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ORIGINAL ARTICLES

Healing effect of a two-herb recipe (NF3) on foot ulcers in Chinese patients with diabetes: A randomized double-blind placebo-controlled study*

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Abstract

Background: In the present study, we examined the effect of a two-herb traditional Chinese medicine (NF3), comprised of Astragali Radix and Radix Rehmanniae, on the healing of diabetic foot ulcer and the possible molecular mechanisms involved.
Methods: This was a prospective randomized double-blind placebo-controlled study. Sixteen diabetic patients were randomized to receive either placebo or NF3 for 6 months. Ulcer healing and sensory changes were examined. Molecular studies included measurement of serum tumor necrosis factor (TNF)-α and RNA microarray investigation.
Results: The daily rate of reduction in ulcer area was 3.55% in the NF3 group and 1.52% in the placebo group ($P = 0.062$). In the index limb, the number of negative tests for sensory neuropathy using monofilament was reduced from 27% to 7% in the NF3 group and from 37% to 35% in the placebo group ($P < 0.001$). In addition, NF3 significantly decreased serum TNF-α levels ($P = 0.034$). Microarray studies revealed concerted changes following NF3 treatment in the expression of genes implicated in fibroblast regeneration, angiogenesis, and anti-inflammation.
Conclusions: In this proof-of-concept study, 6-month treatment with NF3 was associated with improved wound healing and sensation accompanied by concerted changes in gene expression.

Keywords: Astragali Radix, connective tissue growth factor, diabetes, diabetic foot ulcer, keratin, Rehmanniae Radix, RNA microarray, traditional Chinese medicine, ulcer healing.

Significant findings of the study: This study provides the first scientific evidence that NF3 significantly improves wound healing and sensation of patients with diabetic foot ulcers.
What this study adds: This study demonstrates an integrated approach from bench to bedside to bench, which can become a prototype for the scientific development of traditional Chinese medicine to address unmet medical needs, such as diabetic foot ulcer.

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Introduction

Diabetes and obesity affect more than 300 million people worldwide, with Asia in the forefront. Type 2 diabetes is characterized by clustering of cardiometabolic risk factors, notably, hyperglycemia, obesity, dyslipidemia (high triglyceride and low high-density lipoprotein–cholesterol), hypertension, microinflammation, and a prothrombotic state,[1] which interact to cause generalized vasculopathy and neuropathy. The abnormal metabolic milieu increases the lifetime risk of a diabetic patient developing a foot ulcer by 12%–25% compared with those without diabetes.[2] Without prompt and proper medical treatment, these infective ulcers will deteriorate, leading to gangrene and limb amputation.[3] The conventional management of diabetic foot ulcer (DFU) includes rest, antibiotic therapy, surgical debridement, revascularization, and good glycemic control. In China, many herbs have long been used to treat diabetes and its complications. Of these, Rehmanniae Radix and Astragali Radix have often been used in combination with other herbs to form complex formulas, such as "blood-house blood stasis-dispelling decoction with additive ingredients" and "pills of six drugs with Rehmanniae".[4,5]

We have reported on the effectiveness of a recipe containing 12 herbs in promoting the healing of severe chronic non-healing DFU with limb preservation in 85% of treated subjects.[6] In vitro and in vivo studies revealed that five of the 12 herbs were effective in promoting fibroblast proliferation and angiogenesis.[7–10] To minimize potential herb–herb interactions and adverse effects from multiple components in traditional Chinese medicine (TCM) recipes, we have modified the complex 12-herb formula to a simple formula (NF3) containing extracts of two herbs, namely Astragali Radix (Huangqi; AR) and Rehmanniae Radix (Shengdi; RR) in a ratio of 2:1 (NF3).[5,11] These two herbs (AR and RR) were the principal components that elicited viability-promoting effects on primary fibroblasts cultured from DFU tissues.[10] This modified herbal formula has also been shown to promote angiogenesis and wound healing in zebrafish and diabetic rats with wound injury,[12,13] respectively. In the present proof-of-concept study, we hypothesized that the novel two-herb TCM (NF3) would expedite healing of early DFU by promoting vascularization and granulation, as well as modulating the inflammatory response.

Methods

The present study was a randomized double-blind placebo-controlled study conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent. The study was approved on 19 February 2009 by the Joint Clinical Research Ethics Committee of the Chinese University of Hong Kong and New Territories East (NTE) Cluster (CRE-2008.581) and has been registered with ClinicalTrials.gov (ID: NCT01389362).

Production of NF3

The raw herbs AR and RR were purchased from herbal shops in mainland China in Shanxi Province and Hebei Province, respectively. They were authenticated by morphological characterizations and thin layer chromatography in accordance with the Chinese Pharmacopoeia.[14] Voucher herbal specimens were deposited in the museum of Institute of Chinese Medicine, The Chinese University of Hong Kong, with voucher specimen numbers 2008–3200 for RR and 2008–3201 for AR. The herbal preparation used in the present study (NF3) consisted of two herbs, RR and AR. The matching placebo consisted of sodium carboxymethyl cellulose, with color and taste identical to that of NF3 manufactured by a TCM manufacturer (National Engineering Research Center for Modernization of TCM, Guangdong, China) accredited with Good Manufacturing Practice standards. The NF3 powder containing extract granules was formulated into sachets and subjects were instructed to take two sachets daily (5 g/sachet). The chemical fingerprinting of NF3 was conducted previously using HPLC analysis.[15] The chemical composition of NF3 includes calycosin-7-*O*-β-D-glucoside, 4H-1-benzopyran-4-one, formononetin-7-*O*-β-D-glucoside, calycosin, formononetin, etc.

Inclusion and exclusion criteria

To be included in the present study, subjects had to: (i) be men or women aged ≥20 years who were attending the Prince of Wales Hospital (PWH) Podiatry Clinic or had been referred from the community clinics in the NTE cluster; (ii) have type 1 or type 2 diabetes with evidence of mild DFU; (iii) be undergoing treatment with stable doses of medications 4 weeks prior to enrolment; (iv) capable of providing written informed consent; and (v) have agreed to use an adequate method of contraception throughout the study period if they were women of child-bearing age (unless they had been surgically sterilized). The exclusion criteria were: (i) HbA1c ≥10%; (ii) advanced diabetic eye disease, defined as a history of laser therapy, retinal detachment, or vitreous hemorrhage; (iii) pregnancy, breast feeding, or an intention to become pregnant during the study period; (iv) known allergy to ingredients contained in NF3; (v) a history of active gastrointestinal bleeding; (vi) taking NF3 8 weeks prior to study enrolment; (vii) severe liver disease (liver

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enzymes 3× upper limit of normal) and/or kidney disease (serum creatinine ≥150 or 200 μmol/L) and/or any other medical condition considered unsuitable for enrolment by the investigators.

Study design

Subjects were randomized into two groups: (i) a treatment group ($n = 8$), in which subjects were given NF3 as a daily regimen of two sachets (5 g/sachet), one in the morning and one in the evening, for 6 months; and (ii) a control group ($n = 8$), in which subjects received placebo, with a similar color and odor to NF3. Medications for diabetes, hypertension, and hyperlipidemia were continued by all subjects and kept constant as much as possible. Concomitant medications were recorded during the study period. A course of antimicrobial treatment was given for microbiologically proven infection. None of the patients received additional neuroprotective or blood thinning medications.

The primary outcomes were: (i) time to complete healing (in days); and (ii) the daily rate of reduction of wound area. The secondary outcomes were changes in serum inflammatory markers, including high sensitive C-reactive protein (CRP), white blood cell count, tumor necrosis factor (TNF)-α, and nerve function. We also examined adverse events (AEs) associated with the use of NF3. Foot ulcers were defined according to the classification of the Infectious Disease Society of America–International Working Group on Diabetic Foot.[11]

Study implementation

All subjects were assessed by a podiatrist for vascular and neurological lesions of the foot and received standard care for DFU. Following initiation of treatment, patients were evaluated monthly for up to 6 months to monitor the rate of ulcer healing and AEs. At baseline and each follow-up visit, ulcer healing was evaluated and recorded by measuring the length and width of the ulcer. Length was defined as the longest edge-to-edge measurement of the ulcer and width was taken from the longest ulcer dimension perpendicular to the length. Other clinical and laboratory measures, including control of glycemia and risk factors, as well as transcutaneous oxygen pressure ($TcPo_2$) were monitored every 4–6 weeks. Pre- and post-treatment serum TNF-α, high sensitivity (hs) hs-CRP, and RNA analysis were examined.

Skin temperature was measured with a digital thermometer (Model-15-077-8B; Fisher Scientific, Pittsburgh, PA, USA), whereas $TcPo_2$ was determined using a transcutaneous oxygen tension detector (MicroGas 7650; Linde Medical Sensors, Basel, Switzerland) at fixed points around the ulcer. The ankle–brachial index (ABI) test was performed by using a blood pressure (BP) cuff and hand-held Doppler device with a vascular probe. The sensitivity of foot nerves was assessed by the 10-g monofilament test by placing the end of the monofilament over 10 predefined sites on the sole of the foot. Subjects were determined to have passed the test (score = 1) if they identified five or more locations (of 10) correctly; they were deemed to have failed (score = 2) if they identified four or fewer locations correctly.

The vibration perception threshold (VPT) was measured by a hand-held biothesiometer. The biothesiometer tactor was applied perpendicularly to the test site with a constant and firm pressure.

RNA microarray

Three men from each of the NF3 and placebo groups were selected for the cDNA microarray study to evaluate differences in mRNA expression. Peripheral blood samples were collected from each of the subjects in PAXgene Blood RNA tubes (Qiagen, Valencia, CA, USA) to stabilize intracellular RNA and inhibit RNA degradation. The RNA was extracted using the PAXgene Blood RNA kit (Qiagen) and assessed by RNA 6000 nanochips (Agilent, Waldbronn, Germany) and an Agilent 2100 Bioanalyzer. The cRNA was synthesized using an Ambion WT Expression Kit (Life Technologies, Paisley, UK). Hybridization was performed using a GeneChip Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA, USA) containing 764 885 distinct probes corresponding to 28 869 well-annotated genes. The microarrays were read with a GeneChip Scanner 3000 (Affymetrix).

The acquisition and initial quantification of array images were conducted using AGCC software (Affymetrix). Subsequent data analyses were performed using Partek Genomics Suite Version 6.4 (Partek, St Louis, MO, USA). We first performed a one-way ANOVA to identify gene expression with between-group difference at non-adjusted $P < 0.05$, and then calculated relative difference in fold change (FC). Genes with differential expression at FC >1.2 and $P < 0.05$ were selected for validation using quantitative polymerase chain reaction (qPCR) bioinformatics and experimental means. Cluster analyses and principal component analysis were conducted with Partek default settings. The DAVID gene functional classification tool (http://david.abcc.ncifcrf.gov, accessed 10 August 2011) was used to condense the list of genes detected in our sample set into functionally related groups. We used the agglomeration method to cluster the three main gene ontology (GO) charts (Biological Process, Molecular Function and Cellular Component) within a meaningful network context. Comprehensive data and literature mining were also per-

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formed using the Genomatix Pathway System (GePS; http://www.genomatix.de/products/GePS/GePS1.html, accessed 10 August 2011) for extracting gene–gene relationships based on information extracted from public proprietary databases. Because the number of genes with FC 1.2 may not be sufficient for network generation, genes with subtle changes FC >1.15 and $P < 0.05$ were also included.

Real-time qPCR analysis

Real-time qPCR confirmation of the selected genes was performed by using an iScript one-step RT-PCR kit with SYBR Green (Bio-Rad, Hercules, CA, USA) on a CFX96 Real-Time PCR Detection System (Bio-Rad) in accordance with the manufacturer's instructions. The RNA from the remaining subjects in the treatment group ($n = 4$) and the placebo group ($n = 3$) was used for qPCR confirmation of the selected genes. The primer list for qPCR is given in Table S1, available as Supplementary Material to this paper. The endogenous control *18S* ribosomal RNA was used for normalization. The fold difference in post- versus pre-treatment is presented. Differences in gene expression levels between the NF3- and placebo-treated groups were compared by Student's *t*-test. Two-tailed $P < 0.05$ was considered significant.

Statistical analysis

All data were analyzed using SPSS for Windows version 13.0 (SPSS Inc., Chicago, IL, USA). All data are expressed as the mean ± SD or as the median with the interquartile range (IQR) in parentheses, as appropriate. Student's *t*-test or one-way ANOVA was used for variables with normal distribution, whereas skewed data were evaluated using a non-parametric test (Mann–Whitney). Chi-squared or Fisher's exact tests were used for categorical variables. Paired tests were used for within-group comparisons. The time to complete wound closure was compared using Kaplan–Meier methods. Cox's proportional hazards regression model was run on the time-to-healing data with or without adjustment for covariates including age, gender, and the type, number, duration, and severity of ulcers. Two-tailed $P < 0.05$ was considered significant.

Results

Recruitment

Between February 2009 and December 2010, 327 patients were reviewed at the podiatry clinic and 62 patients were referred from the general outpatient clinics in the NTE cluster region. Among these patients, 132 had mild foot ulcers and, after excluding patients with renal impairment, heart failure, liver disease, poor glycemic control, or those who refused to participate, 23 underwent comprehensive assessment, including biochemical testing. In all, 16 patients were randomized to two groups, with eight patients in each group.

Over the 6-month study period, one of eight patients (12.5%) in the NF3 group withdrew compared with three of eight patients (37.5%) in the placebo group. The reasons for withdrawal were acute myocardial infarction in the NF3 group ($n = 1$), and poor healing with leg swelling ($n = 1$), increased salivation ($n = 1$), and toe infection ($n = 1$) in the placebo group.

Outcome measures

Both groups had similar clinical characteristics except for a higher body mass index (BMI) in the NF3 group (Table 1). The duration and location of ulcer were comparable between the two groups before treatment (Tables 1,2). At the end of study, the wound areas decreased in six patients after NF3 treatment compared with only one patient in the placebo group. The mean wound area decreased by 47.8% and 14.1% in the NF3 and placebo groups, respectively; the difference did not reach statistical significance. Patients in the NF3 group showed a steady decrease in wound areas compared with marked fluctuations in the placebo group during clinic visits (Fig. 1). The mean healing time was 125 and 137 days in the NF3 and placebo groups, respectively (Table 2). After adjustment for duration of ulcers and using Cox's proportional hazards regression model, the NF3 group tended to have shorter time to healing than the placebo group (Fig. 2). The survival curves for time to healing showed a consistently higher percentage of healed ulcers in the NF3 group than in the placebo group throughout the study period. At the end of the study period, 52.6% (10/19) of ulcers in the NF3 group were healed compared with 48.0% (12/25) of ulcers in the placebo group. For healed ulcers, the speed of healing, expressed as daily rate of change in ulcer size, was 3.55% in the NF3 group, compared with 1.52% in the placebo group ($P = 0.062$). Clinical assessment of feet did not show between-group or within-group differences in $TcPo_2$, ABI, or VPT (Table 3). Using the 10-g monofilament test, more patients in the NF3 group had normal sensation than in the placebo group ($P = 0.052$), as indicated by an increased number of sensitive sites ($P < 0.001$; Table 3). Serum TNF-α levels were lower after NF3 treatment compared with placebo ($P = 0.034$), with no intra- or between-group differences in hs-CRP (Table 1).

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Table 1 Clinical characteristics, efficacy, and safety data for the NF3 and placebo groups

	NF3 (*n* = 8)	Placebo (*n* = 8)	*P*-value
No. men (%)	4 (50%)	4 (50%)	1.000
Age (years)	74.0 ± 12.0	72.1 ± 12.4	0.763
Disease duration (years)	7.3 ± 6.2	11.8 ± 10.9	0.330
Ulcer duration (days)	56 (36–147)	105 (28–360)	0.302
SBP (mmHg)	134.4 ± 19.1	143.5 ± 20.4	0.371
DBP (mmHg)	64.8 ± 5.3	75.6 ± 4.9	0.001
BMI (kg/m^2)	27.2 ± 3.4	24.1 ± 1.0	0.039
Blood analysis			
Urea (mmol/L)			
Pretreatment	8.51 ± 3.47	7.37 ± 2.94	0.542
Post-treatment	7.96 ± 2.91	6.87 ± 2.23	0.921
Within group *P*-value	0.593	0.519	
Creatinine (μmol/L)			
Pretreatment	106.0 ± 48.7	105.0 ± 27.9	0.963
Post-treatment	108.3 ± 35.1	103.1 ± 24.9	0.754
Within group *P*-value	0.780	0.717	
FPG (mmol/L)			
Pretreatment	6.4 ± 1.4	6.7 ± 2.5	0.783
Post-treatment	7.1 ± 1.4	7.2 ± 3.1	0.924
Within group *P*-value	0.022	0.450	
Total protein (g/L)			
Pretreatment	75.4 ± 4.3	73.9 ± 3.9	0.489
Post-treatment	75.8 ± 6.5	72.9 ± 3.9	0.325
Within group *P*-value	0.869	0.251	
Total bilirubin (μmol/L)			
Pretreatment	11.0 ± 3.1	9.3 ± 3.5	0.337
Post-treatment	10.6 ± 3.2	11.1 ± 4.1	0.787
Within group *P*-value	0.740	0.142	
Albumin (g/L)			
Pretreatment	42.1 ± 2.6	40.0 ± 6.1	0.381
Post-treatment	42.6 ± 2.7	39.6 ± 5.5	0.189
Within group *P*-value	0.752	0.702	
ALT (IU/L)			
Pretreatment	23.5 ± 8.2	22.7 ± 12.8	0.888
Post-treatment	25.5 ± 17.0	22.7 ± 7.6	0.696
Within group *P*-value	0.693	1.000	
ALP (IU/L)			
Pretreatment	71.9 ± 30.9	71.5 ± 21.0	0.983
Post-treatment	90.8 ± 94.6	77.9 ± 23.4	0.732
Within group *P*-value	0.463	0.469	
HbA1c (%)			
Pretreatment	6.4 ± 0.7	6.7 ± 1.4	0.572
Post-treatment	6.5 ± 0.7	6.4 ± 1.4	0.918
Within group *P*-value	0.393	0.568	
TNF-α (pg/mL)			
Pretreatment	2.02 ± 0.76	2.11 ± 2.26	0.920
Post-treatment	1.98 ± 1.52	4.23 ± 1.87	0.034
Within group *P*-value	0.956	0.047	
hs-CRP (mg/dL)			
Pretreatment	10.64 ± 20.16	8.62 ± 16.02	0.835
Post-treatment	9.97 ± 16.44	3.87 ± 4.09	0.675
Within group *P*-value	0.808	0.662	

Unless indicated otherwise, data are given as the mean ± SEM or as the median value with the interquartile range in parentheses.
SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; FPG, fasting plasma glucose; ALT, alanine aminotransferase; ALP, alkaline phosphatase; TNF-α, tumor necrosis factor-α; hs-CRP, high-sensitivity C-reactive protein.

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Table 2 Characteristics of the diabetic foot ulcers before and after treatment in the NF3 and placebo groups

	NF3 (n = 8)	Placebo (n = 8)	P-value
Ulcer area (cm^2)			
Before	0.81 (0.16, 4.16)	0.33 (0.22, 1.67)	0.462
After	0 (0, 1.40)	0 (0, 1.21)	0.772
No. ulcers			
Before	19	25	
Plantar	1 (12.5%)	1 (12.5%)	0.615
Toes	3 (38.5%)	1 (12.5%)	
Foot dorsum	1 (12.5%)	2 (25.0%)	
Ankle	2 (25.0%)	1 (12.5%)	
Other	1 (12.5%)	3 (38.5%)	
After	16	20	
Time to healing (days)	125 ± 60.9	137 ± 56.6	0.262*
Healed wounds			
n	10 (52.6%)	12 (48.0%)	0.761
Healing speed/day* (%)	–3.55 ± 3.20	–1.52 ± 1.43	0.062
Unhealed wound			
n	6 (47.4%)	8 (52.0%)	
Healing speed/day* (%)	–0.14 ± 3.30	–1.49 ± 4.25	0.531
All wounds			
n	16	20	
Healing speed/day* (%)	–2.27 ± 3.56	–1.51 ± 2.80	0.476

Data show the median (interquartile range), mean ± SEM, or the number with percentages in parentheses, as appropriate.
Healing speed/day (%) was calculated as: [(post-treatment wound area – pretreatment wound area)/healing time (days)] ×100.
*Adjusted for wound duration.

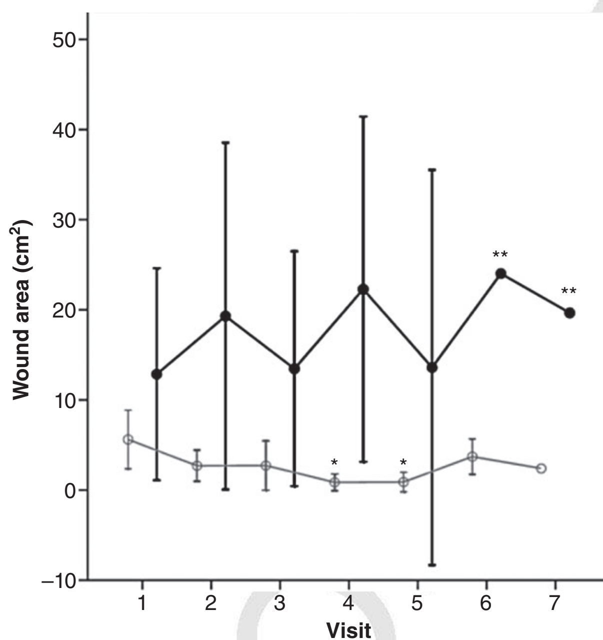

Figure 1 Changes in wound area at each visit (every 4–6 weeks) in diabetic patients with foot ulcer treated with NF3 (○) or placebo (●). The data show a consistent trend for a decrease in wound area (with significant reductions at Visits 4 and 5) after NF3 treatment. No reduction in wound area was observed in the placebo group. Data are expressed as the mean ± SEM or the median ± interquartile range. *P < 0.05, **P < 0.01 compared with corresponding control baseline (repeated-measures ANOVA).

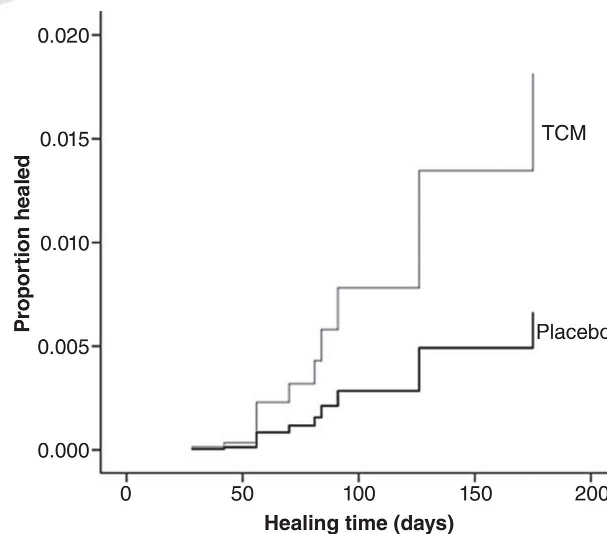

Figure 2 Kaplan–Meier analysis showing the time to healing for ulcers in the placebo and NF3 (TCM) groups, with a tendency for a shorter healing time in the NF3 group (P = 0.262, Cox's proportional hazards regression). Each ulcer was counted as a single point assigned to either the NF3 or placebo group.

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Table 3 Vascular assessment of feet and sensation grading for the NF3 and placebo groups

	NF3 (*n* = 8)	Placebo (*n* = 6)	*P*-value
$TcPO_2$ (kPa)			
Before treatment	54.8 ± 16.0	35.0 ± 13.8	0.025
After treatment	38.4 ± 25.5	43.7 ± 19.9	0.404
% Difference	–17.8%	26.0%	0.071
Within group *P*-value	0.206	0.415	
Limb ABI			
Before treatment	0.78 ± 0.33	0.89 ± 0.22	0.447
After treatment	0.78 ± 0.30	0.85 ± 0.30	0.888
% Difference	4.3%	–2.5%	0.897
Within group *P*-value	0.952	0.734	
ABI grading (*n*)			
Before treatment			0.189
<0.9	5	2	
0.9–1.3	3	5	
>1.3	0	0	
After treatment			0.640
<0.9	5	3	
0.9–1.3	3	3	
>1.3	0	0	
Within group *P*-value	0.614	0.131	
Sensation grading* (*n*)			
Before treatment			0.614
Grade 1	4	3	
Grade 2	4	5	
After treatment			0.131
Grade 1	6	3	
Grade 2	2	3	
Within group *P*-value	0.302	0.640	
VPT left leg			
Before treatment	17.4 ± 7.2	21.8 ± 13.6	0.451
After treatment	22.5 ± 7.7	20.0 ± 7.8	0.642
VPT right leg			
Before treatment	17.7 ± 5.2	20.6 ± 9.8	0.518
After treatment	20.6 ± 7.9	19.4 ± 6.3	0.824
Monofilament test (no. with abnormal sensations)			
Before treatment	2 (25.0%)	3 (37.5%)	0.590
After treatment	0	2 (40.0%)	0.052

Unless indicated otherwise, data show the mean ± SEM.
*Sensation grading was as follows: Grade 1, no loss of protective sensation; Grade 2, loss of protective sensation.
$TcPO_2$, transcutaneous oxygen pressure; ABI, ankle–brachial index; VPT, vibration perception threshold.

Regarding AEs, one patient in the NF3 group (12.5%) reported constipation, and three patients in the placebo group (37.5%) reported AEs of constipation (*n* = 1), coagulopathy (*n* = 1), and swollen leg (*n* = 1), but there were no significant differences between the two groups ($P = 0.570$).

In the safety assessment, no significant changes in liver and renal functions were observed, except for a mild increase in blood glucose levels in the NF3 group ($P = 0.022$). There was no change in therapy during the 6-month observational period (Table 1).

RNA microarray

As shown in Venn diagram in Fig. 3a), total gene expression differed between the NF3 and placebo groups (*n* = 3 in each group; FC > 1.2), with 59 differentially expressed genes (50 upregulated and nine downregulated; see Table S2) in the NF3 group. The differentially expressed genes most relevant to wound healing included connective tissue growth factor (*CTGF*), trefoil factor 2 (*TFF2*), keratin 6B (*KRT6B*), tubulin beta-2C chain (*TUBB2C*), somatostatin (*SST*), Dickkopf homolog 2 (*DKK2*), V-set and immunoglobulin domain containing 4 (*VSIG4*),

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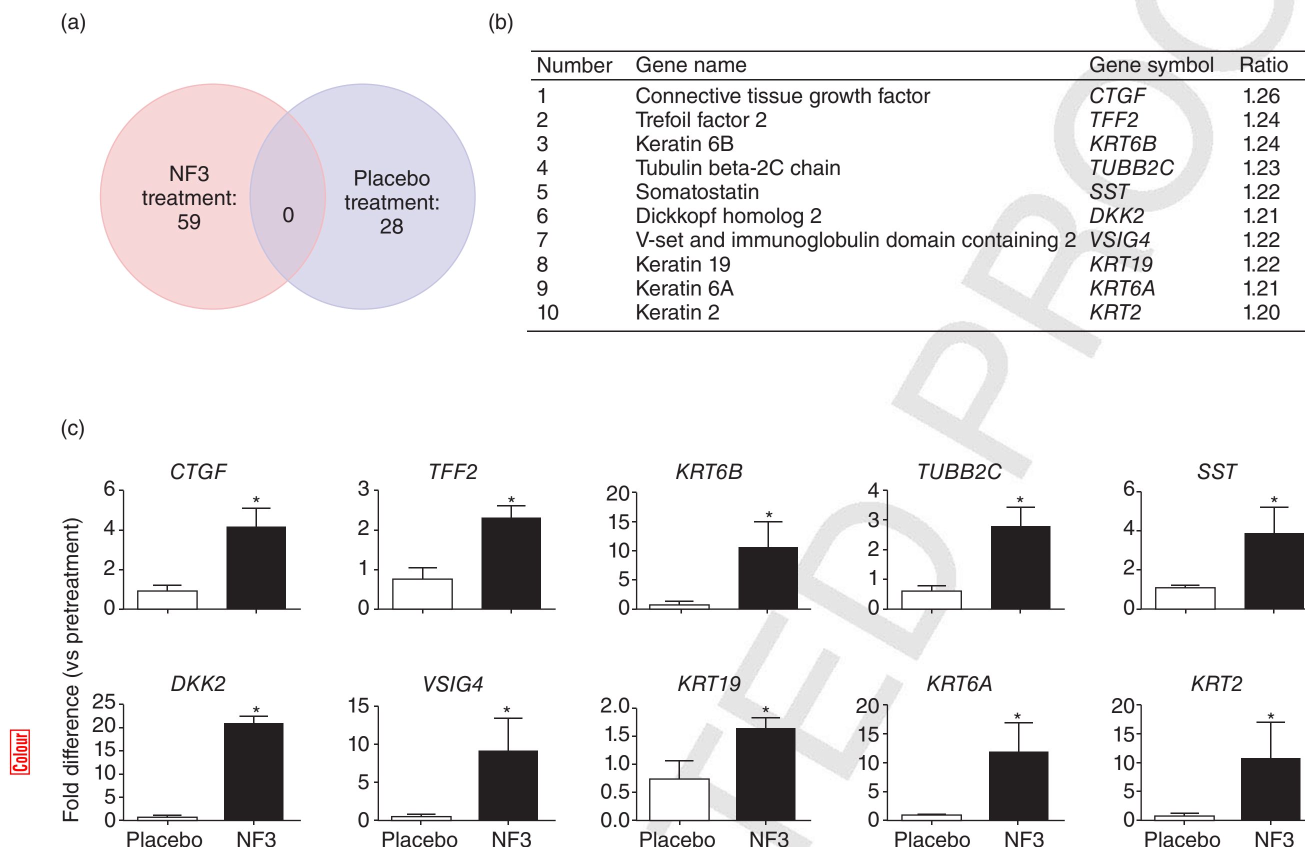

Number	Gene name	Gene symbol	Ratio
1	Connective tissue growth factor	*CTGF*	1.26
2	Trefoil factor 2	*TFF2*	1.24
3	Keratin 6B	*KRT6B*	1.24
4	Tubulin beta-2C chain	*TUBB2C*	1.23
5	Somatostatin	*SST*	1.22
6	Dickkopf homolog 2	*DKK2*	1.21
7	V-set and immunoglobulin domain containing 2	*VSIG4*	1.22
8	Keratin 19	*KRT19*	1.22
9	Keratin 6A	*KRT6A*	1.21
10	Keratin 2	*KRT2*	1.20

Colour

Figure 3 Summary of microarray results. (a) Within the Venn diagram, circles represent the effects of NF3 and placebo treatment. Numbers inside each compartment represent the number of genes that were significant for that effect when compared with their respective pretreatment baseline (fold change <1.2; $P < 0.05$). The intersection of the sets represents genes commonly involved in both treatments. (b) Microarray analysis of genes regulated after NF3 treatment (related to wound healing) with fold changes >1.2. (c) Real-time polymerase chain reaction analyses confirmed differential expression of the 10 representative genes after NF3 treatment (□) compared with placebo (□). Data are the mean ± SEM. $^*P < 0.05$ compared with placebo.

keratin 19 (*KRT19*), keratin 6A (*KRT6A*), and keratin 2 (*KRT2*; Fig. 3b). In the placebo group, there were 28 differentially expressed genes (14 upregulated and 14 downregulated; see Table S2). However, none of the differentially expressed genes was related to the wound healing process within the placebo group. To validate the results from microarray analysis, the differentially expressed genes in the NF3 group were verified by qPCR analyses (Fig. 3c) using the RNA from the remaining subjects in the study ($n = 4$ in the NF3 group; $n = 3$ in the placebo group).

We used DAVID gene functional classification to cluster the differentially expressed genes by NF3 into 47 groups with high stringency. Five groups had an enrichment score >1.2 (range 1.27–3.92; Fig. 4a). The enrichment groups were keratin, intermediate filament, C2 calcium-dependent membrane targeting, membrane fraction, and cell motility. The GO of biological process was represented by the View of GO tree based on GO FAT category. The most significantly enriched biological processes involved positive regulation of protein complex assembly and positive regulation of peptidyl-tyrosine phosphorylation. Using cellular component analysis by GO, the most significant clusters were related to intermediate filament cytoskeleton components, such as keratin filament and dynein complex (Fig. 4b). For genes altered by placebo treatment, only RNA recognition motif *RNP-1* (of 16 groups) had an enrichment score of 1.5. No significant biological processes and cellular components were found in the placebo group in GO analysis.

We then used GePS to generate networks of biologically relevant groups of genes from the microarray data based on curated literature information of protein–protein interactions, coexpression, and genetic regulation. Gene network analysis using differentially expressed genes yielded networks with *CTGF* and SST as the core genes (Fig. S1). Apart from their specific functions in fibroblast regeneration and angiogenesis, from

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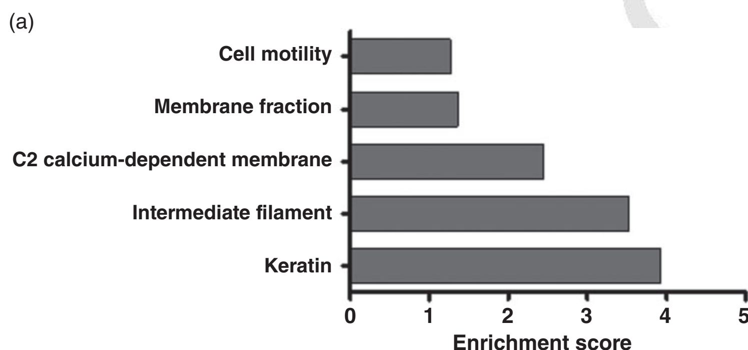
(a)



(b)

GO category	GO term	Score*	%†	*P*-value
GOTERM_CC_FAT	Intermediate filament	14	3.1	0.0001
GOTERM_CC_FAT	Intermediate filament cytoskeleton	14	3.1	0.00013
GOTERM_CC_FAT	Keratin filament	9	2.0	0.00044
GOTERM_CC_FAT	Dynein complex	5	1.1	0.0049
GOTERM_CC_FAT	Cytoskeletal part	32	7.2	0.0069

* Number of differentially expressed genes after NF3 treatment
† Percentage of total number of differentially expressed genes after NF3 treatment

Figure 4 The DAVID gene functional classification tool (http://david.abcc.ncifcrf.gov) was used to condense the candidate genes into functionally related groups that were clustered into a meaningful network context. (a) Analysis chart of gene functional classification based on the DAVID tool. A higher enrichment score for a group indicates that the gene members in the group are involved in more important (enriched) terms after NF3 treatment. (b) Gene ontology (GO) analysis under the "Cellular component" category that showed significant regulation ($P < 0.05$) in response to NF3 treatment.

GePS analysis we found that *CTGF* was frequently cocited and negatively correlated with proinflammatory cytokines, including TNF-α, interleukin (IL)-6, and interferon (IFN)-γ.[16,17] As shown in our clinical data, NF3 treatment decreased serum TNF-α levels, with possibly increased expression levels of *CTGF* to facilitate the wound-healing process of the DFU.

Discussion

In this proof-of-concept study we observed that several efficacy variables, including percutaneous oxygen pressure, vibration sense, and vascular indices, improved or tended to improve at multiple time points, along with reduced serum TNF-α levels, in the NF3 group. This was accompanied by upregulation of specific groups of genes related to anti-inflammation, tissue and vascular proliferation, and cytokines implicated in tissue healing. In addition, NF3 was generally well tolerated with no major adverse clinical or biochemical effects.

Clinical studies

During this 2-year study, after screening nearly 400 patients, only 16 patients were eligible for randomization. This low rate of randomization was due, in part, to wound healing following intensive medical care and education, a chronic or mild nature of the foot ulcer, or the presence of comorbidities, such as heart failure or renal diseases, which precluded the patients for enrolment. We adopted stringent inclusion criteria due to uncertain adverse effects of the new formulation of NF3 in humans and may have excluded patients who would have otherwise benefited. Because 74 patients were needed to detect an absolute 30% difference in healing rate, the negative results were likely to be Type II error. Conversely, the trend towards favorable outcomes in the NF3 group was encouraging despite the small sample size. There was a significant difference in $TcPo_2$ at baseline (pretreatment) between the NF3 and placebo groups ($P = 0.025$). Despite the numerical reduction in $TcPo_2$ in the NF3 group and increase in the placebo group, neither of the changes was significant. Clinical measurements of skin oxygenation can be gadget and ulcer site dependent. These differences in baseline values and variabilities in clinical measures make it difficult to interpret the $TcPo_2$ results.

Apart from minor differences in BMI at baseline, likely due to chance finding, the clinical profile and ulcer characteristics were similar between the two groups, which consisted mainly of elderly subjects. Throughout the study period, the HbA1c level was 6.8% in both groups. Despite this near optimal care and small sample size, the mean ulcer area was decreased by 47.8% in the NF3 group, compared with 14.1% in the placebo group. In patients who had healed ulcers, the daily reduction in

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wound area was 3.55% in the NF3 group, a shorter time to healing compared with the 1.52% daily reduction in wound area in the placebo group.

Rationale for using a multipronged strategy to study effects of TCM

The multicomponent nature of Chinese herbal medicine is complex and targets multiple pathways. In the past, it has been challenging to research the pharmacological activities and active mechanisms of TCM. However, advances in genome research and modern technology, such as microarrays, have provided new concepts and powerful tools to scientifically study the mechanisms of actions of TCM. In our mRNA microarray assay, NF3 increased the expression of genes implicated in fibroblast regeneration, angiogenesis and anti-inflammation, including functionally related genes such as *CTGF*, *TFF2*, *SST*, *VSIG4*, and *DDK2*. Other genes upregulated by NF3 included those implicated in cell motility, keratin, and intermediate filament. These concerted changes may have contributed to the fine-tuning of the epithelial functions and skin plasticity during wound healing. In this vein, our group has also reported concerted changes in microRNA, mRNA, and proteins following treatment with multicomponent TCM resulting in attenuation of fatty liver and sustained glycemic control in Zucker *falfa* rats in support of the pluripotent effects of TCM to improve the internal milieu and restore homeostasis.[18]

Overall molecular mechanism of NF3

Wound healing occurs as a cellular response to injury and involves activation of fibroblasts, endothelial cells, and macrophages. In diabetic ulcers, healing impairment is caused by a number of physiological factors, including diminished fibroblast proliferation, decreased angiogenesis, and a persistent inflammatory response. Therefore, agents that stimulate fibroblast growth, promote angiogenesis, and relieve inflammation may improve diabetic wound healing. Our RNA microarray results demonstrated the efficacy of NF3 in DFU, possibly by restoring the normal response of fibrogenesis (i.e. *CTGF*[19–21]), angiogenesis (i.e. *DKK2*[22,23] and *TFF2*[24,25]) and inflammation (i.e. *SST*[26,27] and *VSIG4*[28,29]). This cascade may lead to enhanced deposition of extracellular matrix and wound closure during the collagen synthesis phase of repair,[30–34] as well as restoration of sensation.[35,36] In support of these molecular mechanisms, clinical measurements also confirmed more rapid wound healing and improved sensation in the NF3 than placebo group, despite the relatively small sample size and near optimal metabolic control in both groups.

Challenges and opportunities

Despite screening nearly 400 diabetic patients with foot ulcers, only 5% were eligible for randomization. These results highlight the benefits of conventional medical treatment. Given the small sample size, there were large interindividual variations in baseline characteristics and treatment responses, although the overall results supported the clinical benefits of NF3 with safety and tolerability. Although microarray studies are a useful investigative tool, their use in human disease is limited by a lack of models with clinically relevant phenotypes and access to human tissues. In the present study, we used peripheral blood to detect biomarkers due to their mediating roles in immune responses, metabolism, and cell communication, and found positive results. Herein, peripheral blood has become a prime tissue for investigation due to the simplicity of its collection. In addition, the subtle biological changes associated with disease or response to injury may leave “footprints” in peripheral blood to provide a glimpse of the microenvironment.[37,38]

Conclusion

In the present proof-of-concept study, we have demonstrated the clinical benefits of NF3 accompanied by molecular changes in systemic circulation that may explain its wound-promoting and anti-inflammatory effects. The positive effects of NF3 on sensory neuropathy require further evaluation. Herein, none of the patients was given supplementary neuroprotective or blood-thinning treatment. Given the complex pathogenesis of DFU, our findings are only preliminary and hypothesis generating. In light of the safety and tolerability of NF3, randomized studies using a larger sample size with less stringent inclusion criteria and a longer duration of follow-up will be needed to confirm these findings. That said, our results have highlighted the usefulness of using molecular tools to study the multifaceted clinical effects of TCM.[18] In short, the combination of randomized clinical studies supplemented by transcriptional profiling of peripheral blood is one possible strategy to discover novel drug targets for unmet needs, such as DFU.

Acknowledgements

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Disclosure

The authors declare there are no conflicts of interest.

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Supporting Information

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Figure S1 A pathway was generated by the GePS program from Genomatix (GePS; http://www.genomatix.de/products/GePS/GePS1.html) that connects differentially expressed genes found after NF3 treatment (fold change >1.15; $P < 0.05$). This network suggested a potential regulatory role of connective tissue growth factor (*CTGF*) and somatostatin (*SST*) in the wound healing process in diabetic foot ulcer patients after NF3 treatment.

Table S1 Reverse transcription–polymerase chain reaction primer pairs for validation of differentially expressed genes (fold change >1.2; $P < 0.05$) between the NF3 and placebo groups in the microarray studies.

Table S2 Microarray analysis of genes in the placebo and NF3 groups and treatment with fold change >1.2 ($P < 0.05$) compared with their respective pretreatment baseline.

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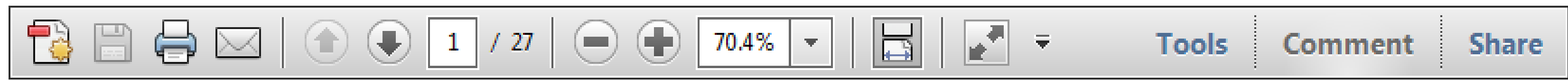

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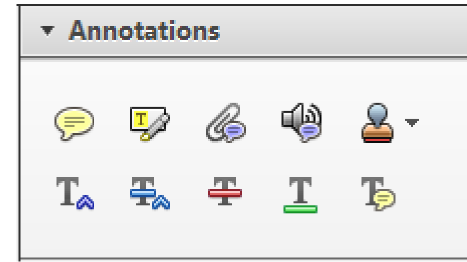

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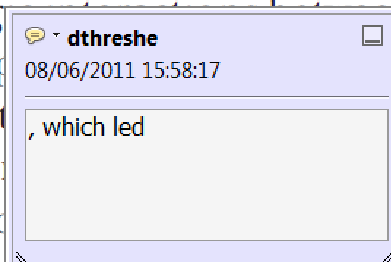

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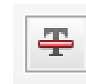

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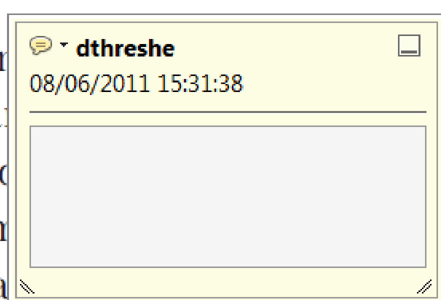

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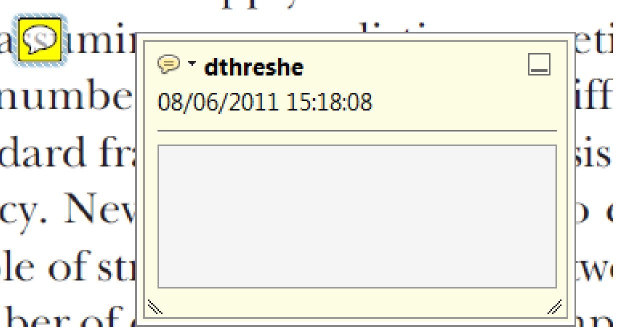

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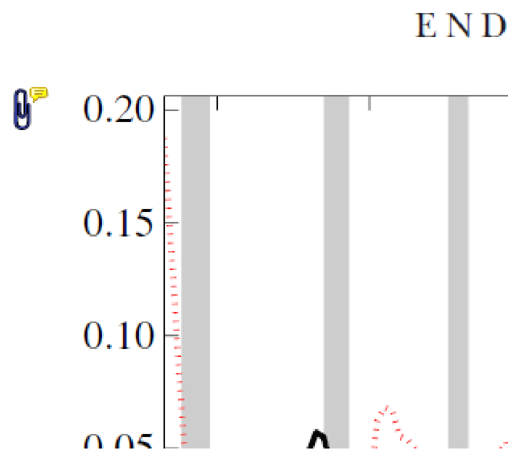

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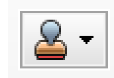

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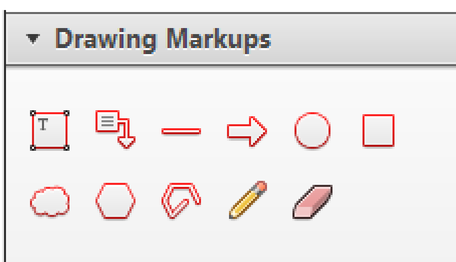

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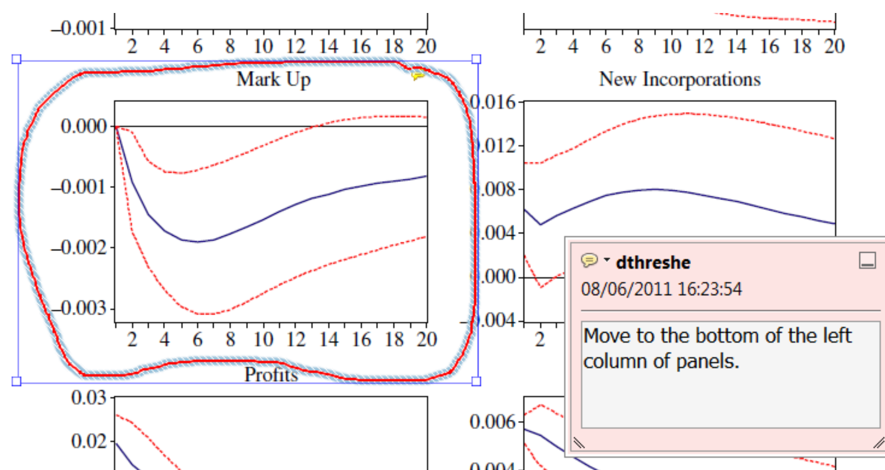

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