mechanically mediated changes at the fibril scale.

Acknowledgements

This work was supported in by the Whitaker Foundation, the DARPA Synthetic Multifunctional Materials Program (Dr. Leo Christodoulou and Dr. Steve Fishman, Program Monitors) and by an NSF PECASE grant (Sastry); this funding is gratefully acknowledged.

References

American Diabetes Association, 1984. Physician's Guide to Non-insulin-dependent Type II Diabetes (NIDDM): Diagnosis and Treatment. American Diabetes Association, Alexandria, VA.

Ayad, S., Boot-Handford, R.P., Humphries, M.J., Kadler, K.E., Shuttleworth, C.A., 1994. The Extracellular Matrix Factsbook. Academic Press, New York, pp. 29–85.

Benazzoug, Y., Borchiellini, C., Labat-Robert, J., Robert, L., Kern, P., 1998. Effect of high-glucose concentrations on the expression of collagens and fibronectin by fibroblasts in culture. Experimental Gerontology 33, 445–455.

Blankenship, J.W., McKinnney, B.C., Roos, P.J., Sandberg, L.B., 1993. Quantitation of lung elastin and collagen in protein and essential fatty acid malnourished rats. Connective Tissue Research 29, 311–318.

Bradley, J.L., King, R.H., Muddle, J.R., Thomas, P.K., 2000. The extracellular matrix of peripheral nerve in diabetic polyneuropathy. Acta Neuropathology 99, 539–546.

Butler, L., Guberski, D.L., Like, A.A., 1983. Genetic analysis of the BB/W diabetic rat. Canadian Journal of Genetics and Cytology 25, 7–15.

Cameron, N.E., Cotter, M.A., 1994. The relationship of vascular changes to metabolic factors in diabetes mellitus and their role in

the development of peripheral nerve complications. Diabetes/Metabolism Reviews 10, 189–224.

Chamberlain, L.J., Yannas, I.V., Hsu, H.P., Strichartz, G.R., Spector, M., 2000. Near-terminus axonal structure and function following rat sciatic nerve regeneration through a collagen–gag matrix in a ten-millimeter gap. Journal of Neuroscience Research 60, 666–677.

DCCT—Diabetes Control and Complications Trial Research Group, 1995. The effect of intensive diabetes therapy on the development of progression of neuropathy. Annals of Internal Medicine 122, 561–568.

Dubovy, P., 2000. Restoration of lamellar structures in adult rat Pacinian corpuscles following their simultaneous freezing injury and denervation. Anatomy and Embryology 202, 235–245.

Feldman, E.L., Stevens, M.J., Russell, J.W., Greene, D.A., 2001. Diabetic neuropathy. In: Becker, K.L. (Ed.), Principles and Practice of Endocrinology and Metabolism. Lippincott Williams & Wilkins Publishers, Philadelphia, PA.

Fung, Y.C., 1993. Bioviscoelastic solids. In: Fung, Y.C. (Ed.), Biomechanics Mechanical Properties of Living Tissues, 2nd Edition.. Springer, New York, pp. 242–320.

Gamble, H.J., Eames, R.A., 1964. An electron microscope study of the connective tissues of human peripheral nerves. Journal of Anatomy 98, 655–663.

Gavin III, J.R., Alberti, K.G.M.M., Davidson, M.B., DeFronzo, R.A., Drash, A., Gabbe, S.G., Genuth, S., Harris, M.I., Kahn, R., Keen, H., Knowler, W.C., Lebovitz, H., Maclaren, N.K., Palmer, J.P., Raskin, P., Rizza, R.A., Stern, M.P., 1998. Report of the expert committee on the diagnosis and classification of diabetes mellitus [American Diabetes Association: Clinical Practice Recommendations 1998]. Diabetes Care 21 (1S), 5S–19S.

Greene, D.A., Feldman, E.L., Stevens, M.J., Sima, A.A.F., Albers, J.W., Pfeifer, M.A., 1997. Diabetic Neuropathy. In: Porte, D., Sherwin, R., Rifkin, H. (Eds.), Ellenberg & Rifkin's Diabetes Mellitus. Appleton & Lange, Stamford, CT, pp. 1009–1076.

Guberski, D.L., 1994. Diabetes-prone and diabetes-resistant BB rats: animal models of spontaneous and virally induced diabetes mellitus, lymphocytic thyroiditis, and collagen-induced arthritis. ILAR News 35, 29–36.

Hulmes, D.J.S., 2002. Building collagen molecules, fibrils, and suprafibrillar structures. Journal of Structural Biology 137, 2–10.

Jakobsen, J., 1976a. Axonal dwindling in early experimental diabetes. 1. A study of cross sectioned nerves. Diabetologia 12, 539–546.

Jakobsen, J., 1976b. Axonal dwindling in early experimental diabetes. 2. A study of isolated nerve fibres. Diabetologia 12, 547–553.

Janssen, K., 1994. Current Protocols in Molecular Biology, Vol. 2. Greene & Wiley, New York (Suppl. 7, Section 14.6.4).

Kessler, D., Dethlefsen, S., Haase, I., Plomann, M., Hirche, F., Krieg, T., Eckes, B., 2001. Fibroblasts in mechanically stressed collagen lattices assume a "synthetic" phenotype. Journal of Biological Chemistry 276, 36575–36585.

Kopelman, P.G., Hitman, G.A., 1998. Diabetes. Exploding type II. The Lancet 352, 19–26.

Like, A.A., Butler, L., Williams, R.M., Appel, M.C., Weringer, E.J., Rossini, A.A., 1982. Spontaneous autoimmune diabetes mellitus in the BB rat. Diabetes 31 (S 1 Pt 2), 7–13.

Lorimer, P., Mezin, P., Moleur, F.L., Pinel, N., Peyrol, S., Stoebner, P., 1992. Ultrastructural localization of the major components of the extracellular matrix in normal rat nerve. The Journal of Histochemistry and Cytochemistry 40, 859–868.

Low, P.A., Dyck, P.J., 1977. Increased endoneurial fluid pressure in experimental lead neuropathy. Nature 269, 427–428.

Low, P.A., Dyck, P.J., Schmelzer, J.D., 1980. Mammalian peripheral nerve sheath has unique responses to chronic elevations of endoneurial fluid pressure. Experimental Neurology 70, 300–306.

Luo, Z.J., King, R.H., Lewin, J., Thomas, P.K., 2002. Effects of nonenzymatic glycosylation of extracellular matrix components on cell survival and sensory neurite extension in cell culture. Journal of Neurology 249, 424–431.

Madia, A.M., Rozovski, S.J., Kagan, H.M., 1979. Changes in lung lysyl oxidase activity in streptozotocin-diabetes and in starvation. Biochimica et Biophysica Acta 585, 481–487.

Muona, P., Jaakkola, S., Salonen, V., Peltonen, J., 1989. Diabetes induces the formation of large diameter collagen fibrils in the sciatic nerves of BB rats. Matrix 9, 62–67.

Muona, P., Peltonen, J., Jaakkola, S., Uitto, J., 1991. Increased matrix gene expression by glucose in rat neural connective tissue cells in culture. Diabetes 40, 605–611.

Muona, P., Jaakkola, S., Zhang, R.Z., Pan, T.C., Pelliniemi, L., Risteli, L., Chu, M.L., Uitto, J., Peltonen, J., 1993a. Hyperglycemic glucose concentrations up-regulate the expression of type VI collagen in vitro. Relevance to alterations of peripheral nerves in diabetes mellitus. American Journal of Pathology 142 (5), 1586–1597.

Muona, P., Kalliomaki, M., Peltonen, J., 1993b. Diabetes-induced connective tissue changes. Duodecim 109 (8), 667–671.

Myers, R.R., Powell, H., 1984. Galactose neuropathy: impact of chronic endoneurial edema on nerve blood flow. Annals of Neurology 16, 587–594.

Myers, R.R., Murakami, H., Powell, H.C., 1986. Reduced nerve blood flow in edematous neuropathies: a biomechanical mechanism. Microvascular Research 32, 145–151.

Nakhooda, A.F., Like, A.A., Chappel, C.I., Murray, F.T., Marliss, E.B., 1977. The spontaneously diabetic Wistar rat: metabolic and morphologic studies. Diabetes 26, 100–112.

O'Connor, P.J., Spann, S.J., Woolf, S.H., 1998. Care of adults with type II diabetes mellitus: a review of the evidence. The Journal of Family Practice 47, s13–s22.

Pavlin, D., Zadro, R., Gluhak-Heinrich, J., 2001. Temporal pattern of stimulation of osteoblast-associated genes during mechanically induced osteogenesis in vivo: early responses of osteocalcin and type I collagen. Connective Tissue Research 42, 135–148.

Pop-Busui, R., Sullivan, K.A., Van Huysen, C., Bayer, L., Cao, X., Towns, R., Stevens, M.J., 2001. Depletion of taurine in experimental diabetic neuropathy: implications for nerve metabolic, vascular, and functional deficits. Experimental Neurology 168, 259–272.

Reddy, G., Stehno-Bittel, L., Hamade, S., Enwemeka, C.S., 2001. The biomechanical integrity of bone in experimental diabetes. Diabetes Research and Clinical Practice 54, 1–8.

Reif, S., Lu, R.B., Tano, M., Terranova, V., Young, C., Fisher, J., Petell, J., Lebenthal, E., 1993. Perinatal food restriction in rats reduces the content but not concentration of liver extracellular matrix proteins. Journal of Nutrition 123, 811–816.

Reihsner, R., Menzel, E.J., 1998. Two-dimensional stress–relaxation behavior of human skin as influenced by non-enzymatic glycation and the inhibitory agent aminoguanidine. Journal of Biomechanics 31, 985–993.

Sanders, J.E., Goldstein, B.S., 2001. Collagen fibril diameters increase and fibril densities decrease in skin subjected to repetitive compressive and shear stresses. Journal of Biomechanics 34, 1581–1587.

Sensi, M., Pricci, F., Pugliese, G., De Rossi, M.G., Petrucci, A.F.G., Cristina, A., Morano, S., Pozzessere, G., Valle, E., Andreani, D., Di Mario, U., 1995. Role of advanced glycation end-products (AGE) in late diabetic complications. Diabetes Research and Clinical Practice 28, 9–17.

Sima, A.A.F., Lattimer, S.A., Yagihashi, S., Greene, D.A., 1986. Axoglial dysjunction: a novel structural lesion that accounts for poorly

reversible slowing of nerve conduction in the spontaneously diabetic bio-breeding rat. Journal of Clinical Investigation 77, 474–484.

Sima, A.A.F., Zhang, W., Xu, G., Sugimoto, K., Guberski, D., Yorek, M.A., 2000. A comparison of diabetic polyneuropathy in type II diabetic BBZDR/Wor rats and in type I diabetic BB/Wor rats. Diabetologia 43, 786–793.

Spanheimer, R., Zlatev, T., Umpierrez, G., Digirolamo, M., 1991. Collagen production in fasted and food-restricted rats: response to duration and severity of food deprivation. Journal of Nutrition 121, 518–524.

Sternberg, M., Cohen-Forterre, L., Peyroux, J., 1985. Connective tissue in diabetes mellitus: biochemical alterations of the intercellular matrix with special reference to proteoglycans, collagens and basement membranes. Diabetes and Metabolism 11, 27–50.

Stevens, M.J., Lattimer, S.A., Feldman, E.L., Helton, E.D., Millington, D.S., Sima, A.A.F., Greene, D.A., 1996. Acetyl-L-carnitine deficiency as a cause of altered nerve myo-inositol content, NAD-ATPase activity, and motor conduction velocity in the streptozotocin-diabetic rat. Metabolism 45, 865–872.

Thomas, P.K., Berthold, C.H., Ochoa, J., 1993. Microscopic anatomy of the peripheral nervous system. In: Dyck, P.J., Thomas, P.K., Griffin, J.W., Low, P.A., Poduslo, J.F. (Eds.), Peripheral Neuropathy, 3rd Edition.. Saunders, London, pp. 28–91.

Thomas, P.K., Tomlinson, D.R., 1993. Diabetic and hypoglycemic neuropathy. In: Dyck, P.J., Thomas, P.K., Griffin, J.W., Low, P.A., Poduslo, J.F. (Eds.), Peripheral Neuropathy, 3rd Edition.. Saunders, London, pp. 1219–1250.

Ushiki, T., Ide, C., 1986. Three-dimensional architecture of the endoneurium with special reference to the collagen fibril arrangement in relation to nerve fibers. Archivum Histologicum Japonicum 49, 553–663.

Ushiki, T., Ide, C., 1990. Three-dimensional organization of the collagen fibrils in the rat sciatic nerve as revealed by transmission- and scanning electron microscopy. Cell and Tissue Research 260, 175–184.

Wang, H., Layton, B.E., Sastry, A.M., 2003. Normal and diabetic nerve collagens: an atomic force microscopy study on Sprague–Dawley and BioBreeding rats. Diabetes—Metabolism Research and Reviews 19, 288–298.

Winegrad, A.I., Greene, D.A., 1976. Diabetic polyneuropathy: the importance of insulin deficiency, hyperglycemia and alterations in myoinositol metabolism in it pathogenesis. The New England Journal of Medicine 295, 1416–1420.

Woodiwiss, A.J., Kalk, W.J., Norton, G.R., 1996. Habitual exercise attenuates myocardial stiffness in diabetes mellitus in rats. American Journal of Physiology 271, H2126–2133.

Wright, A., Nukada, H., 1994. Sciatic nerve morphology and morphometry in mature rats with streptozocin-induced diabetes. Acta Neuropathologica (Berlin) 88, 571–578.

Yue, D.K., Swanson, B., Mclennan, S., Marsh, M., Spaliviero, J., Delbridge, L., Reeve, T., Turtle, J.R., 1986. Abnormalities of granulation tissue and collagen formation in experimental diabetes, uraemia and malnutrition. Diabetic Medicine 3, 221–225.

Zhang, L., Ma, D., Wang, F., Zhang, Q., 2002. The modification of scaffold material in building artificial dermis. Artificial Cells, Blood Substitutes, and Immobilization Biotechnology 30, 319–332.