Corneal Confocal Microscopy Detects Early Nerve Regeneration After Pancreas Transplantation in Patients with Type 1 Diabetes.

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OBJECTIVE - Corneal confocal microscopy is a rapid, non invasive clinical examination technique which quantifies small nerve fibre pathology. We have employed it to assess the neurological benefits of pancreas transplantation in Type 1 diabetic patients.

RESEARCH DESIGN AND METHODS - 20 patients with Type 1 diabetes undergoing simultaneous pancreas and kidney transplantation (SPK) and 15 control subjects underwent assessment of corneal sensitivity and small nerve fibre morphology using corneal confocal microscopy.

RESULTS - Corneal sensitivity (1.54 ± 0.28 v 0.77 ± 0.02, P<0.0001), nerve fibre density (NFD) (13.8 ± 2.1 v 42 ± 3.2, P<0.0001), nerve branch density (NBD) (4.04 ± 1.5 v 26.7 ± 2.5, P<0.0001) and nerve fibre length (NFL) (2.23 ± 0.2 v 9.69 ± 0.7, P<0.0001) were significantly reduced and nerve fibre tortuosity (NFT) (15.7 ± 1.02 v 19.56 ± 1.34, P=0.04) was increased in diabetic patients prior to pancreas transplantation. Six months after SPK, 15 patients underwent a second assessment and showed a significant improvement in NFD (18.04±1.48 v 9.25 ±1.87, P= 0.001) and NFL (3.60 ± 0.33 v 1.84 ± 0.33 , P=0.002) with no change in NBD (1.38 ± 0.74 v 1.38 ± 1.00, P=1.0 ), NFT (15.58 ± 1.20 v 16.30 ± 1.19, P=0.67) or corneal sensitivity (1.23 ±0.39 v 1.54±0.42, P=0.59).

CONCLUSIONS - Despite marked nerve fibre damage in Type 1 diabetic patients undergoing pancreas transplantation, small fibre repair can be detected within 6 months of pancreas transplantation using corneal confocal microscopy. Corneal confocal microscopy is a novel non-invasive clinical technique to assess the benefits of therapeutic intervention in human diabetic neuropathy.

Abbreviations: CCM: Corneal Confocal Microscopy; SPK: Simultaneous Pancreas and Kidney Transplantation; NCCA: Non Contact Corneal aesthesiometer, NFD: Nerve fibre density; NBD: Nerve branch density; NFL: Nerve fibre length; NFT: Nerve fibre tortuosity
Introduction

Somatic polyneuropathy is one of the commonest long-term complications of diabetes and is the main initiating factor for foot ulceration and lower extremity amputation (1, 2). As 80% of amputations are preceded by foot ulceration, an effective means of detecting and treating peripheral neuropathy would have a major medical, social and economic impact. With the exception of optimal glycemic control, there are currently no licensed treatments that prevent, slow or arrest the development of neuropathy (1). The development of new treatments is of paramount importance, but they are hampered by a lack of clinically relevant surrogate endpoints favoured by regulatory authorities (1). We have relied on tests which quantify predominantly large nerve fibre dysfunction which were principally developed to aid diagnosis and not to assess nerve repair and hence a therapeutic response (3). Thus nerve conduction studies are useful but only detect an abnormality in large myelinated nerve fibers, whilst thermal and pain thresholds assess thinly myelinated (Aδ) and unmyelinated (C) fiber function. Heart rate variability during respiratory stimuli indicates parasympathetic vagal efferent function and blood pressure change during orthostatic manipulation evaluates sympathetic vasomotor efferents.

Although, these tests correlate with axonal loss (4, 7), there are major shortcomings when they are employed to define therapeutic efficacy in clinical intervention trials (8). These tests do not target the specific fibre types which may benefit from the therapeutic intervention and demonstrate a limited ability to detect regeneration and repair. Only sural nerve biopsy with electron microscopy (9,10) and the assessment of intraepidermal nerve fibre density using skin-punch biopsies (11, 12) directly assess nerve damage and repair, however, both are invasive procedures.

No available therapy (including glycemic control) has previously been shown to result in an improvement in diabetic neuropathy. Even in the most dramatic example of “curing” type 1 diabetes with pancreas transplantation, in 115 patients followed over 10 years, neurological function, nerve conduction studies, and autonomic function were only prevented from worsening and failed to show an improvement (13). This is in keeping with the lack of improvement in heart rate variability, 43 months after SPK (14). Neuropathy is of course extremely severe at this stage, as evidenced recently by the demonstration of severe intra-epidermal nerve fiber depletion in pancreas transplant recipients, suggesting that long-term follow-up is necessary to assess post-transplant nerve fiber regeneration (15).

Our previous work suggested that small nerve fibres are the earliest to undergo damage and retain the ability to repair longer than large fibers (9,10). More recently we have employed corneal confocal microscopy to demonstrate that corneal nerve fibre damage is directly related to the severity of somatic neuropathy (16,17) and to intraepidermal nerve fibre pathology (18). These data led us to propose that CCM, a non-invasive and reiterative test might be an ideal surrogate endpoint for evaluating therapeutic efficacy in clinical trials of human diabetic neuropathy (19). To test this hypothesis we have assessed corneal sensitivity and employed corneal confocal microscopy, to evaluate corneal nerve fibre morphology, at
baseline and 6 months after SPK in patients with Type 1 diabetes.

Research Methods and Design

Study groups
20 Type 1 diabetic patients undergoing SPK and 15 non-diabetic healthy control subjects were studied at baseline and 15 diabetic patients underwent repeat assessment 6 months after SPK transplantation.

Corneal Sensitivity
The non-contact corneal aesthesiometer (NCCA) uses a puff of air expressed through a bore of 0.5mm diameter with an electronic pressure sensor which displays the force exerted in millibars (mbars). The stimulus jet is mounted on a slit lamp positioned 1 cm from the eye and aligned to the centre of the cornea. Corneal sensitivity was assessed using our established methodology (20).

Confocal Microscopy
Patients underwent examination with a Tomey Confoscan corneal confocal microscope model P4 (Erlangen, Germany). One eye of each subject was selected at random and anaesthetized with one drop of benoxinate hydrochloride 0.4% (oxybuprocaine hydrochloride, Minims). The objective lens of the confocal microscope was disinfected (isopropyl alcohol 70% v/v, Swabs) and a drop of Viscotears liquid gel (carbomer 940, Ciba Vision Ophthalmics) was applied onto the tip of the lens and advanced forwards until the gel touched the cornea, allowing optical but not physical contact between the objective lens and corneal epithelium. The entire cornea was scanned in approximately 2 minutes and en face two dimensional images (lateral resolution approximately 1 to 2 µm and final image size of 768 pixels x 576 pixels) were acquired. Three to five high quality images of Bowman’s layer were examined as it contains the main nerve plexus. The investigator who examined the cornea and who undertook morphometric measurements of the images was masked with respect to the identity of the patient. The following parameters were quantified to define corneal nerve fibre damage and repair: (i) Nerve fibre density (NFD) the total number of major nerves per mm$^2$ of corneal tissue; (ii) Nerve fibre length (NFL) the total length of all nerve fibres and branches per mm$^2$ of corneal tissue; (iii) Nerve branch density (NBD) the number of branches emanating from each nerve trunk per mm$^2$ of corneal tissue (16); (iv) Nerve fibre tortuosity (NFT) a parameter mathematically derived from the images (17). Measures (i) and (iii) were determined using morphometric software incorporated within the Tomey instrument, measure (ii) was determined using third party image analysis software (Scion Image for Windows, Scion Corporation, Frederick, Ma., USA) and measure (iv) was calculated using a MATLAB function (MATLAB, Mathworks, USA, version 6.5) that was created for this purpose (17). Corneal nerve morphology was quantified fully in each patient in approximately 30 minutes. To estimate the error in measuring NFD, NFL and NBD we acquired images and determined each of these parameters on 15 subjects on two occasions separated by at least 48 h. The coefficient of variation of these parameters was: 12% for NFD, 9% for NFL and 24% for NBD.

Statistics
SPSS 11.05.0 for Windows was used to compute the results. The data are expressed as Mean ± SEM and the analysis includes descriptive and frequency statistics. One-way analysis of variance (ANOVA) with Scheffe Post-hoc tests was used to study differences between means. A P value of
Results
Baseline evaluation
20 Type 1 diabetic patients aged (41 ± 1 yrs), with diabetes duration (27 ± 2 yrs) and HbA1c (%) (8.9 ± 1.4) undergoing SPK and 15 age matched (46 ± 3 yrs) healthy control subjects were examined. Corneal sensitivity was significantly reduced in diabetic patients (1.54 ± 0.28 mbar) compared to control subjects (0.77 ± 0.02 mbar), P<0.0001. Corneal nerve fibre density (NFD) (13.8 ± 2.1 v 42 ± 3.2, P<0.0001), nerve branch density (NBD) (4.04 ± 1.5 v 26.7 ± 2.5, P<0.0001) and nerve fibre length (NFL) (2.23 ± 0.2 v 9.69 ± 0.7, P<0.0001) were significantly reduced and nerve fibre tortuosity (NFT) (15.7 ± 1.02 v 19.56 ± 1.34, P=0.04) was increased in diabetic patients (Fig.1b) undergoing pancreas transplantation compared to control subjects (Fig.1a) (Table 1).

Post Transplantation
15 patients underwent repeat assessment 6 months after SPK (Table 2). The HbA1c (%) fell significantly into the normal range (5.5 ± 0.1 v 8.6 ± 0.4, P=0.007), confirming successful pancreas transplantation. There was a trend for improvement in corneal sensitivity (1.23 ± 0.39 v 1.54 ± 0.42, P=0.59). Corneal NFD (18.04 ±1.48 v 9.25 ±1.87, P= 0.001) (Fig.2a) and NFL (3.60 ± 0.33 v 1.84 ± 0.33 , P=0.002) (Fig.2b) improved significantly. No change was observed for either NBD (1.38 ± 0.74 v 1.38 ± 1.00, P=1.0) or NFT (15.58 ± 1.20 v 16.30 ± 1.19, P=0.67).

Conclusion
The natural history of nerve damage in diabetic patients with Type 1 diabetes is not entirely clear. Longitudinal data from the Rochester cohort supports the contention that the duration and severity of exposure to hyperglycemia are related to the progression and hence severity of neuropathy rather than its onset (21). A recent study of patients with Type 1 diabetes showed a high prevalence of diabetic neuropathy which progressed in a significant proportion of patients and was related not only to glycaemic control but also to conventional cardiovascular risk factors such as hypertension and lipids (22). The replacement of functioning islet beta-cells by pancreas transplantation has been considered to be the most logical treatment for patients with Type 1 diabetes to normalize blood glucose and ameliorate long term complications. Although pancreas transplantation takes approximately 5 years to prevent progression and 10 years to reverse the lesions of diabetic nephropathy (23), a recent study has demonstrated an improvement and/or stabilization of diabetic retinopathy after a median follow up of only 17 months (24). For diabetic neuropathy, the largest and longest follow up series to date has shown that pancreas transplantation improved both motor and sensory nerve conduction as well as sudomotor function in the hand and foot within 1 year, which was maintained throughout follow up for 10 years (13,25). However, autonomic function did not improve (13), as confirmed by another recent study (14). At this stage most patients receiving transplantation will have severe nerve fibre damage as evidenced by marked depletion of intraepidermal nerve fibres (15). Also concomitant uremia which often coexists in these patients may contribute to nerve damage and limit repair, although renal transplantation in patients with diabetic nephropathy has not been shown to halt progression of neuropathy.
(26) and there was no difference in outcomes when comparing pancreas alone with combined pancreas and renal transplantation (13). To determine a therapeutic response to an intervention in diabetic neuropathy a standardized set of end points which include clinical and neurophysiological evaluation combined with quantitative sensory and autonomic function testing have been recommended (1,27,28). Pancreas transplantation has previously been shown to improve large nerve fiber conduction and in particular upper limb motor and sensory action potentials as well as sudomotor function within 1 year (13) but with no impact on autonomic function (13,14). Our studies show no relationship between quantitative sensory tests evaluating small fibre function and unmyelinated fibre pathology (9). Thus, whilst nerve conduction studies and quantitative sensory testing are useful and well validated measures to help diagnose and assess progression of diabetic neuropathy, their utility in evaluating a therapeutic response may be limited (29). Whilst more detailed and reproducible measures which accurately quantify small fibre neuropathy such as skin or nerve biopsy will reduce this variability, they are invasive (9-12). Our recent work using the corneal confocal microscope suggests that this non-invasive and reiterative test might be an ideal surrogate endpoint for use in clinical trials of human diabetic neuropathy and therefore may be appropriate for assessing the benefits of pancreas transplantation (16-18). The cornea is richly innervated by the ophthalmic division of the trigeminal nerve, via the anterior ciliary nerves. Corneal nerves have been studied by detailed light and electron microscopy (30-31) and immunohistology (32-33). Corneal confocal microscopy provides a novel approach to study corneal nerve morphology (34). The main advantage of this technique is that it enables a non-invasive in vivo evaluation of the human cornea at 700x magnification, with excellent resolution and contrast. Initial studies were descriptive and hence limited for the purposes of interpreting small fibre degeneration and regeneration. However, a study from our group in control subjects refined and significantly improved the quantification of C and A-δ corneal nerve fibers (35) showing high concordance with previous histological studies (30). One may argue that corneal innervation has little relevance to diabetic somatic neuropathy, characterized by a distal loss of nerve fibres innervating the lower limbs, and therefore has limited application as a measure of peripheral neuropathy in diabetic patients. However, diabetic patients have a reduction in corneal sensitivity, and a reduction in corneal nerve fibre bundles, which correlates with the Michigan Neuropathy Screening Instrument, a quantitative measure of somatic neuropathy (36). We have also shown a progressive reduction in corneal sensitivity (20) and significant corneal nerve pathology which relates to the severity of neuropathy assessed using neurophysiology, quantitative sensory testing (16,17) and in particular intrepidinal nerve fibre density (18).

In the present study we have demonstrated a highly significant loss of corneal nerve fibres in Type 1 diabetic patients undergoing pancreas transplantation which confirms previous studies demonstrating a severe neuropathy in patients undergoing pancreas transplantation (13-15). However, despite this considerable baseline damage, we have shown a significant improvement in corneal nerve fibre density and length.
within 6 months of transplantation, indicating an early repair process with the restoration of euglycemia. These findings are in contrast to previous studies in diabetic nephropathy (23), retinopathy (24) and particularly neuropathy (13,14,25) where at best a prevention of progression in nerve damage was shown only after several years of euglycemia. However, these latter studies focused heavily on electrophysiology and quantitative sensory assessment which predominantly assessed large fibre function and, to a lesser extent, small fibre function. Where small fibre function was assessed in the form of sudomotor function, it is of relevance that a significant improvement was demonstrated within 1 year of SPK (13).

Our study using corneal confocal microscopy focused on detailed pathology as opposed to function of the small fibres and demonstrated repair despite significant baseline damage. These observations support the view that in clinical intervention trials for diabetic neuropathy, perhaps the focus should be on assessment of small fibre damage and repair (1, 8). Until recently this could only be provided by costly, time consuming and most importantly invasive procedures such as nerve (10) and skin biopsy (11, 12). We now show that corneal confocal microscopy, a non-invasive and hence reiterative test might be an ideal surrogate endpoint for assessing the benefits of pancreas transplantation and indeed for assessing therapeutic efficacy of other therapies in clinical trials of human diabetic neuropathy.

**Acknowledgements**

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References


Table 1. Corneal Confocal nerve fibre morphology in control subjects (n=15) and in Type 1 diabetic patients (n=20) undergoing SPK. (Data presented as Mean ± SEM with significant difference). *Non Contact Corneal Aesthesiometer (NCCA) (mbar); Nerve fibre density (NFD) (number/ mm²); Nerve branch density (NBD) (number/mm²); Nerve fibre length (NFL) (mm/ mm²); Nerve fibre tortuosity (NFT)*

<table>
<thead>
<tr>
<th></th>
<th>Age</th>
<th>NCCA</th>
<th>NFD</th>
<th>NBD</th>
<th>NFL</th>
<th>NFT</th>
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<tr>
<td>Control</td>
<td>46 ± 3</td>
<td>0.77 ± 0.02</td>
<td>42.04 ± 3.2</td>
<td>26.73 ± 2.5</td>
<td>9.69 ± 0.7</td>
<td>19.56 ± 1.34</td>
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<tr>
<td>Pre-SPK</td>
<td>41 ± 1.8</td>
<td>1.54 ± 0.28</td>
<td>13.88 ± 2.1</td>
<td>4.04 ± 1.5</td>
<td>2.23 ± 0.28</td>
<td>15.76 ± 1.02</td>
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P<0.0001  P=0.04

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Table 2. HbA1c and corneal confocal nerve fibre morphology at baseline and 6 months after SPK in 15 patients with Type 1 diabetes. (Data presented as Mean ± SEM with significant difference). Non Contact Corneal Aesthesiometer (NCCA) (mbar); Nerve fibre density (NFD) (number/mm²); Nerve branch density (NBD) (number/mm²); Nerve fibre length (NFL) (mm/mm²); Nerve fibre tortuosity (NFT).

<table>
<thead>
<tr>
<th></th>
<th>HbA1c</th>
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<th>NBD</th>
<th>NFL</th>
<th>NFT</th>
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<tr>
<td>Pre SPK</td>
<td>8.6 ±0.4</td>
<td>1.54 ± 0.42</td>
<td>9.25 ± 1.87</td>
<td>1.38 ± 0.74</td>
<td>1.84 ± 0.33</td>
<td>16.30± 1.19</td>
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<tr>
<td>6 months post SPK</td>
<td>5.5 ±0.1</td>
<td>1.23 ±0.39</td>
<td>18.04± 1.48</td>
<td>1.38± 1.0</td>
<td>3.60± 0.33</td>
<td>15.58± 1.20</td>
</tr>
<tr>
<td>P=0.007</td>
<td>P=0.59</td>
<td>P=0.001</td>
<td>P =1.0</td>
<td>P=0.002</td>
<td>P=0.67</td>
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Figure 1. Image of corneal nerves in Bowman’s layer, showing 6 nerve fibers with a typical beaded appearance, mild tortuosity, and adequate branching in a control subject (a) compared to marked loss of nerve fibres with 1 nerve in a patient undergoing pancreas transplantation (b) and improvement in nerve fibre density with increased numbers of nerves, 6 months after transplantation (c).
Figure 2. Nerve fiber density in diabetic patients pre SPK at baseline compared to age matched non-diabetic control subjects (P<0.0001) with a significant improvement after SPK (P=0.001) (a) and nerve fiber length in diabetic patients pre SPK at baseline compared to age matched non-diabetic control subjects (P<0.0001) with a significant improvement after SPK (P=0.002) (b). (Results expressed as Mean ± SEM).