Better islet function and cardiovascular autonomic function in type 2 diabetic patients with pure small fibre neuropathy than with mixed neuropathy

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Abstract

Background

The clinical characteristics and outcomes of small fibre neuropathy (SFN) in Chinese patients with type 2 diabetes have not been thoroughly described, and we investigated metabolic and neurological indexes and the prognosis of type 2 diabetic patients based on skin biopsy.

Methods

Thirty-four healthy Chinese volunteers were recruited for skin biopsy to establish the reference range of intraepidermal nerve fibre density (IENFD). Eighty-nine patients with type 2 diabetes from the Department of Endocrinology at Nanjing Drum Tower Hospital between December 2015 and April 2020 were included in the final study. Metabolic and neurological indexes were evaluated at baseline. Diabetic cardiovascular autonomic function was tested through cardiovascular autonomic reflex tests (CARTs). Seventeen pure SFN subjects and 9 mixed diabetic polyneuropathy (DPN) subjects were reassessed after the follow-up.

Results

Levels of HbA1c and postprandial blood glucose were lower (P=0.005 and P=0.041, respectively), while postprandial C-peptide and insulin were higher (P=0.001 and P=0.019, respectively) in the pure SFN group than in the mixed DPN group. Regarding the CARTs, the mixed DPN group obtained the highest score, indicating the worst cardiovascular autonomic neuropathy (CAN). Among the four CART items, postural BP change was lower while deep breathing max-min was higher in the pure SFN group than in the mixed DPN group (P=0.023 and P=0.040, respectively). A partial correlation
showed that there was a negative correlation between IENFD of the distal leg and CART scores (r=−0.513, P=0.001) after adjusting for age and duration of diabetes. Only vitamin B12 (p=0.028) and motor nerve conduction velocity (MCV) of the common peroneal nerve (p=0.045) were increased in the 17 patients with pure SFN after the follow-up. However, MCVs of the common peroneal nerve (p=0.025) and tibial nerve (p=0.047) were decreased at the final visit in the mixed DPN group.

**Conclusions**

Better islet function and cardiovascular autonomic function were observed in patients with pure SFN compared with mixed DPN. CART scores were negatively correlated with IENFD in the distal leg even after adjusting for age and duration of diabetes. The metabolic and neurological indexes remained relatively stable in the follow-up of pure SFN subjects.

**KEYWORDS**

Small fibre neuropathy, diabetic polyneuropathy, cardiovascular autonomic neuropathy, type 2 diabetes mellitus, skin biopsy

**Background**

A recent epidemiological survey indicated that approximately 1.56 billion people suffer from diabetes in mainland China[1]. The prevalence of diabetic polyneuropathy (DPN) is approximately 30% among those with diabetes, and it increases the risk of foot ulcers and amputation[2]. Small fibre neuropathy (SFN) is a type of DPN with a diameter below 7 μm, and it usually impairs unmyelinated C-fibres and thinly myelinated small Aδ-fibres[3]. The main effects associated with SFN are autonomic neuropathy and paraesthesia related to pain, numbness, coldness, and burning sensation, while the nerve conduction velocity (NCV) is usually normal. Skin biopsy is an effective method to diagnose SFN with high specificity and sensitivity. Although skin biopsy is considered the “gold standard” for SFN, it has not been widely used at the Department of Endocrinology in China due to the invasive nature of the procedure. Moreover, we found that SFN was also common in diabetic patients with large nerve fibre neuropathy manifested as abnormal skin biopsy results and NCV. In this study, diabetic patients receiving skin biopsies were enrolled to analyse the metabolic and neurological characteristics, including cardiovascular autonomic function, among different groups. Additionally, we explored correlated factors that may have an impact on intraepidermal nerve fibre density (IENFD) for the early recognition of patients with diabetic neuropathy. In addition, the follow-up of patients with pure SFN and mixed DPN was conducted to understand their prognosis.
Methods

Subject recruitment

A total of 103 diabetic patients complaining of symptoms related to SFN, aged between 20 and 80 years, were enrolled from the Department of Endocrinology at Nanjing Drum Tower Hospital between December 2015 and April 2020. Finally, 89 patients with type 2 diabetes were included in this study. Diabetes was diagnosed according to the WHO criteria[4]. Pure SFN was defined as abnormal IENFD and normal NCV. Mixed DPN was defined as abnormal IENFD and NCV at the same time. Exclusion criteria included patients with type 1 diabetes, peripheral vascular disease, foot ulcer, history of stroke, thyroid disease, vitamin B12 deficiency, and other causes of peripheral neuropathy. (Figure 1)

Neuropathy Symptom Score (NSS) and Numerical Pain Rating Scale (NPRS)

All the participants received the NSS and the NPRS. For the NSS, patients were asked about symptoms in the lower limbs such as burning, numbness or tingling, when the symptoms usually happened and ways to alleviate the discomfort. The maximum score was 9. A symptom score of 3-4 was defined as mild symptoms; 5-6, moderate symptoms; and 7-9, severe symptoms[5]. An 11-point NPRS was used to evaluate pain intensity in patients with DPN, where 0=no pain and 10=worst possible pain[6].

Nerve conduction velocity (NCV) test

The NCV test was performed by a specialized technician at a room temperature of 26°C, and NCV was considered abnormal if more than two nerve conduction velocities were less than the reference value. A patient with abnormal NCV and a symptom or sign of neuropathy was defined as having confirmed diabetic sensorimotor polyneuropathy (DSPN)[7].

Cardiovascular autonomic reflex tests (CARTs)

CARTs are used for the assessment of cardiovascular autonomic neuropathy (CAN), including blood pressure variability in response to standing up (postural BP change), heart rate variability (HRV) during deep breathing (HRV_deep breathing), HRV during the Valsalva manoeuvre (Valsalva ratio) and HRV during the lying-to-standing test (30:15 test). For each test, the grade was as follows: normal (score = 0), borderline (score = 0.5), or abnormal (score = 1). The patients with a total score ≥ 2 were defined as patients with CAN.
**SUDOSCAN test**

SUDOSCAN (Impeto Medical, Paris, France) is a non-invasive mobile device to measure autonomic neuropathy in relation to sudomotor function. The patients placed palms and feet on detecting stainless steel electrodes, and a low direct voltage was applied. Quantitative results were measured as hand and foot electrochemical skin conductance levels (HESC and FESC, μS). The severity of sudomotor dysfunction was classified as follows: none (ESC>60), moderate (40≤ESC≤60), or severe (ESC<40).

**Skin biopsy**

After local anaesthesia with lidocaine, skin biopsy was performed at the proximal thigh (10 cm above the lateral malleolus) and distal thigh (20 cm above the greater trochanter) by using a 4-mm disposable punch. Then, the incisions were packed with gauze. The skin usually began to scab in two or three days. Aseptic techniques and timely treatment were required during the skin biopsy.

**Frozen section preparation**

Skin punch biopsies were dehydrated in 30% sucrose solution before fixation with 4% paraformaldehyde for 24 h. Then, biopsy specimens were embedded in OCT compound (SAKURA Tissue-Tek® O.C.T. Compound) and frozen at -20°C. Tissue blocks were cut into 50-μm slices on a frozen section machine, and each microslide contained 3 discontinuous slices.

**Immunofluorescence and epidermal nerve counting**

Microslides covered with skin biopsy slices were left at room temperature (RT) for 30 min and then rinsed in PBS for 10 min three times. PBS was used for rinsing. Slices were permeabilized for 4 h with 0.3% Triton-X100 and incubated with a rabbit monoclonal antibody against PGP9.5 (1:1000; Abcam (ab108986) overnight at 4°C). PBST (PBS containing 0.1% Tween 20) was used for rinsing, followed by Cy3-conjugated goat anti-rabbit antibody (1:200; Servicebio, 2 h at RT). Nuclei were stained with DAPI solution for 10 min at RT in the dark (50 μl for each sample; Servicebio). An antifade solution was applied to the slices by the coverslip and sealed with clear nail polish.

Images were acquired using laser scanning confocal microscopy (TCS-SP8, Leica, Germany) and overlaid by LAS X software (Leica, Germany). Each slice was scanned
through the Z-axis every 5 μm. The quantification of IENFD was performed according to European Federation of Neurological Societies (EFNS) guidelines, and nerves crossing the basement membrane of the epidermis were regarded as effective counts[8]. IENFD = the number of skin nerve fibre counts / the length of the corresponding section (mm).

**Statistical analysis**

Data are presented as the mean ± standard deviation (SD) or mean (range). All analyses were performed using SPSS 22.0 (Chicago, IL). For continuous variables, Student’s t-test and one-way ANOVA were used for normally distributed variables, while the Wilcoxon rank-sum test and Kruskal-Wails H test were used for nonnormally distributed variables. The chi-square test or Fisher’s exact test was used for dichotomous variables. ImageJ software 1.50 (National Institutes of Health, Bethesda, Maryland, USA) was used to count the length of the epidermis. P-values ≤0.05 were considered statistically significant.

**Results**

**Establishment of IENFD reference range**

Thirty-four healthy Chinese volunteers, including 18 men and 16 women, were recruited for this study. The mean age of the subjects was 51.7±14.5 years, and the mean HbA1c was 5.4±0.4%. The mean IENFD at the proximal thigh was 20.7±6.0/mm (mean ± SD; lower 5th percentile: 10.0/mm). The mean IENFD at the distal thigh was 11.3±3.5/mm (mean ± SD; lower 5th percentile: 8.1/mm).

**Comparison of normal IENFD between different sexes and ages**

There were no differences in the distal thigh and proximal thigh measures between males and females. Moreover, the subjects were sorted into groups based on their age: 20~50 years and 51~80 years. No significant differences were seen between the two groups (Tables 1 and 2).

**Clinical characteristics of subjects participating in the skin biopsy**

Figure 2 shows representative PGP9.5 immunofluorescence in human skin biopsy. IENFD was significantly lower in a diabetic patient with SFN (Figure 2a) than in a
healthy subject (Figure 2b).

Patients with type 2 diabetes were classified into the non-DPN, pure SFN and mixed DPN groups according to the established IENFD reference range and NCV test (Table 3). There were no differences in age, sex, body mass index (BMI), or the duration of diabetes among the three groups. The percentages of family history of diabetes, smoking, and alcohol intake were also similar. Blood pressure and resting heart rate were higher in the mixed DPN group than in the non-DPN group (all P ≤0.05).

Additionally, the levels of HbA1c and postprandial blood glucose were lower (P=0.005 and P=0.041, respectively), while postprandial C-peptide and insulin were higher (P=0.001 and P=0.019 for each) in the pure SFN group than in the mixed DPN group. Regarding microvascular complications, the albumin-to-creatinine ratio (ACR) was higher in the mixed DPN group than in the non-DPN group (P=0.016). The value of 25(OH)-VitD was lower in the mixed SFN group than in the non-DPN group (P=0.014). No differences were seen in the lipid profiles, or eGFR, folate, or vitamin B12 levels among the three groups (Table 4).

Neuropathy examinations including IENFD, NSS, NPRS, CART scores, SUDOSCAN and NCV tests were performed on the subjects with type 2 diabetes. The IENFD of the proximal and distal leg was significantly lower in the pure SFN group and the mixed DPN group than in the non-DPN group (P=0.004 and P=0.000, respectively), but there was no significant difference between the pure SFN group and the mixed DPN group. Motor nerve conduction velocity (MCV) was the lowest in the mixed DPN group, while NSS, NPRS and average hands and feet ESC in SUDOSCAN did not show significant differences among the three groups.

For the CARTs, the mixed DPN group obtained the highest score, indicating the worst CAN. Among the four items of the CARTs, postural BP change was lower, while deep breathing max-min was higher in the pure SFN group than in the mixed DPN group (P=0.023 and P=0.040, respectively). The percentage of CAN was significantly higher in the mixed DPN group than in the non-DPN group and the pure SFN group (P=0.003) (Table 5).

Follow-up of pure SFN and mixed DPN subjects

To understand the outcome of pure SFN and mixed DPN subjects, follow-up was conducted after the collected baseline, and the follow-up ended on October 28th, 2020. Seventeen pure SFN patients were included with an average age of 55.0±7.5 years, diabetes duration of 9.0±5.7 years, BMI of 25.0±2.9 kg/m², and a median follow-up of 15 (12, 20) months. Nine mixed DPN patients were included with an average age of 62.9±11.1 years, duration of 15.3±9.5 years, BMI of 24.4±3.1 kg/m², and a median follow-up of 12 (8.5, 16.5) months. Biochemical and neurological indexes were
assessed during the follow-up.

For both groups, no changes were seen in glucose and lipid profiles, ACR and eGFR at the final visit. Among micronutrients, only vitamin B12 was increased at the final visit in the pure SFN group (p=0.028). Regarding neurological indexes, the NSS, NPRS, CARTs, SUDOSCAN and NCV tests were conducted during the follow-up. The MCVs of the common peroneal nerve (p=0.025) and tibial nerve (p=0.047) were decreased in the mixed DPN group. However, the MCV of the common peroneal nerve showed an upward trend in the pure SFN group (p=0.045) (Table 7).

**Discussion**

**Application of skin biopsy in the diagnosis of diabetic small fibre neuropathy**

In the present study, we reported an overall evaluation of the metabolic indexes in Chinese type 2 diabetic patients with SFN based on skin biopsy. In addition, neurological tests, including cardiovascular autonomic function, were assessed in these subjects.

IENFD is an important parameter for evaluating the severity of SFN by calculating the number of skin nerves per unit length[9]. However, its reference range varies based on race, districts, and staining methods[10]. First, the reference range of IENFD in our district was established by enrolling healthy subjects receiving skin biopsy. The lower 5th percentile of IENFD at the proximal thigh was 10.0/mm, and for the distal thigh, it was 8.1/mm. Kennedy et al[11, 12] reported that the lower 5th percentile of IENFD at the distal leg was 20.0/mm in subjects aged 20–59 years, while the number was 11.8/mm in subjects over 60 years. However, conversely, McArthur et al[13] found no sex or age effect in the IENFD of healthy subjects. There was no significant difference in IENFD between 20~50 years and 51~80 years in our study, so we used a unified cut-off value to diagnose SFN.

Although the IENFD in patients in the pure SFN group was significantly lower than that in the non-DPN group, there was no significant difference between the pure SFN group and the mixed DPN group, which indicated that SFN may have a unique pathophysiology rather than simply being an “early stage” of polyneuropathy.

**Clinical factors related to pure SFN and mixed DPN**

The pure SFN group manifested better islet function, including lower HbA1c and PBG accompanied by higher postprandial insulin and C-peptide compared to the mixed DPN group. However, the eGFR and ACR showed no differences between the two
groups, indicating that a longer follow-up period was needed to assess diabetic microangiopathy.

We noticed that the level of vitamin D was lower in the mixed DPN group than in the non-DPN group, which was in line with several previous studies[14-16]. Possible mechanisms include vitamin D protecting Schwann cells against advanced glycation end product (AGE)-induced apoptosis[17] and promoting proangiogenic molecules[18]. Further studies are needed to focus on vitamin D in the protection against diabetic neuropathy and the related molecular mechanisms.

**Association of CARTs and diabetic small fibre neuropathy**

Several studies have paid more attention to diabetic cardiovascular autonomic neuropathy (DCAN) in recent years. As an important type of autonomic neuropathy, DCAN can increase the mortality associated with cardiovascular diseases[19-21]. However, it usually has an insidious onset and is easily neglected by clinicians. CARTs are the gold standard for screening for DCAN[22]. Heart rate variations, including deep breathing, the Valsalva manoeuvre, and lying-to-standing, are used to test parasympathetic function. Orthostatic hypotension indicates impairment in sympathetic function[23].

In this study, the item of deep breathing max-min, a marker of the excitability of the parasympathetic nervous system (PNS), was lower in the pure SFN group than in the non-DPN group, although their total CART scores showed no difference. Additionally, partial correlation showed a negative correlation between IENFD of the distal leg and CART scores even after adjusting for age and duration of diabetes. In general, CARTs are vital to early screening for DCAN in diabetic patients considering long-term severe complications.

**Follow-up and outcome of pure SFN and mixed DPN subjects**

After the baseline study of SFN confirmed by skin biopsy, we conducted a follow-up of patients with pure SFN and mixed DPN. No changes were seen in islet function or lipid and kidney profiles between the two groups. Vitamin B12 and MCV of the common peroneal nerve were increased at the final visit in the pure SFN group. Conversely, the MCVs of the common peroneal nerve and tibial nerve were decreased in the mixed DPN group.

In a previous study, MacDonald S et al[24] reported a retrospective analysis of 101 patients with biopsy-confirmed SFN, and the average follow-up was 6.2 years. They found that small fibre neuropathy tended to be stable and rarely affected ambulation and employment status. However, electrophysiological tests were not assessed in
MacDonald’s study, and the aetiology of SFN was diverse, including hyperglycaemia, immune dysfunction, vitamin B12 deficiency, and so on. Løseth S et al[25] evaluated the progression of 35 type 1 and 24 type 2 diabetic neuropathy patients, and there was minimal progression of large fibre neuropathy in both groups.

In our study, the MCV of common peroneal nerve increased in the pure SFN group. The reason for the improvement of large fibre neuropathy in pure SFN patients is still unknown, and it may partly due to the individual pathophysiological mechanism of SFN. Although HbA1c and fasting C-peptide ameliorated in the mixed DPN group, the MCV of lower limbs deteriorated at the final visit. The phenomenon was also consistent with the previous studies related to intensive blood glucose control and neurological complications, which meant tighter blood glucose control did not bring to lighter neuropathy [26-28]. Therefore, more attention should be paid to the early recognition of SFN in diabetic patients with a high risk of large fibre neuropathy.

Regarding vitamin B12, the differences between the two groups may be attributed to the usage of nutritional supplements in some patients. Additionally, we noticed that age at the initial visit was higher in the mixed DPN group, which meant that the large fibre nerve was probably vulnerable because of an age-related factor. Thus, a longer follow-up time is needed in future studies related to SFN.

Limitations and strengths of this study

The study has several limitations that should be addressed. First, the number of healthy subjects was relatively small, and due to heterogeneity in IENFD, the diagnosis of small fibre neuropathy could only be defined by the reference range determined from the local district. Second, the subjects were recruited from the inpatient department, which may not represent the overall population of patients with small fibre neuropathy. Third, the follow-up was relatively short to assess the features of patients with pure SFN and mixed DPN.

In summary, the strength of the study was the initial and thorough assessment of SFN based on skin biopsy in a relatively large population of Chinese diabetic patients. Additionally, we found a negative correlation between CART scores and IENFD in the distal leg even after adjusting for the age and duration of diabetes. Finally, metabolic and neurological indexes remained stable in the pure SFN subjects with type 2 diabetes during the follow-up.

Conclusion

In this study, we reported the clinical characteristics of Chinese diabetic patients with
small fibre neuropathy based on skin biopsy. Better islet function and cardiovascular autonomic function were observed in those with pure SFN compared with mixed DPN. CART scores were negatively correlated with IENFD in the distal leg even after adjusting for age and duration of diabetes. It seems that the metabolic and neurological indexes were relatively stable in the follow-up of pure SFN subjects. Additionally, it is necessary to attach importance to early screening of CAN in diabetic patients.

**Additional files**

**Abbreviations**

ACR: albumin-to-creatinine ratio; AGES: advanced glycation end products; BMI: body mass index; BP: blood pressure; CAN: cardiovascular autonomic neuropathy; CARTs: cardiovascular autonomic reflex tests; CRP: C-reactive protein; DBP: diastolic blood pressure; eGFR: estimated glomerular filtration rate; FESC: feet electrochemical skin conductance; FPG: fasting plasma glucose; HDL-C: high-density lipoprotein cholesterol; HESC: hands electrochemical skin conductance; HOMA-IR: homeostasis model assessment of insulin resistance; MCV: motor nerve conduction velocity; NCV: nerve conduction velocity; NPRS: Numerical Pain Rating Scale; HRV: heart rate variability; NSS: Neuropathy Symptom Score; IENFD: intraepidermal nerve fibre density; LDL-C: low-density lipoprotein cholesterol; PBG: 2-hour postprandial blood glucose; PCP: 2-hour postprandial C-peptide; PINS: 2-hour postprandial insulin; RHR: resting heart rate; SBP: systolic blood pressure; TC: total cholesterol; TG: triglycerides.

**Authors’ contributions**

D.Z and Y.B. conceived and designed the study. C.L., W.N, C.C., and J.J. conducted the skin biopsy. C.L. performed all data analysis and drafted the manuscript. W.C. performed the NCV test and W.W. contributed to the revision of the manuscript. All the other authors critically reviewed and approved the final manuscript.

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Acknowledgements

Special thanks to Hongyu Huang, Quan Zhang, and Lu Zhou for photographing stained slices and Dr. Lan Wang for the theoretical guidance in neurology. Donghui Yang participated in patient-related instrumental examinations.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analysed in the current study are available from the corresponding authors upon reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The study (NCT04071535) was approved by the Ethics Committee of Drum Tower Hospital Affiliated to Nanjing University Medical School and was performed according to the Declaration of Helsinki.

Funding

This work was supported by National Natural Science Foundation of China Grant Awards [81570737, 81570736] and the Key Research and Development Program of Jiangsu Province of China [BE2015604].

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