Wounds are a ubiquitous part of any surgical practice and often are challenging to treat. They can be broadly described as acute or chronic, have a varied etiology, and may be associated with increased morbidity, mortality, and healthcare cost.

Wound healing is a complex, active, multifactorial process. Wounds that have not shown any progress toward healing for 1 month or more can be defined as chronic; however, this definition does not take into account wound size or underlying etiology. What causes a wound to become chronic is still not clear. Acute and chronic wounds have been the source of intense debate and much research regarding the roles of substances such as transforming growth factor (TGF) beta I and II receptors and the failure to eliminate overexpression.
of fibroblasts, which can cause undue scarring. Although many factors have been implicated that prevent or facilitate wound healing, randomized clinical trials (RCTs) that address improved wound healing are few and far between. No single intervention, such as topical hyperbaric oxygen or electrical stimulation, has been shown to unequivocally improve wound healing.

In resource-rich nations, wound healing has evolved as a result of specialized nursing, improved newer wound care agents, and community-based wound care management teams bringing care to the patient, thereby reducing hospital load. Countries with national health services also have shown that community-based wound management teams can help decrease the costs to the healthcare provider. However, as noted in a 2006 European Endocrine Panel report, the emphasis and recommendations of the panel focus on preventing problems such as dry, cracked feet related to autonomic neuropathy and subsequently reduce major amputations, rather than on healing wounds already present.

Given the implications of a predicted increase in the number of persons with diabetes, and therefore chronic wounds and their economic consequences, there is need to investigate how to increase the chances of wounds healing more rapidly. Theoretically, stem cells could provide an answer. Stem cells have been shown in animal models (in vivo and in tissue sections) to have the potential to reduce healing time because they are able to differentiate into the cell types required in wound healing. It is known that bone marrow-derived stem cells can be recruited locally and transform into keratinocytes and other cells, promoting local granulation without cell-to-cell fusion. If this does not occur, it may be due to the fact that the stem cells did not completely engraft, but rather, fused with the normal epithelial cells, fibroblasts, and myocytes already present, as well as stem cells that circulate peripherally. In theory, the local stimulus should cause these pluripotent cells to differentiate into the required fibroblasts, myofibroblasts, and keratinocytes, as well as dermal appendage precursors. If this does not occur, it may be due to the fact that the stem cells did not completely engraft, but rather, fused with the normal epithelial cells, fibroblasts, and myocytes already present, and prolonged cell lifespan while increasing their size and function.

The purpose of this prospective, randomized, clinical study was to compare the rate of healing chronic lower limb wounds in patients with diabetes mellitus (DM) whose wounds were treated with topically applied and locally injected bone marrow-derived cells or whole blood (control).

**Background**

According to data derived from a cross-sectional, community-based survey of wounds in urban and rural areas of northern India, the prevalence of chronic wounds in the community is estimated to be 4.48 per 1,000 and 15.0 per 1,000 for acute and chronic wounds combined. In India, the number of people older than 20 years of age with diabetes is predicted to increase 151% between 2000 to 2030. This population has a 30- to 40-fold greater chance of major complications (such as non-fatal myocardial infarctions, strokes, end-stage renal disease, and major limb amputations) compared to the normal population, and 15% of people with diabetes are estimated to have foot ulcers at any given time. It is also estimated that 40 to 77 per 10,000 people with diabetes will have a major amputation in their lifetime that could be avoided 65% of the time with adequate foot care. India is expected to have the world’s largest population of people living with diabetes by 2030, adding to an already burdened health system.

Impediments to chronic wound healing may include the presence of necrotic tissue and bacterial overgrowth. Regular debridement can act as a stimulant for recruitment of progenitor cells and local cytokines. Once these are released, they can attract stem cells from the bone marrow as well as stem cells that circulate peripherally. In theory, the local stimulus should cause these pluripotent cells to differentiate into the required fibroblasts, myofibroblasts, and keratinocytes, as well as dermal appendage precursors. If this does not occur, it may be due to the fact that the stem cells did not completely engraft, but rather, fused with the normal epithelial cells, fibroblasts, and myocytes already present, and prolonged cell lifespan while increasing their size and function.

Case reports and case series describing the injection or application of autologous bone marrow-derived cells onto chronic wounds have shown promising results, although in some cases it has taken up to 3 years for complete wound resolution. Animal models also have shown promising...
Results with fascial and cutaneous wound healing. Cells engrafted onto the wounded backs of diabetic nude mice have been shown to incorporate into the healing tissue, as demonstrated by the formation of glandular structures and endothelial cell tubes, detected by the presence of allogeneic bone marrow-derived stem cells that had been labeled with green fluorescence protein. Bone marrow-derived stem cell grafting also been performed successfully in human volunteers.9

Materials and Methods

Study enrollment. The study was conducted among patients >18 years of age seen in the surgical outpatient department of the Christian Medical College, Vellore and persons referred to the Department of General Surgery for chronic wound management between July 2006 and July 2008. Because the latter center is a tertiary-level institution, only patients with lower limb wounds were seen in the Department of General Surgery. Chronic wounds in other anatomical locations such as upper limbs are treated by specialist units such as the Hand Reconstruction Unit; chest wounds are managed by the department of Cardiothoracic Surgery and decubitus ulcers by the department of Physical Medicine and Rehabilitation. A chronic wound management program with multidisciplinary input is not in place at this center. All patients with chronic lower limb wounds of >3 months’ duration other than chronic venous or arterial insufficiency were referred for inclusion.

After the study purpose and methods were explained, patients interested in participating were provided an explanation of the study and a consent form in their own language. Patients who provided written informed consent were further counseled by the principal investigator and additional inclusion/exclusion criteria were reviewed.

Inclusion and exclusion criteria. Patients eligible to participate included persons with wounds that could not heal by primary or tertiary intention because, for example, they could not be closed surgically either per-primar or using skin grafts for reasons such as site (e.g., plantar ulcers) and patient preference. Exclusion criteria included wounds >15 cm in any one dimension and the presence of active infection requiring emergency operative intervention. Also excluded were patients in whom distal pulses were not clinically palpable below the level of the popliteal vessel (as confirmed by a consultant surgeon); patients who were pregnant; patients diagnosed with hematological or Hansen’s disease; patients receiving chemotherapy; and persons with poorly controlled blood sugar levels (defined by any single random blood sugar value >160 mg%).

Once approved for study enrollment, patient confidentiality was maintained by assigning a unique patient identification number. This study received approval from the Institutional Review Board of the institution. It also was approved by and registered with the Clinical Trials Registry of India: CTRI/2009/091/000250.

Sample size and randomization. The sample size for two independent groups was calculated to be 63 persons each for the treatment and control groups. However, given the rates of chronic wounds in the community (4.48 out of 1,000), it was calculated that a total number of 30 cases and 30 controls would suffice for a study power of 80%.

Randomization was carried out in blocks of 10 using serially numbered opaque envelopes opened by a third party in front of the investigator at the time of intervention. Randomization was done using a computer-generated random number sequence program.

Procedures. Before any procedure was performed, the numbered opaque envelope was opened by a third party in front of the investigator. The procedure to be performed was read to the interventionist and shown to the clinician and to the study participant at the time of intervention.

The longest and widest dimensions of the wound were measured from the edge of the wound to the nearest mm with a cm ruler in a designated minor surgical procedure room by the principal investigator. The wound also was photographed using a Nikon S5 camera (Nikon Corporation, Tokyo, Japan) in auto focus mode and with a flash. The distance of the camera from the wound was kept as close to 1 m as possible. The wound area was positioned to allow for easy reproducibility in follow-up photographs.

Treatment group. Patients randomized to the treatment group were given local anesthesia and sedation; 10 cc of bone marrow was aspirated from the posterior superior iliac spine. The bone marrow aspirate was centrifuged at 3,600 rpm for 20 minutes and the buffy coat pipetted off.

Once the aspirate was prepared for injection, the wound bed was prepared by gently scraping it to encourage fresh surface bleeding.21 The buffy coat then was loaded into a 5-cc syringe and a 26 G needle, 2.5 cc was injected into the edges of the wound at four points farthest from the center of the wound and then at four intermediate equal positions between the initial injection sites.

The remaining 2.5 cc of the stem cell concentrate was sprayed onto the wound and covered with a saline-moistened gauze dressing and several layers of cotton pads. Participants were asked to return for follow-up after 48 hours. At that time, the dressings were removed and the wound measured and photographed. All subsequent dressing changes used saline-moistened gauze and cotton bandages and were performed by the principle investigator. Patients assigned to the treatment group did not receive any other treatments or supportive measures such as offloading casts or negative pressure or any other special dressings.

Control group. The procedure for the control patients was exactly the same as for the treatment group in terms of consent, randomization, and follow-up. However, instead of undergoing a bone marrow aspiration and local application of the centrifuged cells, following scraping the wound bed their wounds were injected with autologous peripheral
Once the wound bed was prepared, 5 cc of peripheral blood was drawn from the brachial vein of the left forearm and injected into the wound in exactly the manner outlined for bone marrow concentrates in the case group. Follow-up dressing change and wound assessment procedures were the same as for persons in the treatment group.

Patients in the control group continued using their regular wound care treatments, including offloading casts, negative pressure therapy, and regular dressings. Saline-moistened gauze dressings were provided after the procedure for both groups and at each follow-up visit. Dressings were changed daily only in the treatment group, while the control group reverted to their previously instituted treatment plan, including offloading casts.

Follow-up. All patients were assessed 48 hours, 1 week, 2 weeks, 4 weeks, 6 weeks, and 3 months following the first application of centrifuged bone marrow aspirate or peripheral blood (control). At each visit, the longest and widest dimensions of the wound were measured from the edge and the wound photographed. All follow-up dressing changes were performed by the principle investigator. No special wound care was required for any of the harvest sites or injection areas. None of the patients complained of pain either at the site of injection or harvest areas during follow-up visits and no wound infections were noted in either arm of the study.

Study endpoints in both arms of the study were: 1) complete closure, 2) wound suitable for surgical closure (sutures or skin graft), or 3) end of the study period (3 months).

Data entry and analysis. All data were entered into a spreadsheet (Microsoft Excel Office 2007™). Wound size was calculated by multiplying wound length and width. Statistical significance was set at 0.05.

Statistical testing was performed using the Student’s independent two-tailed t-test for unequal sample sizes with equal variance to compare wound healing rates among cases and controls. This was done to assess whether the rate of healing in the cases was significantly more than the controls over specified time intervals. All statistical analysis was performed using SPSS 16.0™ (Chicago, IL) with the assistance of the Department of Biostatistics, CMC, Vellore.

Results

Forty-eight (48) patients participated in the study; 25 were randomized to treatment and 23 received the control. The majority of patients had type 2 DM—84% and 95% of patients in the treatment and control groups, respectively. All participants had random blood sugar, plasma glucose levels <160 mg %—considered abnormal—at least one single reading (neither group had daily blood sugar readings), were taking an oral hypoglycemic agent or prescribed insulin, and received regular follow-up care for DM treatment; all patients with diabetes were well-controlled on their regular medication through follow-up with the Diabetic Endocrinology Unit, but for the purpose of this study, regular blood sugar levels were not available.

The remaining patients (three in the treatment and one in the control group) had a chronic wound from neglected trauma. One patient in the control arm had bilateral lower limb wounds. All wounds were below knee: three (13%) in the treatment group and two (8%) in the control group had wounds on the bottom of the foot and 12 (52.2%) in the treatment group and 15 (62.5%) in the control group had foot ulcers. Most patients completed the study and patient average age did not differ between the two treatment arms (see Table 1).

Average wound age was 14.28 months in the treatment and 10.21 months in the control group. Average wound area reduction at 2 weeks was 17.4% in the treatment compared to 4.84% in control group (P <0.05). At 12 weeks, the difference in wound area reduction was no longer statistically significant (36.4% in the treatment versus 27.32% in the control group, P >0.05) (see Table 1 and Figure 1).
The proportion of wounds considered healed was higher in the treatment (40%) than in the control group (29.2%, \(P < 0.05\)). Three patients in the treatment group (30% of this group’s healed cases) and one control patient (14.3% of healed wounds in this group) had a skin graft.

Apart from the two cases lost to follow-up due to distance from the clinic, patient adherence with the protocol of care was good. Typical healing progress is shown in Figure 2a,b.

Discussion
The purpose of this study was to compare the rate of healing lower limb chronic wounds in patients with DM using a study treatment and a control. Although three previous reports have shown enhanced wound healing with the use of autologous bone marrow, an increase in wound healing rate was not conclusively or objectively demonstrated. The current study showed that applying bone marrow-derived stem cells to chronic wounds increases the rate of wound healing compared to control. The difference between the two treatments is most pronounced during the first 2 weeks after the injection and then slowly decreases over time (see Figure 1).

In the control group, an initial decrease in the wound area indicated a response to the start of treatment. This decrease perhaps could be attributed to the injection of peripheral blood, which also contains a small percentage of circulating bone marrow-derived stem cells and other factors, or a response to the initial debridement. However, even though over time the decrease in wound area in both treatment groups leveled off, the overall reduction in wound area in the treatment group remains higher than that in the control group.

The observed healing rates in this study are comparable to results of a prospective, longitudinal, intent-to-treat study of a patient population (n = 101) with a wide variety of non-healing ulcers treated using an intensive, multipronged strategy and dedicated wound care providers with results reviewed weekly. During an average of 7.9 weeks, 41.6% healing was observed with 31.6% of patients achieving >50% volume reduction at 7.9 weeks. Even though almost all of the patients in the current study had DM and persons in the treatment group did not receive supportive treatments while those in the control group followed prescribed standard treatment schedules (eg, offloading), 40% of patients in the study treatment arm healed within 12 weeks. Results of a meta-analysis of diabetic neuropathic ulcers treated with standard treatment protocols showed a weighted mean healing rate (ie, percent healed) of 30.9% (95% CI 26.6-35.1) at 20 weeks; a similar analysis at the 12-week point showed a mean healing rate of 24.2% (19.5-28.8). This is less than the healing rate in current study in patients who received bone-marrow injections but

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### Table 1. Study patient, wound, and outcome variables

<table>
<thead>
<tr>
<th>Total follow-up completed</th>
<th>Treatment group (n=25)</th>
<th>Control group (n=23)</th>
<th>(P) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lost to follow-up</td>
<td>2</td>
<td>0</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Average patient age</td>
<td>54 years, 3 months (range: 33–76 years)</td>
<td>58 years, 7 months (range: 28–69 years)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Male: Female ratio</td>
<td>17:8</td>
<td>15:9</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Number of bilateral wounds</td>
<td>0</td>
<td>2</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Average wound age (months)</td>
<td>14.28 (range: 3–123 months)</td>
<td>10.21 (range: 1–37 months)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Maximum wound age (months)</td>
<td>123</td>
<td>37</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Average initial wound size (cm²)</td>
<td>65.32 cm² (SD 0.72)</td>
<td>48.83 cm² (SD 0.37)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Average decrease in wound size at 2 weeks (%)</td>
<td>17.4% (SD 0.40)</td>
<td>4.84% (SD 0.36)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Average decrease in wound size at 12 weeks (%)</td>
<td>36.4% (SD 0.48)</td>
<td>27.32% (SD 0.32)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Number of wounds healed within 3 months</td>
<td>10 (30% skin graft, 85.7% secondary intention)</td>
<td>7 (14.3% skin graft, 85.7% secondary intention)</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

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**Figure 1.** Average wound area during the course of the study.
comparable to the control group where a 27% decrease in wound area was noted at 12 weeks.

Although the initial effect of autologous bone marrow injection into the chronic wounds is evident, the mechanisms of this effect are not clear. If the stem cells were dividing and differentiating\textsuperscript{17,20,28} into the fibroblasts, keratinocytes, and dermal appendages, the wound area curve should not have slowed. Furthermore, the wounds should have epithelialized and closed on their own\textsuperscript{9} instead of requiring skin grafting. It is more probable that the bone marrow-derived cells were fusing with existing epithelial cells, fibroblasts, and myocytes, prolonging their life and enhancing their function. Evidence for this hypothesis is seen in animal studies\textsuperscript{19,24} where the green fluorescent protein (GFP)-labeled cells have been shown to fuse with the dermal cells in wounds on the backs of diabetic mice.

The other possible mechanisms that may help speed healing (see Figure 1) over this short period of 2 to 4 weeks following the intervention are local cytokine factors\textsuperscript{11,15,29} such as TGF-alpha, fibroblast growth factor (FGF), cluster of differentiation (CD) 34+, and CD 14, as well as a local increase in the oxygen tension of the wound.\textsuperscript{3,5,12} The increase in the wound oxygen tension could be the effect of the direct local application to the center of the wound, which is usually low in oxygen tension; a local increase in oxygen tension could have stimulated new granulation tissue (see Figure 3).

All control wounds were chronic; six closed spontaneously. This can be explained by the fact that the largest area in this subgroup was 8.32 cm\textsuperscript{2} and based on a meta-analysis\textsuperscript{8} of 11 clinical trials with 160 patients, complete healing could be assumed by 20 weeks.

Evidence from a large, prospective, multicenter trial\textsuperscript{30} of patients with diabetes and foot ulcers suggests that the healing rate of diabetic foot wounds at 4 weeks is a robust indicator of healing. In the current study, wounds reached a healing plateau at that time, suggesting that any intervention attempting to improve wound healing should be implemented before this period. It is also possible that healing slowed because no supportive measures, such as wound offloading, were implemented.

Because the effects of the one-time intervention in the form of the autologous bone marrow injection are maximal in the first 2 weeks after application and then taper off (see Figure 1), it seems that a second application at the end of this period should be considered. The main purpose of this study was to evaluate the effect of topically applied and locally
injected bone marrow-derived cells on the rate of healing chronic lower limb wounds in patients with DM. The results indicate that this treatment increases the healing rate of chronic wounds, at least during the first 2 to 4 weeks of treatment, compared to whole blood injections (control) with no adverse events. Additional studies to elucidate the treatment mode of action are warranted. Specifically, at this time it is not known how the bone marrow-derived cells behave once injected into the wound, which cell lineage contributes most to the increased rate of healing or granulation tissue formation, or if repeat applications of bone marrow-derived cells would sustain the increased rate of healing. Finally, controlled clinical studies are necessary to confirm the efficacy of this treatment modality in other patient populations and to evaluate its effectiveness when used in conjunction with needed supportive treatments.

Study Limitations

A number of factors limited this study. First, during the study period, researchers were unable to recruit the optimal sample of 30 patients in each group, reducing the ability to detect statistically significant differences. In addition, financial constraints prevented the confirmation of the patients’ clinically assessed vascular status using Doppler ultrasound techniques or toe pressure measurements and no histopathological examination of tissue before and after the bone marrow injection was performed. Finally, wound assessments and dressing changes were completed by the primary investigator, who was not blinded to treatment allocation.

Conclusion

This study, conducted based on a case series with promising results, was designed to ascertain whether it was possible for autologous bone marrow-derived stem cells to improve healing in chronic wounds in persons with DM. Because this was designed to be a translational clinical study, the authors deliberately did not attempt to further process the bone marrow-aspirate or to demonstrate accelerated healing at a cellular level. Compared to the control group, wounds treated with a one-time autologous bone marrow local injection and application exhibited a significantly greater decrease in wound area after 2 weeks. However, this rapid healing rate did not persist over the entire follow-up period and was no longer statistically significant at 12 weeks. The healing rate did not persist over the entire follow-up period with no statistical difference compared to whole blood injections (control) with no adverse events. Additional studies to elucidate the treatment mode of action are warranted. Specifically, at this time it is not known how the bone marrow-derived cells behave once injected into the wound, which cell lineage contributes most to the increased rate of healing or granulation tissue formation, or if repeat applications of bone marrow-derived cells would sustain the increased rate of healing. Finally, controlled clinical studies are necessary to confirm the efficacy of this treatment modality in other patient populations and to evaluate its effectiveness when used in conjunction with needed supportive treatments.

References