IMMUNOMODULATORY ROLE OF *TINOSPORA CORDIFOLIA* AS AN ADJUVANT IN SURGICAL TREATMENT OF DIABETIC FOOT ULCERS: A PROSPECTIVE RANDOMIZED CONTROLLED STUDY

HARSHAD PURANDARE, AVINASH SUPE

ABSTRACT

BACKGROUND: Chronic diabetic patients with wounds have deficient growth factors and impaired local and systemic cellular immunity. Treatment with growth factors is expensive with risk of infection transmission, and these factors may not achieve optimum wound concentration. We evaluated the role of generalized immunomodulation in diabetic ulcers by using Tinospora cordifolia as an adjuvant therapy and studied its influence on parameters/determinants of healing, on bacterial eradication and on polymorphonuclear phagocytosis. MATERIALS AND METHODS: A prospective double-blind randomized controlled study lasting for over 18 months in 50 patients. The ulcer was classified by wound morphology and severity with Wound Severity Score (Pecoraro-Reiber system). Mean ulcer area, depth and perimeter were measured and swabs taken for culture. Blood was collected to assess polymorphonuclear % phagocytosis (PMN function by Lehrer-Cline C. albicans method). Medical therapy, glycemic control, debridement, wound care were optimized. At 4 weeks, parameters were reassessed. PMN function was reviewed at 3 months. RESULTS AND ANALYSIS: Forty-five patients completed the trial: study group – 23 (M:F = 17:1; mean age = 56.3 years; mean ulcer duration = 21.1 days); control group 22 (M:F = 19:3; mean age = 56.3 years; mean ulcer duration = 30.4 days). Net improvement was seen in 17 patients (73.9%) in the study group; while in the control group, in 13 patients (59.1%); P = 0.292. Specific parameters included rate of change of ulcer area - cm²/day (study - 0.15; control - 0.07; P = 0.145); rate of change of ulcer perimeter - mm/day (study - 0.09; control = - 0.07; P = 0.089); change of depth - mm (study - 2.2; control - 1.4; P = 0.096); change of wound score (study - 14.4; control - 10.6; P = 0.149); total number of debridements (study - 1.9; control - 2.5; P = 0.03) and change in % phagocytosis (study - 3.9; control - 2.3; P = 0.048). CONCLUSION: Diabetic patients with foot ulcers on T. cordifolia as an adjuvant therapy showed significantly better final outcome with improvement in wound healing. Reduced debridements and improved phagocytosis were statistically significant, indicating beneficial effects of immunomodulation for ulcer healing.

Key words: Diabetic foot ulcers, immunomodulation

Department of Surgery, Seth G. S. Medical College ! and K. E. M. Hospital, Mumbai, India!

Correspondence:
Dr. Avinash Supe, !
Sangeeta Towers, 6th Floor, Chembur, Mumbai - 400 071, !
India. E-mail: avisupe@vsnl.com!

Diabetic ulcers of the lower extremity are ! a major concern, preceding most of the ! amputations in general surgery. More than ! 7 million people in India suffer from diabetic ! foot ulcers, with a recurrence rate of 53%. The ! expenditure on diabetic foot may amount to 25-! 30% of a patient's annual income.[1,2]!

Diabetes mellitus is a secondary ! immunodeficiency disorder.[3] There is impaired ! chemotactic, phagocytic, lytic and intracellular ! killing capacity of the neutrophils, macrophages ! and monocytes; defective cellular immunity ! and T-cell-dependent IgG/IgM response and ! impaired complement activity.[4] Non-healing ! diabetic foot ulcers have deficient !synthesis ! growth factors such as PDGF, βFGF, TGF-! β, GM-CSF, as these factors are trapped ! in pericapillary fibrin cuffs or destroyed by ! metalloprotein enzymes.

Recombinant REGRANEX (Becaplermin; ! rhPDGF-BB), approved by the US FDA ! (1997), is found to impair wound contraction, ! epithelialization and wound strength in some ! cases.[5] Though a 40% increase in healing ! of noninfected neuropathic foot ulcers was ! observed, a 30% recurrence was noted at 6 ! months.[6] Treatment of such immunodeficiency ! by immunomodulators or growth factors is ! technologically expensive. The average cost ! of US$10/day is substantial in the Indian ! setting. Based on the Ayurvedic concept of ! use of plant extracts to improve body's natural ! resistance, we used immunomodulation to ! overcome shortcomings of specific targeted ! immunopotentiation. Macrophages play ! a key role in phagocytosis, production of ! cytokines and growth factors and potentiation ! of neutrophil-platelet functions. They were ! the target of our therapy. The aqueous extract ! of the creeper Tinospora cordifolia (family ! Menispermaceae), purified and biostandardized, ! is well tolerated and induces leukocytosis with neutrophilia and improves PMN-macrophage ! phagocytic, lytic and intracellular killing activity ! by the enhancement of GM-CSF activity.[7] ! Clinical studies show reduced complications ! in intra-abdominal sepsis, enteric fistulas and ! obstructive jaundice with early resolution in ! pulmonary tuberculosis, cirrhosis, Chronic ! Suppurative Otitis Media (CSOM), burns sepsis, ! as also lymphatic and hemopoetic malignancies, ! chemotherapy-induced neutropenia and fungal ! infections.[8] GM-CSF increases the levels and ! activity of platelet- and neutrophil-derived PDGF ! and acts in a positive feedback loop, a vital ! step in wound healing. The global stimulatory ! role of GM-CSF and actions of T. cordifolia to ! stimulate GM-CSF have led to this study, which ! aims to evaluate the efficacy of T. cordifolia ! as an adjuvant in the treatment of diabetic ! foot ulcers. We also aim to study its influence ! on determinants of wound healing, bacterial ! eradication and phagocytic function of PMN ! cells.

MATERIALS AND METHODS

A double-blind prospective randomized placebo-! controlled study lasting for over 18 months ! (August 1999 to February 2001) was carried out ! at a reputed public hospital after authorization ! from the hospital's Ethics Review Committee. Patients admitted to the surgical wards were ! screened as per the inclusion-exclusion criteria, ! and 50 patients entered the study.

Inclusion criteria

1. ! Adult patients of either sex with diabetic foot !

ulcer not less than 4 cm in diameter!

2. !Ulcers of Wagner's system's Grade I ! or Grade II; and digital, ray or forefoot ! amputations with non-healing ulcer[9]!

Exclusion criteria

1. !Patients with ulcer of any other etiology!
2. !Patients with local or systemic disease or ! on therapy that may interfere with wound ! healing!
3. !Ulcer with radiographic evidence of ! osteomyelitis or involving joint spaces or ! bones (Wagner's Grade III and IV)[9]!

On inclusion into the trial, a detailed history ! was taken with emphasis on the duration of ! ulcer and diabetes, its treatment, previous ! ulcerations, amputations and other associated ! illnesses. A detailed clinical examination was ! performed. The ulcer included in the trial was ! classified by wound morphology, severity and ! location with a total wound severity score ! according to a published grading system by ! Pecoraro and Reiberg[10] [Table 1] 1 week after ! the initial debridement. The mean target ulcer ! area and wound perimeter were calculated from ! wound contour tracing over a sterile transparent ! plastic film. Ulcer depth was measured by ! means of a blunt-tip calibrated probe from ! the level of wound surface. Swabs were ! taken for Gram staining and culture. Wound ! photographs were taken at a standard focal ! length. Clinical assessment of neuropathy and ! peripheral vascular disease with flow-pressure ! assessment using an arterial color Doppler ! was done. Baseline investigations including a ! complete hemogram, differential count, ESR, ! fasting and postprandial blood sugar, liver and ! renal function tests were also performed.

Blood samples were also collected for the ! assessment by polymorphonuclear (PMN) ! function test: % phagocytosis evaluation by an ! in vitro Lehrer and Cline technique of Candida ! albicans phagocytosis using trypan blue dye ! exclusion method.[11] Ten ml of the patient's ! heparinized blood diluted with normal saline ! (1:1) on Ficoll Hypaque gradient, centrifuged at ! 200 rpm for 40 min, and the PMN-RBC pellet ! were separated and mixed with 1 ml Dextran-! 70 and 1 ml heterologous plasma. The RBC ! sediment and PMN cells were counted and ! adjusted to 1 x 10^6 cells/ml with 90% viability. The test organism was C. albicans obtained ! by overnight cultures in nutrient broth adjusted ! to 1 x 10^7 spores/ml by spectrophotometric ! absorbance. In the assay system, 2 × 10^6 ! PMN were suspended in Minimum Essential !

Table 1: Pecoraro wound grading score

	Score			
Parameter	*0*	*1*	*2*	*3*
Pain	Nil	Minimal	Moderate	Severe
Itching	Nil	Minimal	Moderate	Severe
Odour !	Nil !	Minimal, !	Moderate !	Severe, foul smell !
Discharge !	Nil !	Minimal, serous !	Moderate, sero-purulent !	Copious, purulent !
Edema	Nil	Minimal	Moderate	Severe
Granulation Tissue !	UniformLy Pink !	Pale, heaped at places !	Unhealthy !	Absent !
Slough !	Nil !	Minimal, <25% !	Moderate patchy, !	Abundant uniform,!
!	!	ulcer area !	25-50%ulcer area !	>50% ulcer area !
Margin !	Healing !	Blue epithelialized !	Partially blue !	Angry red non-healing !
Erythema	Nil	Minimal	Moderate	Angry red
Induration	Nil	Minimal	Moderate	Severe
Fibrosis	Nil	Minimal	Moderate	Uniform
Ulcer Depth !	<5 mm !	5 –10 mm !	10 –20 mm !	>20 mm !

Medium (MEM) (Himedia, containing 100 ! µg/ml each of penicillin and streptomycin) and ! supplemented with 20% AB serum to which ! 1 x 10^6 (0.1 ml) spores of C. albicans were ! added, and the suspension was incubated ! at 37°C in 5% CO_2 atmosphere for 1 h. The ! tube was then centrifuged at 2,000 rpm for ! 10 min, and cytosmears were prepared, fixed ! and stained with Giemsa. One hundred PMN ! cells were scanned and the cells with ingested ! organisms were counted to obtain percentage ! phagocytosis.

On the basis of a predesigned randomization ! schedule, each patient was administered the ! drug supplied in prepacked numbered bottles for ! a period of 1 month. The individuals concerned ! with conducting the trial were blinded to the ! randomization. Medical therapy was optimized ! and all the patients were administered the drug ! (study drug or placebo) along with conventional ! therapy for the treatment of the ulcer as well ! as diabetes. All slough, pus pockets, clefts, ! necrotic tissue and infected granulation tissue ! in the ulcer were removed by a sharp surgical ! debridement. Regular debridements were done ! as per further wound assessment during the ! trial. The total number of debridements needed ! was assessed as a parameter of response. ! Standard regimen of daily wound care included ! gentle cleansing with half strength 1.5% ! hydrogen peroxide solution with ample amount ! of saline. Superficially infected wounds were ! dressed with topical antibiotics, followed by ! a gauze dressing. Empirical broad-spectrum ! systemic antibiotic coverage was initially ! achieved with oral antibiotic therapy using ! Amoxycillin-Clavulanate. Specific antibiotic ! coverage was reconsidered after culture results ! were obtained. Ambulation was minimized and ! protective footwear advised. Patients were ! started on insulin and the dose adjusted. The ! patients were assessed on a weekly basis ! till complete healing (either satisfactory or ! complete) was achieved. The wound severity ! score was determined as mentioned earlier. ! Wound swabs were sent for culture at 2 and 4 ! weeks.

A 4-week period was considered to be adequate ! for clinical and parametric shifts in the wound to ! be obvious for assessment of the effect on the ! progress of the wound, as also for avoiding the ! attrition of the sample size due to the failure of ! the patient to attend the OPD.[12] At the end of ! 4 weeks, a final wound severity score, the final ! ulcer area and perimeter were determined; and ! blood samples were taken at the end of the trial ! to measure total and differential count, liver and ! renal function and PMN function. For optimum ! results, !final PMN function assessment was ! done at 3 months.[13]!

Evaluation

The rate of ulcer healing was calculated by two ! related methods[14]:!

- **Rate of change of wound area:** Leugers ! coefficient of healing (centimeters squared ! per day) was obtained as the slope of! the graph of linear regression of wound ! area epitheliazed versus time (duration of treatment). A positive rate contributed to ! early healing.

- **Rate of linear advancement of wound margin:** Change in wound perimeter ! (millimeters per day), calculated by Gilman's ! formula!

d = ΔA / p, where!

'ΔA' = A_1 - A_2, in which A_1 is the initial and A_2 !

is the final wound surface area. 'p' = $p_{1+}p_2$, in ! which p_1 is the initial and p_2 is the final wound ! perimeter. The rate of closure (mm/day) is then ! obtained as a linear regression.

The difference in the initial and final !wound ! severity score was calculated. An ulcer was ! considered to be healing when it demonstrated ! an overall progress in net tissue repair process ! as seen by a fall in the severity score or by a ! positive rate of healing or both. An ulcer was ! non-healing if there was no healing progress ! or if net wound expansion occurred.

At the end of the trial, patients were divided ! into two groups - Group A = drug, Group B ! = placebo - as per the randomization chart. ! The data obtained from the two groups was ! analyzed for comparability by validation tests ! like the Fisher's F test. The various parameters ! mentioned above were compared between the ! two groups for statistical significance using the ! Chi-square (χ^2) test, the unpaired 't' test and ! the ANOVA (Analysis of Variance) test.

RESULTS AND ANALYSIS [TABLE 2]

Of the 50 patients enrolled, 45 patients ! completed the trial - 23 in the drug study group ! and 22 in the placebo group. In the study group, ! 17 were males (73.9%) and 6 females (26.1%); ! while in the placebo group of 22 patients, there ! were 19 males (86.4%) and 3 females (13.6%). The mean age for the entire study group was ! 56.29 years (range: 32.4-80.6 years; study ! group = 56.26 years; placebo group = 56.32). The mean duration of diabetes in those patients ! receiving the study drug was 5.95 years (range: ! 0-18 years) while in those of the placebo group ! was 8.27 years (range: 0-22 years).

In patients receiving the study drug, the mean ! duration of ulcer prior to inclusion in the trial was ! 21.08 days (range: 12-35 days); while in those ! receiving the placebo, it was 30.36 days (range: ! 21-44 days). The Chi-squared and Fischer's ! tests indicate comparable groups for inferential ! analysis. As many as 66% of the ulcers were ! involving the forefoot and midfoot, suggesting ! proneness to trauma.

Four patients (8.89%) had more than one ulcer, ! out of which only one ulcer was included in the ! trial as per the protocol. The total number of ! patients with repeated diabetic foot lesions was ! 10 (22.22%). Six patients (13.33%) were freshly ! detected to be diabetics.

Analyzing the final outcome, viz., net improvement ! or failure, of the 23 patients in the study drug !

Table 2: Results and analysis

Parameter	*Group*	*Mean*	*Sd*	*Sum of squares*	*P_*	*Standard error*	*Confidence Intervals range*	
							High	*Low*
Rate of change of ulcer !	Drug !	0.149 !	0.996 !	21.82 !	0.145 !	0.21 !	0.36 !	(0.06)!
area (R1) !	Placebo !	-0.069 !	0.894 !	16.77 !	!	0.19 !	0.12 !	(0.26)!
Rate of change of ulcer !	Drug !	0.093 !	0.036 !	0.029 !	0.089 !	0.01 !	0.10 !	0.09!
perimeter (R2) !	Placebo !	-0.073 !	0.055 !	0.064 !	!	0.01 !	(0.06) !	(0.08)!
Change of ulcer depth !	Drug !	2.17 !	1.33 !	-- !	0.096 !	0.28 !	2.45 !	1.89 !
!	Placebo !	1.36 !	1.314 !	!	!	0.28 !	1.64 !	1.08 !
Change in Wound Severity !	Drug !	14.39 !	8.39 !	1549.49 !	0.149 !	1.75 !	16.14 !	12.64!
Score !	Placebo !	10.59 !	8.88 !	1657.32 !	!	1.89 !	12.48 !	8.70 !
Number of Debridements !	Drug !	1.91 !	0.9 !	-- !	0.03 !	0.19 !	2.10 !	1.72 !
!	Placebo !	2.49 !	1.48 !	!	!	0.32 !	2.81 !	2.17 !
Change in % phagocytosis !	Drug !	3.94 !	3.942 !	341.83 !	0.048 !	0.82 !	4.76 !	3.12 !
!	Placebo !	2.34 !	7.806 !	1279.45 !	!	1.66 !	4.00 !	0.68 !

group, 17 (73.9%) improved; while in the placebo ! group of 22 patients, 13 (59.1%) improved (*P* = ! 0.292). Though not statistically significant, better ! healing and net improvement were obtained in ! patients of the study drug group.

When the wounds were evaluated in terms of ! the two measured healing rates, viz., R_1 - rate ! of change of ulcer area in cm^2/day; and $R_{2!}$ - rate of change of ulcer perimeter in mm/day, ! improvement was more obvious in the group ! receiving the drug. The mean R_1 in the drug group ! was 0.149 cm^2/day (95% confidence !interval: ! 0.06-0.36), while that in the placebo group was ! -0.069 cm^2/day (95% confidence !interval: !0.12-! 0.26), indicating a net increase in the ulcer area ! in the placebo group in comparison with the ! drug group (*P* = 0.145). Similarly R_2, the mean ! change of perimeter, in the patient group was ! 0.093 mm/day (95% confidence !interval: !0.09-! 0.1), while that in the placebo group was -0.073 ! mm/day (95% confidence interval: 0.06-0.08) (*P* ! = 0.0890). Thus, better results were obtained in ! the drug group, both the parameters indicating an ! expansion of the wound either in width or in depth ! in the placebo group, indicating non-healing.

Change in the wound depth was analyzed ! by applying the unpaired 't' test to the mean ! difference in the wound depth in the drug ! and the placebo group. It was found that d_1 = ! 2.17cm, 95% confidence interval: 1.89-2.45; ! and $d_{2!}$= 1.36 cm, 95% confidence !interval: ! 1.08-1.64 respectively, a bigger positive value ! indicating a better improvement in the ulcer with ! reduction in its depth. The obtained '*P*' value of ! 0.096 indicates better improvement in the drug ! group. Thus the change in the depth of ulcers is ! one of the important determinants of healing. The Wound Severity Score was measured as ! a qualitative parameter in wound assessment, ! a positive difference indicating healing. The ! difference in the initial and final wound score ! was calculated (drug score = 14.39, 95% ! confidence interval: 12.64-16.14; placebo score ! = 10.59, 95% confidence interval 8.70-12.48; *P* ! = 0.149, analyzed by ANOVA test), indicating ! that the drug group showed better improvement ! in the wound scores.

The total number of debridements needed ! per patient was considered as a measure of ! wound status, more debridements indicating ! a progressively worsening wound. The mean ! number of debridements (drug = 1.91, 95% ! confidence interval 1.72-2.10; placebo = 2.49, ! 95% confidence interval 2.17-2.81; *P* = 0.030) ! is significantly reduced in the drug group.

Finally, analyzing the change in percentage ! phagocytic function of PMN cells as a quantitative ! measure of immune function improvement, the ! values of 3.94% (95% confidence interval 3.12-! 4.76) and 2.34% (95% confidence interval 0.68-! 4.0) in the drug and placebo group respectively ! (*P* = 0.048) suggest a significant effect of drug ! on the immune function.

No difference was observed in bacterial ! clearance from wounds in both the groups [53% ! persistence of the same strain, especially in ! patients with polymicrobial spectrum - 34 out ! of 45 patients (75.55%)]; also, no difference ! was observed with respect to neutrophil count. Gram-negative bacteria formed 70% of the ! bacterial isolates.

DISCUSSION

Recent molecular studies indicate a defect in immune functions in diabetics. Growth factors and cytokines regulating proteolytic activity and matrix synthesis are reduced in chronic wounds. These chronic wounds have a persistent bacterial load, possibly related to local immune dysfunction and growth factor deficiency. The development of REGRANEX (recombinant PDGF-BB) evoked interest in immune therapy in diabetic non-healing ulcers. It showed beneficial effect, primarily in noninfected neuropathic ulcers. However, therapy was extremely costly (approximately US$9.70 per day), and long-term effects on wound contraction and wound strength, as well as recurrences, were not satisfactory. Though these products are still unavailable in our country, their usage is bound to be highly cost-ineffective from both their clinical benefit and economic standpoint.

The modulation and stimulation of GM-CSF and related cytokines thus improving the macrophage function were considered as a therapeutic modality in non-healing diabetic ulcers. The biostandardized aqueous extract of stem of T. cordifolia, an Ayurvedic agent, has a proven stimulatory effect on GM-CSF and is extremely economical (approximately Rs. 11 per day).

Sixty percent of the ulcers involve the distal plantar aspect of the foot, indicating a proneness to minor trauma, explained on the basis of occupational activity, higher incidence of foot deformities, neglected footwear precautions and the higher incidence of lean non-obese diabetics in India, further complicated by a very poor knowledge of foot care and improper treatment of diabetes itself.

A net final outcome of better healing in the drug group correlated with a better rate of change of ulcer area (R1) and a better rate of change of ulcer perimeter (R2). Moreover, the drug group showed better improvement in the wound score. These mentioned parameters are an assessment of qualitative as well as parametric evaluation of overall wound healing. An improvement in the wound score is an indirect indicator of increased phagocytic activity as well as of enhancement of granulation tissue formation in a chronic wound. Positive values of R1 and R2 in the drug group with a corresponding improvement in wound scores suggest an overall positive outcome, though both these parameters did not reach statistical significance in the current study. The ulcer heals with wound contraction as well as the development of proliferating granulation tissue from its floor, r with reduction in its depth. This is seen as a change in the wound depth and is an important determinant of wound healing. At cellular level, the above changes are probably related to enhanced macrophage and monocyte activity, leading to a substantiation of growth factor and increase in scavenging activity.

A statistically significant lower number of debridements were needed in the drug group, which correlates with better improvement in wound depth. This indicates halting of progression of infection to deeper planes of the wound. Improved scavenging and defense mechanisms involving PMN and macrophages play a vital role in this aspect.

A statistically significant improvement in the percentage phagocytic function of PMN cells in

the drug group is a quantitative and functional corroboration of the role of this drug in cellular immune potentiation.

Though the results of this study showed no significant difference between the two groups, T. cordifolia has been known to induce an increase in circulating neutrophils. Coexistent polymicrobial bacterial infection and chronic local sepsis probably account for this disparity.

Vasculopathy and neuropathy, with their effects on microcirculation, further confound results and support non-healing, as seen in 66.67% of patients in the drug group and 77.33% of patients in the placebo group. These had variable degrees of neuropathy and/or vasculopathy, and non-healing was more common in patients in both the groups who had these changes. T. cordifolia, unfortunately, does not seem to have an effect on these neuropathic, autonomic and microvascular dysfunctions. Infection was another important determinant of healing in both the groups. No difference was observed in bacterial clearance from wounds in them though polymorphonuclear functions were enhanced, possibly due to the chronicity and polymicrobial nature of most infections. Almost 45% of the patients with improved score still showed bacterial persistence.

CONCLUSION

The overall beneficial effect of Tinospora cordifolia appears to be primarily dependent on enhancement of scavenging and desloughing action of PMN cells and macrophages. These immunomodulated cells with their improved phagocytic function create a favorable environment in an otherwise non-healing wound. This probably facilitates a series of pro-healing processes like growth factor activation, angiogenesis and granulation tissue formation - all processes inhibited and suppressed in chronic wounds. The role of such immunomodulated cells in direct or indirect growth factor stimulation is not proven in this study and is only speculative, based on the observation of better wound healing in the drug group.

As this study was conducted with an observation period of only 1 month to facilitate in-patient assessment and prevent attrition of the sample size through loss of follow-up, a number of parameters were possibly not altered due to chronic and non-healing nature of these wounds to an extent obvious enough to cause statistically significant observations. A longer duration would definitely aid a more comprehensive evaluation of the effects of the drug.

Though not proven beyond doubt, the initial results of this study are promising. Considering the cost-effectiveness of the drug and the fact that almost 70% of our patients depend on traditional or alternative medicine, further study with a larger patient group and a longer follow-up is definitely indicated. T. cordifolia is also seen to have some degree of anti-hyperglycemic effect; and if accepted, oral T. cordifolia may be the future of immunotherapy in diabetic wounds, aimed at an overall enhancement of immune function.

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