Loss of cutaneous unmyelinated and myelinated fibers in streptozotocin-induced diabetic rats: a time course study

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Abstract: Background: Quantification of density of intraepidermal nerve fibers (IENFD), targeting mainly unmyelinated C fibers using skin biopsies, has emerged as a promising method for assessment of diabetic peripheral neuropathy. However, detailed histological changes in unmyelinated fibers in combination with large myelinated fibers in rat models have not been adequately explored. Methods: The current study assessed both cutaneous unmyelinated and myelinated fiber changes, as well as pain-related behavior, in an 8-week streptozotocin (STZ)-induced rat model of diabetic mellitus (DM). Mechanical allodynia and thermal hyperalgesia were evaluated at baseline and weekly after injections with STZ. Quantification of IENFD, dermal nerve fiber (DNF) density, and density of intrapapillary myelinated endings (IMEs) was conducted at weeks 4, 6, and 8 post-injection. Results: Mechanical allodynia started at 1-week post-injection. It progressed through week 5, then plateaued at week 8. Thermal hyperalgesia occurred at week 4 and only lasted through week 5 in DM rats. IENFD reduction had already occurred at week 4. However, both DNFL and IME densities were still comparable at that time. IME density in the DM rats started to decrease right after week 4. It was significantly reduced at week 8. DNFL reduction in the DM rats did not occur until week 8. Conclusion: Present results show early persistent mechanical allodynia and delayed temporary hyperalgesia in this STZ-induced diabetic rat model. Significant IENF loss preceded changes in IMEs and DNFs, suggesting that IENFD quantification might be a potential marker for detection of early diabetic neuropathy.

Keywords: Diabetic neuropathy, mechanical allodynia, thermal hyperalgesia, intraepidermal nerve fiber density (IENFD), dermal nerve fibers (DNF), intrapapillary myelinated endings (IME)

Introduction

Diabetic peripheral neuropathy (DPN) is a common complication of diabetes, affecting nearly half of all diabetic patients over the course of the disease [1]. Patients with DPN may have a variety of symptoms ranging from hyperalgesia, allodynia, and spontaneous pain to progressive hypoalgesia in their limbs [2]. However, the reasons why pain symptoms occur in certain groups of patients and the relationships between pain generation and peripheral nerve damage remain unclear [3]. Due to length-dependent characteristics of diabetic neuropathy, the distal parts of the limbs are first affected. This could affect both small-caliber fibers (such as unmyelinated C fibers and thinly myelinated Aδ fibers, which receive thermal and noxious stimuli [4]) and large myelinated Aβ fibers innervating cutaneous mechanoreceptors, known as Meissner corpuscles (MCs)[5, 6]. Understanding the underlying pathological changes of DPN is critical in advancing optimal treatment.

Skin biopsies have become a commonly used alternative approach to assess pathophysiological changes in distal part of the limbs [7]. Quantification of density of intraepidermal nerve fibers (IENF) (the peripheral termini of nociceptive unmyelinated C fibers and thinly myelinated Aδ fibers) by skin biopsies has now become the gold standard method for assessment of diabetic peripheral neuropathy [7, 8]. Reduction of IENF densities (IENFD) was found in both diabetic patients and animal models, suggesting that loss of IENFD could be an early indication of
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diabetic neuropathy [4, 9-11]. Despite intensive efforts in studying IENF, few studies have evaluated large myelinated fibers. One study found a significant reduction of intrapapillary myelinated endings (IMEs) (large myelinated fibers innervating MCs) in patients with diabetic neuropathy [6]. Dermal nerve fiber (DNF) quantification has been used to detect nerve fiber bundles in the superficial dermis [12]. However, there remains a lack of studies concerning parallel histological changes of both myelinated and unmyelinated fibers in diabetic neuropathy.

Therefore, the present study aimed to compare the progression of both cutaneous unmyelinated and myelinated fiber changes (IENF, DNF, and IMEs densities) in an 8-week streptozotocin (STZ)-induced diabetic rat model. Additionally, pain-related behavioral changes (mechanical allodynia and thermal hyperalgesia) were assessed.

Materials and methods

Animals

Fifty 4-week old Sprague-Dawley rats were purchased from Shanghai Laboratory Animal Center of China Academy of Science. They were housed in a hooded cage at room temperature (RT) with a 12-hour light-dark cycle. They were provided food and water ad libitum. Experimental diabetes was induced in 28 rats by single intraperitoneal injections of 60 mg/kg STZ per rat (Sigma-Aldrich, St Louis, MO, USA) after 24-hour fasting. The STZ was dissolved in a 0.1 M sodium citrate buffer, with pH 4.5 (the DM group). The remaining 22 rats were injected with sodium citrate, without STZ, serving as normal controls (control group). Fasting blood glucose levels were measured 72 hours after injections and every two weeks thereafter, ensuring a constant hyperglycemic status during the study. Experimental diabetes was established for rats with a fasting blood glucose level >16.6 mmol/L. These rats were included in the DM group.

All experimental procedures were approved by the Animal Care and Use Committee of Fudan University and were in accordance with NIH Guidelines for the Care and Use of Laboratory Animals.

Behavioral testing

Mechanical allodynia and thermal hyperalgesia were assessed before STZ injections, establishing the baseline level. They were assessed weekly, after injections, for 8 weeks. Mechanical allodynia was assessed by measuring the 50% hind paw withdrawal threshold using calibrated von Frey filaments, according to the up-down method of Dixon. Briefly, rats were placed in wire-mesh bottomed plastic chambers. They were acclimated for 30 minutes. A series of 8 calibrated von Frey filaments of increasing stiffness levels (0.4, 0.6, 1.4, 2, 4, 6, 8, and 15 g, Stoelting, Wood Dale, Illinois, US) were applied to the mid-plantar hind paw of each rat, perpendicularly, with sufficient force to cause a slight bending of the filament against the paw. It was held there for 4-6 seconds, with 10-minute intervals between applications of 2 filaments. The test started with the 2 g filament. A sharp withdrawal of the paw or immediate paw flinching upon removal of the filament was considered a positive response. Stimuli were applied consecutively, either in an ascending order in the absence of positive response or in a descending order if a positive response was observed. Testing consisted of five more stimuli after the first change in response occurred. The pattern of response was converted to a 50% paw withdrawal threshold (50% PWT) using the method of Dixon [13].

Paw withdrawal latency (PWL) to radiant heat was measured to assess thermal hyperalgesia, using the Model 390 Paw Stimulator Analgesia Meter (IITC/Life Science Instruments, USA). The rats were placed on a glass platform in transparent plastic chambers and allowed to acclimate for at least 30 minutes. Radiant heat was applied to the plantar surface of the hind paws until the rat removed its paw from the glass. Both hind paws were tested, independently, with a 10-minute interval. PWL was defined as the time from application of the heat to paw removal. The heat had a constant intensity set to elicit a 10-12 second PWL in rats in the control group. The maximal time duration for heat application was 20 seconds, avoiding tissue damage. A total of 5 tests were conducted. The longest and shortest PWL scores for each hind paw were excluded. Final PWL was calculated as the average of the remaining 3 PWLs [14].
**Immunohistochemistry of skin biopsies**

Biopsy samples were obtained at weeks 4, 6, and 8 after injections for immunohistochemistry examinations. The rats were given an overdose of chloral hydrate. Glabrous skin in the middle of their hind limbs was separated and dissected. The glabrous skin was chosen because, unlike hairy skin, it not only has unmyelinated C fibers and thinly myelinated Aδ fibers, but also large myelinated Aβ fibers innervating MCs or Merkel cells. Dissected glabrous skins were immediately fixed in Zamboni’s fixative (2% paraformaldehyde-picric acid) at 4°C for 24 hours. They were then cryoprotected in 20% sucrose -0.1 M phosphate buffer (PH 7.4) for 24-48 hours. Next, 40 μm-thick sections transverse to the epidermis were cut and stored at -20°C for further immunohistochemistry analysis.

Six sections from a single footpad of a rat were randomly chosen for immunohistochemistry analysis using a free-floating protocol [15, 16]. Sections were blocked with 4% normal goat serum in 0.01 M PBS - 0.3% Triton X-100 for 2 hours at RT, then incubated at 4°C overnight in rabbit polyclonal anti-PGP 9.5 antibody (1:1000, Chemicon). This was conducted to stain all nerve fibers or mouse anti-myelin basic protein (MBP) primary antibody (1:200, Chemicon), observing the myelinated nerve fibers. After rinsing with PBS, the sections were incubated with biotinylated goat anti-rabbit IgG or biotinylated goat anti-mouse IgG (1:1000, Vector) at RT for 1 hour. They were quenched in 30% methanol/1% hydrogen peroxide for 30 minutes and incubated in avidin-biotin complex (Vector) for 1 hour. The final reaction product was revealed by the blue chromogen/peroxidase substrate (Vector SG substrate kit). Sections were then mounted, air-dried, dehydrated in graded alcohol, cleared in xylene, and cover-slipped.

**Quantification of nerve fiber loss**

Quantification of IENFD, DNF, and IMEs densities was conducted at weeks 4, 6, and 8 post-STZ injection.

IENFD is defined as the number of IENF per length of a section (IENF/mm). It was quantified and calculated by an observer blinded to the grouping of the rats using bright-field immunohistochemistry, according to the methods of Lauria et al. [16]. The number of PGP 9.5 positive IENFs in each section was counted under a light microscope at high power magnification (400×). Individual IENFs crossing the dermal-epidermis junction were counted. Secondary branching within the epidermis was not included in the counting. Images were used to measure the length of the epidermal surface. IENFD was calculated in at least 3 sections from 1 footpad, arriving at an average IENFD.

Quantification of DNF was also conducted, as described previously. Briefly, a dermal area of interest (AOI) 200 μm below the dermal-epidermal junction was drawn and the length of each DNF was measured. DNF density is defined as the sum of the length of each DNF (DNFL) divided by the area of AOI (mm²) [12].

Loss of myelinated fibers was evaluated by quantification of density of IMEs innervating MCs. The number of IMEs was counted using bright-field immunohistochemistry on at least 3 MBP-stained sections for each footpad of a rat. The density of IMEs is defined as the number of IMEs per length of the epidermis (IMEs/mm) [5].

**Statistical analysis**

SPSS 17.0 for Windows was used for data analysis. Results are presented as mean ± standard deviation (SD). For parameters with multiple time points, repeated measure analysis of variance (ANOVA) was applied. For inter-group comparisons, two-tailed student’s t tests were used for data with normal distribution and Mann-Whitney U-tests were used for data with non-normal distribution. P<0.05 indicates statistical significance.

**Results**

**Establishment of an STZ-induced diabetic rat model**

Male Sprague-Dawley rats were divided into 2 groups, the DM group (n=28) and control group (n=22) as described previously. In this study, a single injection of STZ induced persistent hyperglycemia (fasting blood glucose >16.6 mmol/L) in 82.1% of injected rats, indicating that the hyperglycemia model was successfully established in the DM group. Rats in...
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The DM group grew substantially slower than those in the control group and weighed less (Figure 1A). Hyperglycemia in DM rats occurred around 1 week after injection. Fasting blood glucose levels increased progressively throughout the study period, while fasting blood glucose levels in control rats was maintained below 10 mmol/L throughout the study period (Figure 1B).

**Mechanical allodynia and thermal hyperalgesia in DM rats**

Eight DM rats and 6 control rats were tested for mechanical allodynia and thermal hyperalgesia. Mechanical allodynia, defined as significantly reduced 50% paw withdrawal threshold (50% PWT), was reduced in the DM group, compared to the control group (P<0.001). This variation changed with time (P=0.039). Mechanical allodynia occurred 1 week after STZ injections in the DM group (50% PWT: 9.15±1.81 g vs 9.85±2.02 g, P=0.51 at baseline, and 6.81±2.54 vs 10.55±2.14 g, P=0.013 at week 1 for DM and controls, respectively). It progressed through week 5 (50% PWT: 3.7±0.93 g vs 10.56±1.62 g, P<0.001, at week 5 for DM and controls, respectively), then plateaued afterward (50% PWT: 3.81±0.93 g vs 10.23±2.10 g, P<0.001, at week 8 for DM and controls, respectively) (Figure 2A).

Mean paw withdrawal latency was not different between the DM group and control group (P=0.059). Thermal hyperalgesia was not obvious in the DM group, except for weeks 4 and 5, in which significantly decreased PWL was observed. This indicated the presence of thermal hyperalgesia (PWL 13.06±1.96 s vs 13.52±1.30 s, P=0.51 at week 0, 10.44±1.34 s vs 13.09±1.51 s, P=0.005 at week 4 and 10.01±1.66 s vs 12.53±0.57 s, P=0.009 at week 5 for DM and controls, respectively) (Figure 2B).

**Progressive skin denervation in DM rats**

Fifteen rats in each group (4 for week 4, 4 for week 6, and 7 for week 8) were used for quantification of IENFD, DNF, and IMEs densities.

Figure 3A and 3B show PGP 9.5-stained sections, identifying IENF and DNF. Moderately to heavily stained nerve fibers were seen abundantly near the dermal-epidermal junction (Figure 3A). These epidermal nerve fibers travelled in bundles in the dermis layer, penetrated...
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the dermal-epidermal junction, and terminated at the surface of the epidermis in a perpendicular fashion (Figure 3B). Compared to the control group, significantly reduced IENFD was observed in the DM group at week 4 (30.23±2.38 vs 35.19±2.82 fibers/mm for DM and controls, respectively, P<0.05). IENF loss progressed through week 8 (27.39±2.25 vs 35.23±3.54 fibers/mm, P<0.01 at week 6, and 26.18±4.2 vs 34.84±4.31 fibers/mm, P<0.001 at week 8). At week 8, IENFD in the DM group was 24.9% less than that in the control group (Figure 4).

PGP 9.5 stained nerve bundles were also present in the subepidermal area. DNF densities (DNFL/mm²) were comparable between the two groups at weeks 4 and 6. At week 8, the DM group had a significantly reduced DNF density, compared to the control group (7.27±0.92 vs 8.61±1.15 mm/mm² for DM and controls respectively, P<0.05) (Figure 4), indicating damage and loss of superficial dermal nerve fibers at week 8 in DM rats.

MBP-stained sections identified abundant MBP-positive myelinated fibers in the upper dermis of the footpad. Some ascended into the dermis papillary and innervated the Meissner corpuscles (Figure 3C-E). IMEs densities and integrity levels were comparable between the two groups at week 4 (8.85±1.52 vs 10.45±1.57 fibers/mm, P=0.195 for DM and controls, respectively). However, IME densities in DM rats decreased progressively over the next several weeks. At week 8, significantly reduced IMEs densities were observed in the DM group (7.1±1.38 vs 11.51±0.89 fibers/mm, P<0.001 for DM and controls, respectively) (Figure 5).

IENFD reduced prior to DNFL and IMEs density

At week 4, significant reduction of IENFD had already taken place in DM rats and progressed through week 8. In contrast, DM rats and control rats had comparable DNFL and IMEs densities at week 4. IMEs densities in DM rats started to decrease after week 4. At week 8, IMEs densities in DM rats were significantly reduced.

Figure 3. Normal appearance of epidermal and dermal nerve fiber profiles in glabrous skin from hind paw of the rats. (A, B) were PGP-9.5 immuno-stained sections. (A) In low magnification field (×100), moderately to heavily stained nerve fiber bundles were seen abundantly near the dermal-epidermal junctions and run through the subpapillary dermis. (B) In high magnification field (×400), IENF can be seen cross the dermal-epidermal junctions and run towards the skin surface with multiple branches. Arrow heads-nerve bundles in the dermis; Arrows-IENF. (C-E) were MBP immune-stained sections. (C) In low magnification field (×100), MBP stained myelinated nerve fibers can be seen abundantly in the upper dermis of the footpad. (D, E) In high magnification field (×400), myelinated fibers can be seen running in the upper dermis (D), ascending into the dermis papillary and innervated the Meissner corpuscles (E). Arrow heads-dermis myelinated fibers, Arrows-intrapapillary myelinated endings. The bar represents 50 um.
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Figure 4. Changes of IENFD and DNF densities in DM and control groups at different time points. (A-F) PGP9.5-immunostained skin sections of control and diabetic rats 4 weeks (A, B), 6 weeks (C, D), 8 weeks (E, F) after modeling. (G, H) Bar graphs show differences of IENFD (G) and DNFL (H) between DM and control group in 4 weeks, 6 weeks, and 8 weeks after modeling. Data are expressed as means ± standard deviation (SD), \( P \) represents differences between the DM and control groups, \( * P < 0.05, ** P < 0.01, *** P < 0.001 \). The bar represents 50 μm.

Significant DFNL reduction in DM rats also did not occur until week 8. Epidermal unmyelinated fibers were damaged prior to myelinated fibers in the dermis papillary and nerve fiber bundles in the subepidermal area. In week 8, all kinds of cutaneous nerve fibers were reduced. These results suggest that cutaneous nerve fiber loss began with unmyelinated fibers and progressed to generalized nerve fiber denervation in this painful diabetic neuropathy model.

Discussion

The present study examined pain-related behavioral and cutaneous pathological changes in a STZ-induced diabetic rat model during an 8-week study period. Two key results were found: (1) STZ-induced DM rats exhibited early persistent mechanical allodynia and delayed temporary hyperalgesia; and (2) Significant IENF loss preceded changes in IMEs and DNF, indicating that unmyelinated fibers were damaged earlier in diabetes and IENFD might be a potential marker for detection of early diabetic neuropathy.

Behavioral results in mechanical sensitivity profiles were consistent with most previous studies, showing significant mechanical allodynia early in the disease course [17-19]. However, thermal hyperalgesia only appeared for a short period in the current study. Behavioral changes of diabetic rodents are complicated and vary in different rat or mice models. It begins with an acute metabolic phase, featuring the slowing of nerve conduction and hyperalgesia. These are usually reversible. With the progression of the disease course, more severe functional abnormalities develop in concert with onset of progressive structural changes in the nerves [20]. Different pathogenic mechanisms may produce multiple manifestations of neuropathy. The current DM rat model showed definite mechanical allodynia and thermal hyperalgesia in week 4 to week 6, making it more useful to simulate the clinical
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A reduction in epidermal innervations in rat models of diabetic neuropathy [9, 17, 21]. The current study, however, is the first to investigate concurrent changes of myelinated fibers. One study of diabetic mice showed that densities of myelinated fibers were also reduced with the behavior of mechanical sensory loss. Pathological changes could be improved with neurotrophin treatment [22]. The current study used IMEs as a target to evaluate the myelinated Aβ fibers innervating MCs. It was found that this specific kind of fiber was preserved in the early stage of diabetes. It began to show a tendency toward decline in week 6. DNFL reflects damage of nerve bundles, which includes both unmyelinated and myelinated fibers in the subepidermal area [12]. DNFL reduction appeared even later in week 8. Data suggests an early apparent degeneration of unmyelinated nerve axon in the epidermis. This is consistent with the ‘dying-back phenomenon’ of diabetic neuropathy. The early preservation of myelinated fibers indicated that myelin may have the function of protecting nerve axons against metabolic injuries.

Functional consequences of cutaneous nerve fiber loss are complicated. The manifestation of patients with painful diabetic neuropathy instead of insensate neuropathy.

Present results showed that significant IENF loss preceded changes in IMEs and DNFL, indicating that intraepidermal unmyelinated fiber loss happened before the impairment of myelinated fibers. Previous studies have also found a reduction in epidermal innervations in rat models of diabetic neuropathy [9, 17, 21]. The current study, however, is the first to investigate concurrent changes of myelinated fibers. One study of diabetic mice showed that densities of myelinated fibers were also reduced with the behavior of mechanical sensory loss. Pathological changes could be improved with neurotrophin treatment [22]. The current study used IMEs as a target to evaluate the myelinated Aβ fibers innervating MCs. It was found that this specific kind of fiber was preserved in the early stage of diabetes. It began to show a tendency toward decline in week 6. DNFL reflects damage of nerve bundles, which includes both unmyelinated and myelinated fibers in the subepidermal area [12]. DNFL reduction appeared even later in week 8. Data suggests an early apparent degeneration of unmyelinated nerve axon in the epidermis. This is consistent with the ‘dying-back phenomenon’ of diabetic neuropathy. The early preservation of myelinated fibers indicated that myelin may have the function of protecting nerve axons against metabolic injuries.

Functional consequences of cutaneous nerve fiber loss are complicated. The current study observed an early occurrence of mechanical allodynia in diabetic neuropathy, in accord with previous reports. These studies placed the onset of mechanical allodynia at 1 week after STZ-injections and fully-developed allodynia by 2-8 weeks [17-19]. Some studies have suggested that mechanical allodynia could possibly result from a direct effect of hyper-
glycemia on the peripheral nervous system, rather than from a structural deficit [18]. In the current study, mechanical allodynia progressed through week 5, then plateaued until the end of the 8-week study. Thermal hyperalgesia appeared in week 4 and week 5. Pathological examinations also showed IENF reduction in week 4, indicating that IENF loss might be relevant to the maintenance of mechanical allodynia and generation of thermal hyperalgesia. However, the progression of IENF loss and IMEs loss from week 5 to week 8 was not paralleled by the behavioral manifestation. With the progressive loss of cutaneous nerve fibers, mechanical allodynia plateaued and thermal hyperalgesia behavior even disappeared. This indicates a dissociation between fiber loss and behavioral manifestation. Previous studies have also reported an onset of behavioral deficits prior to quantifiable intraepidermal fiber loss [9, 23]. In addition, a consensus has not yet be reached concerning whether IENFD is different between patients with painful and painless diabetic neuropathy and whether IENF loss could possibly increase the risk of neuropathic pain [3, 24]. This dissociation indicates that structure changes may not be functionally relevant. In addition to overt fiber loss, it is likely that metabolic damage leading to dysfunction of intact fibers may contribute to pain behavior. This dysfunction could include electrophysiological or neurochemical changes [21, 25]. Future studies should investigate the expression of functional proteins related to pain on the nerve terminals, such as voltage-gated sodium channels or TRPs.

The current study was limited by the short observational duration. Additionally, the first measured time point for skin pathological examinations was set at week 4 post-STZ injections, in accord with most previous reports. Therefore, whether IENFD changes happen before week 4 deserves further investigation. More behavioral phenotype and skin pathological studies concerning structural and pain-related functional proteins are warranted in both DPN patients and animal models.

Conclusion

In present study, STZ-induced diabetic rats developed persistent mechanical allodynia throughout the 8-week test period. However, thermal hyperalgesia developed much later and had a shorter duration. Free intraepidermal nerve fibers were significantly reduced in week 4, while dermal nerve fibers and intrapapillary myelinated fibers were damaged later in week 8. Significant IENF loss preceded changes in IMEs and DNF, suggesting that IENFD quantification might be a potential marker for detection of early diabetic neuropathy.

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Disclosure of conflict of interest

None.

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