

A Prospective, Randomized Clinical Study Evaluating the Effect of Transdermal Continuous Oxygen Therapy on Biological Processes and Foot Ulcer Healing in Persons with Diabetes Mellitus

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Abstract

Hypoxia is a major factor in delayed wound healing. The aim of this prospective, randomized, clinical trial was to compare outcomes of treatment in persons with chronic diabetic foot ulcers (DFUs) randomly assigned to transdermal continuous oxygen therapy (TCOT) for 4 weeks as an adjunct to standard care (debridement, offloading, and moisture). Nine patients (age 58.6 ± 7.1 , range 38–73 years) received TCOT (treatment group) and eight patients (age 59.9 ± 12.6 , range 35–76 years) received standard care alone (control group). Most patients (12) were male, and all had a Wagner I or II foot ulcer for an average of 14 (control group) or 20 months (treatment group). Weekly wound measurements and wound tissue biopsies were obtained and wound fluid collected. Levels of pro-inflammatory cytokines and proteases in wound fluid samples were analyzed using Luminex-based multiplex assays. Tissue-resident macrophages were quantified by immunohistochemistry. At week 4, average wound size reduction was 87% (range 55.7% to 100%) in the treatment group compared to 46% (15% to 99%) in the control group ($P < 0.05$). Changes in cytokine levels (IL-6, IL-8) and proteinases (MMP-1, -2, -9, TIMP-1) at weeks 2 to 4 in wound fluid correlated with clinical findings. CD68+ macrophage counts showed statistically significant reduction in response to TCOT compared to the control group ($P < 0.01$). The results of this study show that TCOT may facilitate healing of DFUs by reversing the inflammatory process through reduction in pro-inflammatory cytokines and tissue-degrading proteases. Additional research to elucidate the effects of this treatment on complete healing and increase understanding about the role of wound fluid analysis is needed.

Keywords: clinical study, foot ulcer, diabetes mellitus, cytokines, healing

Index: *Ostomy Wound Management* 2013;59(11):19–26

Potential Conflicts of Interest: This study was supported by a grant from Ogenix, Inc, Norwood, MA.

Dr. Driver and Dr. Yao are equal contributors to this work.

Wound healing is a complex biological process that requires the successful mobilization and integration of cells for repopulation of the wound.^{1–3} Activated inflammatory cells consume oxygen at a high rate.^{4,5} Coupled with the impaired microcirculation associated with various conditions such as ischemia, the inflammatory process results in hypoxia.^{6,7} Chronic ischemia is a pathological condition that

inhibits normal wound healing and poses a major challenge in the management of diabetes.^{8–11}

The efficacy of topical oxygen (TO) treatment as an adjunct to wound healing in order to resolve the ischemia-mediated hypoxia and restore the wound healing kinetics has been studied since the 1960s.^{12,13} In a small pilot study, Heng et al¹⁴ reported a modified technique for administering

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hyperbaric oxygen with the use of disposable polyethylene bags for the treatment of six patients with 27 chronic arterial leg ulcers; results were compared to arterial ulcers without treatment. The results showed 18 of 27 ulcers (five out of six patients) healed within 6 to 21 days with 50% to 90% reduction in wound size from the baseline after a 3-week period. No one in the control group healed. The treated ulcers were reduced by $7.8\% \pm 1.15\%$ per day compared with $-0.5\% \pm 0.37\%$ in the control patients. Subsequent research using systemic hyperbaric oxygen showed this approach was effective in the management of severe foot infections in diabetic patients.^{15,16} Gordillo et al¹² conducted a nonrandomized study of patients receiving hyperbaric (n = 32) or TO (n = 25) treatments. Hyperbaric oxygen (HBO) seemed to benefit some wounds while not benefit others. Overall, HBO did not result in statistically significant improvements in wound size in the given population over the time monitored in this study ($P = 0.15$, $R^2 = 0.068$). TO treatment significantly improved the wound size ($P < 0.001$, $R^2 = 0.414$). On the other hand, results of later studies did not confirm these findings. For example, Leslie et al's¹⁷ randomized, controlled clinical trial involved a 2-week, topical hyperbaric oxygen treatment of diabetic foot ulcers (DFUs) in 12 patients compared to 16 control patients (standard of care [SOC]). The results revealed progressive and significant reductions in the ulcer areas in both groups on days 7 and 14 ($P < 0.05$ in each group). Ulcer depths also significantly reduced in both groups at day 7 and day 14 ($P < 0.05$ in each group) with no difference between the two groups. Currently, there is no consensus on the benefits of TO treatment, although the technology still holds strong promise based on the pathway of wound healing mechanism through resolution of the hypoxia-mediated inflammatory process that is inherent to DFUs.

Transdermal continuous oxygen therapy (TCOT) provides a continuous delivery of a very low dose (3 mL/hour) of 99.8% pure oxygen directly to the wound site, allowing uninterrupted treatment 24 hours per day, 7 days per week, while TO is administered in two daily 90-minute sessions.¹⁷⁻¹⁹ In a murine diabetic wound model, full-excisional dorsal skin wounds were treated for 10 weeks continuously with pure oxygen (>99.9%) at low flow rates (3 mL/hour). After 6 days, oxygen treatment resulted in a mean reduction of the original wound size by 60.2% as compared with only 45.2% in control mice. After 10 days, oxygen-treated wounds were 83.1% closed compared with 71.2% in wounds on control mice. Reepithelialization was completed after 10 days in 57% of wounds receiving low-flow oxygen treatment.¹⁸ A prospective pilot study¹⁹ in patients with chronic wounds (N = 9) evaluated the application of TCOT. After 4 weeks of treatment, mean wound surface area and wound infection checklist scores were significantly reduced ($P < 0.05$). Signs of bacterial damage also were reduced.

TCOT technology addresses two major issues, which may explain the unpredictability of the previous approaches. First,

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Key Points

- The authors of this study compared the use of transdermal continuous oxygen therapy (TCOT) in patients with diabetes mellitus and foot ulcers to standard care alone.
- During the 4-week study, the percent reduction in wound size was significantly greater in the treatment than in the control group.
- Wound fluid analysis also showed some differences at different assessment times.
- Additional studies of longer duration are needed to confirm the clinical effects observed, and more studies may elucidate the effect of treatments on potential biomarkers of healing.

because of the very low flow rate, the device does not dry out the wound, and a moist wound environment is maintained. Second, continuous delivery of TO creates a constant reversal of the hypoxic state, altering the micro-environment of the wound and enhancing the healing process. Dynamic interactions among cytokines, chemokines, growth factors, and extracellular matrix (ECM) degrading enzymes (proteinase) are integral to chronic wound healing. Previous prospective study²⁰ has shown these were markers associated with status of wound healing.

To evaluate the efficacy of TCOT on nonhealing DFUs, a prospective, randomized clinical study was conducted to determine wound healing and biological markers of tissue response (cytokines, proteinases, and growth factors), as well as the cellular component of the tissues.

Materials and Methods

Patients. Participants with chronic nonhealing DFUs were consecutively enrolled in the study after they provided signed informed consent in the Department of Surgery Limb Preservation and Wound Care Research Clinic at Boston University between November 2010 and March 2011. Study protocol was approved by the Institutional Review Board. Participants were chosen based on the inclusion/exclusion criteria at screening (see Table 1). Eligible participants then were randomized into one of two groups using a block randomization scheme: treatment group patients received SOC including debridement (once a week), boot offloading, and moisture together with TCOT therapy; control group patients received SOC only.

Following screening, all enrolled subjects had a 1-week washout period on SOC and returned for a baseline visit (week 1) for data and sample collection and treatment based on group assignment. With regard to sample collection, visits not completed within ± 2 days were considered a "missed" visit and the participant was scheduled for the subsequent visit.

Table 1. Patient inclusion and exclusion criteria on screening

Inclusion Criteria
Age 18–90 years
Diabetes mellitus, type 1 or 2
Chronic diabetic foot wound (0.5–15 cm ² area)
Wagner grade 1 or 2
TcPO ₂ >30 mm Hg OR ankle brachial index >0.6.
Ulcer size 0.5 cm ² –15 cm ²
Exclusion Criteria
Treatment with noncontact ultrasound during the 4 weeks before this study
Lower extremity malignancy (either limb)
Critical limb ischemia
Local infection of limb with target ulcer
Systemic infection
Pregnancy
End stage renal disease
Severe congestive heart disease
Severe liver disease
Venous leg ulcer with or without diabetes mellitus
Known/suspected lidocaine allergy

Treatment. SOC was provided per treatment protocols for all DFUs in both the control and treatment groups at the Boston University Medical Center Department of Surgery. TCOT, performed by physician investigators, included tissue oxygen insufflation with EpiFlo® oxygen generators provided by Neogenix, LLC (Beachwood, OH). The peri-incisional skin was cleaned with normal saline 0.9% and dried with sterile gauze. A cannula from each EpiFlo generator was positioned over a hydrocolloid dressing (DuoDERM®, ConvaTec, Princeton, NJ) with the tip of the cannula protruding approximately 0.5 cm beyond the dressing. The cannula was secured to the hydrocolloid dressing with Steri-Strip™ tape (3M Health Care, St. Paul, MN). The entire site then was sealed under a Tegaderm™ transparent film dressing (3M Health Care), leaving a 2-inch margin between the tip of the cannula and the edge of the transparent dressing. The transparent film dressing around the extension cannula was pinched to ensure a leak-proof seal. Each new, single-use unit is designed to provide transdermal, continuous oxygen delivery 24 hours per day for up to 15 days. After 15 days, the used unit was discarded and therapy continued with a new unit. The study lasted for 4 weeks (from week 1 through week 5).

Clinical evaluations, wound fluid, and tissue specimen collection. Clinical data including demographics, medical/surgical history, medication, baseline wound characteristics (ie, length and width, ulcer type, location, Wagner grade), and

Table 2. Baseline characteristics of patients

	Control (n=8)	TCOT (n=9)	P value
Age (years)	59.6±7.1	59.9±12.6	>0.05
Gender (n)			
Male	6	6	>0.05
Female	2	3	
Race (n)			
Black	2	2	>0.05
White	5	3	
Hispanic	1	4	
Body weight (lb)	230.6±44.9	199.4±91.7	>0.05
Ulcer age (month)	14.3±26.8	20.7±21.1	>0.05
Ankle brachial index	1.00±0.56	1.02±0.53	>0.05
Pain score	2.8±2.6	1.4±2.7	>0.05
Ulcer volume (cm ³)	1.5±0.6	1.3±0.7	>0.05
Wagner grade			
I	6	3	>0.05
II	2	6	
History of infection (n)			
Yes	6	8	>0.05
No	2	1	
History of amputation (n)			
Yes	1	3	>0.05
No	7	6	

response to treatment were collected by trained study staff at weeks 1, 2, 3, 4, and 5 using case report forms. Wounds were measured by length, width, and depth with a ruler. Wound volume was calculated as length (cm) × width (cm) × depth (cm) at the screening visit (week 0), treatment visits (weeks 1 through 4), and at the post-treatment follow-up visit (week 5). Collected data were de-identified and entered into a database using Microsoft® Access 2003.

Biological samples were collected once every week during the study visit at weeks 1 through 4. Wound fluid was collected using a filter paper (PerioPaper, Oraflow Inc, Smithtown, NY) for 30 seconds as described in prior publications,²⁰ and ulcer tissue was obtained through tissue punch biopsies. Samples were processed according to established protocols and stored at -80° C until time of analysis.

Biological markers of healing in wound fluids. Wound fluid specimens from the filter strips were retrieved by high-speed centrifugation in 100-μl phosphate buffered saline and used for analyses. Once reconstituted, repeated freeze-thaw cycles were avoided and assays were performed consecutively. In order to determine the tissue response to treatment, several panels of biological markers were selected. The analyses

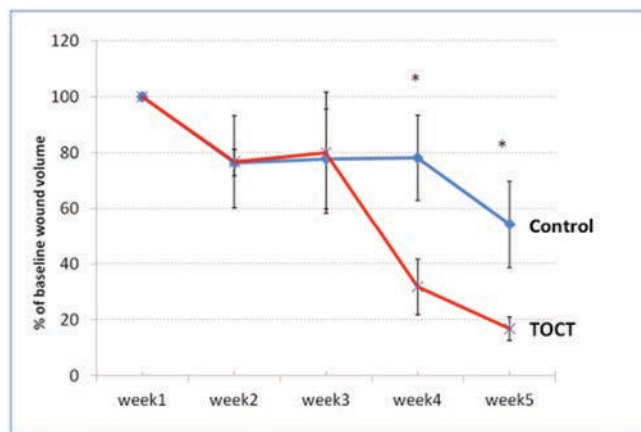


Figure 1. Clinical wound healing (change in wound volume) in response to TCOT. (Y-axis represents percent change of baseline wound volume, * $P < 0.05$)

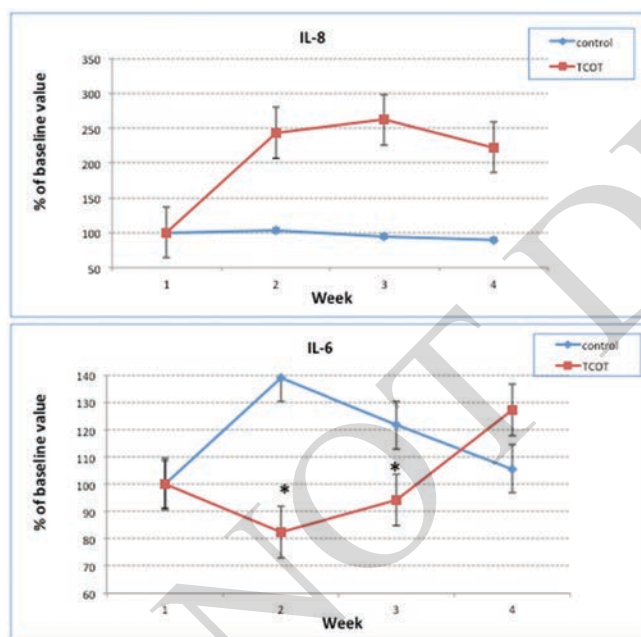


Figure 2. Change in inflammatory cytokines in response to TCOT. (Y-axis represents percent change of baseline cytokines, * $P < 0.05$)

tested represent a wide array of biological processes. These include markers of inflammation, tissue turnover, and wound healing — specifically, cytokines (regulate the inflammatory process: IL-6, IL-1 β , TNF- α , IL-8, and IL-10), tissue degrading matrix metalloproteinases (MMP-1, 2, 8, and 9), tissue inhibitors of metalloproteinases (TIMP-1, 2, and 3), fibroblast growth factor- β (bFGF), transforming growth factor

(TGF- β 1), and nitrous oxide (NO) were measured.

These markers were measured by multiplex xMAP immunoassay (Luminex, Austin, TX) using commercially available panels from Millipore (Chicago, IL) according to the manufacturer's protocols. NO was measured using enzyme-linked immunosorbent assay (ELISA). Data were evaluated against standard curves generated for each analysis, obtained as pg/mL, and reported as percent change over baseline.

Immunohistochemistry. Inflammatory response at the tissue level was studied in biopsied tissues. Tissues were fixed in 4% formaldehyde as previously explained²¹ and kept frozen at -80° C until analysis. Five (5) μ m-thick sections were obtained using a cryostat; every fifth section was analyzed first for the tissue morphology using the standard hematoxylin-eosin stain, and every sixth section was used for the analysis of CD68+ macrophage numbers as determinants of inflammatory infiltrate. CD68 antigen is the most frequently used for the identification of macrophages in immunohistochemistry.²⁰ The protocol followed the recommendations of the vendor (R&D Systems, Minneapolis, MN). FITC-labeled CD68-positive cells were counted using a fluorescence microscope. The data were reported as macrophage numbers per mm² of wound area analyzed.

Statistical analysis. The data were summarized using descriptive statistics. Continuous variables were summarized with mean and standard deviation. Categorical variables were summarized with frequency and percentage. Chi-square test or Fisher's exact test was performed to compare proportions for categorical variables. Correlation coefficients between two continuous variables were calculated, and repeated measures analysis using mixed model on clinical wound reduction was conducted using SAS 9.2 (SAS Institute, Cary, NC). Actual values of cytokines, proteinase, and growth factors were log-transformed before analysis due to skewed distribution. Original data were expressed as percent change compared to baseline (week 1).

Results

Baseline characteristics of patients. Table 2 shows baseline characteristics of subjects in each group. Eight patients were enrolled in the control group and nine in the TCOT group. There were no significant differences ($P > 0.05$) in demographics, clinical features of the ulcers, and comorbidities between the two groups before enrollment, indicating that randomization was successful in this study.

Clinical wound reduction by treatment. Figure 1 demonstrates the clinical healing of the wounds in the test and control groups. Analysis of the longitudinal data using repeated measure mixed models showed a significant difference in percent volume reduction in wound size for TCOT group at week 5 compared to control group (21.8% $\pm$ 20.0% of week 1 versus 49.2% $\pm$ 52.3% of week 1, respectively, $P < 0.05$), demonstrating the clinical efficacy of the TCOT.

Impact of TCOT on inflammation, tissue healing, and

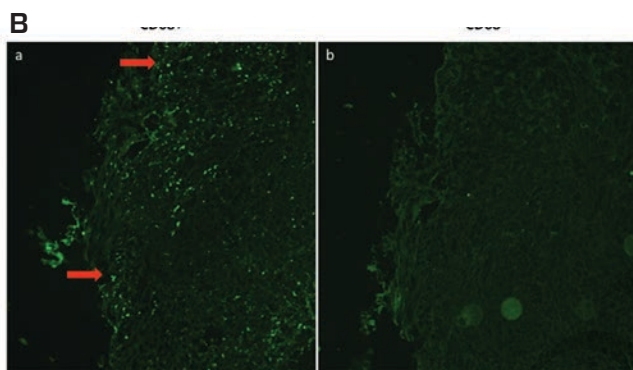
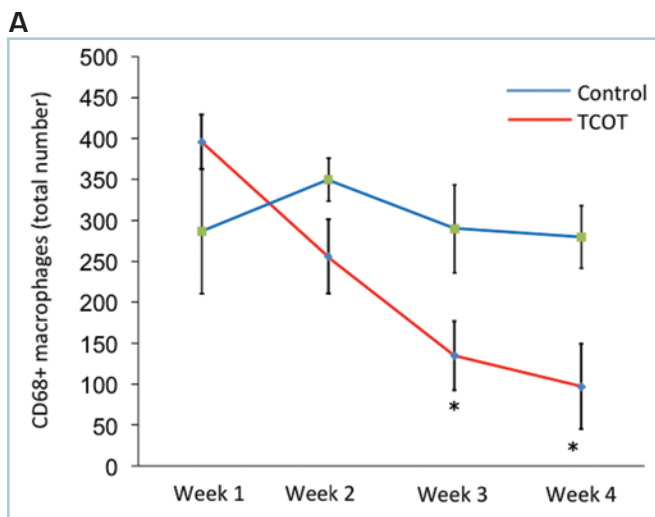


Figure 3 . Macrophage in diabetic foot ulcers
A) Macrophage counts in tissues (* $P < 0.05$);
B) Representative images for the macrophage infiltration in diabetic foot ulcers.

regeneration. Figure 2 demonstrates the levels of IL-8 and IL-6 in wound fluids during the course of therapy. Average levels of IL-8 increased up to 260% of baseline (week 1) in the treatment group and remained level in the control group, whereas the average levels of IL-6 increased to $139.2\% \pm 52.9\%$ of baseline (week 1) after 2 weeks in the control group and decreased to $82.4\% \pm 25.2\%$ of baseline (week 1) in the treatment group ($P < 0.05$), suggesting the macrophage-induced inflammatory cytokine response would be a target for the TCOT. Levels of IL-1 β , TNF- α , and IL-10 were not detectable in most of the analyzed samples.

Macrophage counts were relatively unchanged in the control group but reduced from 410 ± 49 at week 1 to 97 ± 52 at week 4 in the treatment group ($P < 0.01$) (see Figure 3A). Figure 3B shows representative images for the macrophage infiltration after 3 weeks in one of the wounds.

Changes in MMP-1 and TIMP-1 levels were modest, with the largest increase from 215% to 288% of baseline (week 1) observed in MMP-1 levels in the treatment group after week 1. TIMP-1, an inhibitor of MMP-1, also increased in the treatment group. None of the differences was statistically

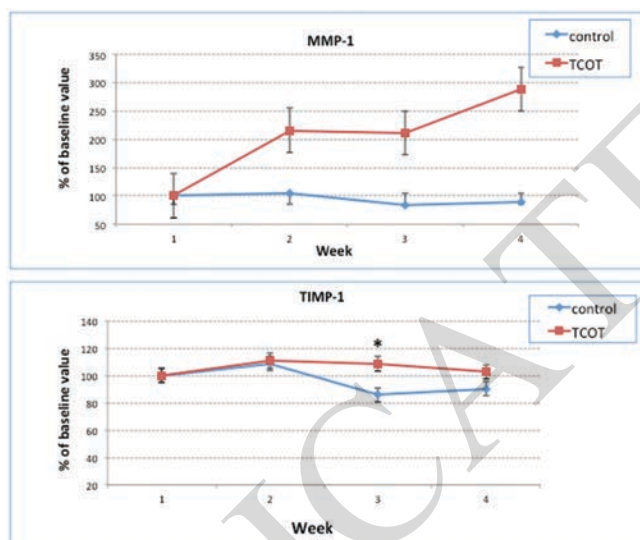


Figure 4. MMP-1 and TIMP-1 levels in diabetic foot ulcers.
(Y-axis represents percent change of baseline MMP or TIMP levels,* $P < 0.05$)

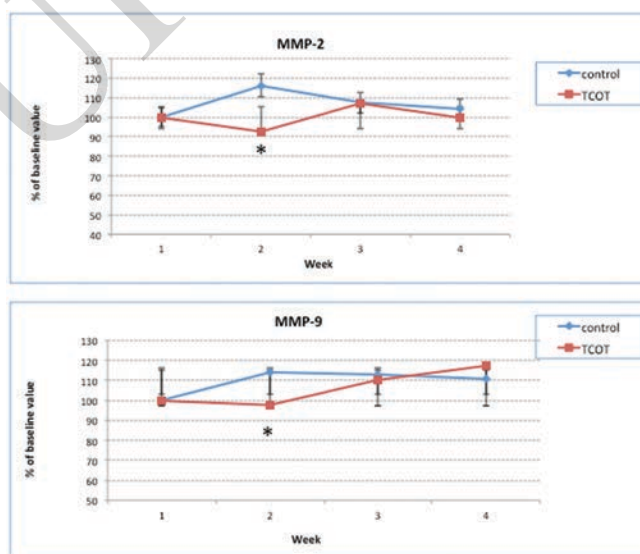


Figure 5. MMP-2 and MMP-9 levels in diabetic foot ulcers.
(Y-axis represents percent change of baseline MMP or TIMP levels,* $P < 0.05$)

significant. Average MMP-2 and MMP-9 levels were both significantly lower in the treatment than in the control group after 2 weeks ($P < 0.01$ and $P < 0.05$, respectively) but increased during the next 2 weeks. By contrast, in the control group average MMP-2 levels increased during the first 2 weeks and then decreased to baseline (week 1) levels. MMP-8, TIMP-2, and TIMP-3 levels were not detectable in the fluid of treatment or control group wounds.

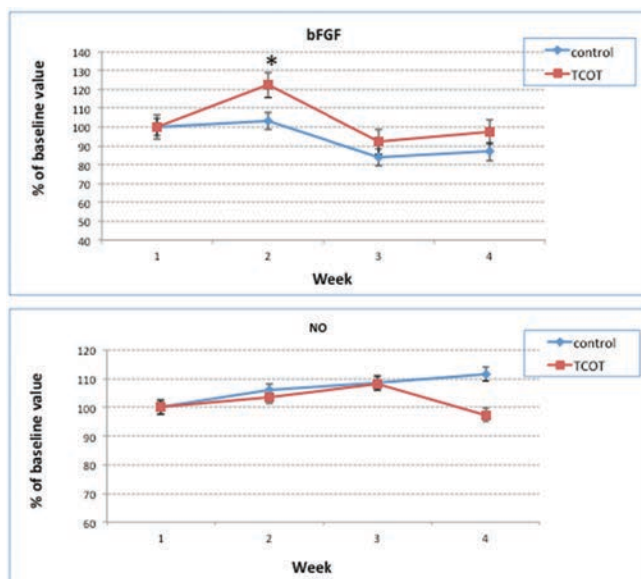


Figure 6. bFGF and nitrous oxide (NO) in response to treatment. (Y-axis represents percent change of baseline bFGF or NO levels, * $P < 0.05$)

Average bFGF levels in wound fluids increased during the first 2 weeks in the treatment group, followed by a drop to baseline levels at week 3 and 4. Percent change in control wound fluid bFGF levels was minimal and generally negative (see Figure 6). Changes in NO levels also were minimal and not significantly different between the two treatment groups. TGF- β 1 levels were not detectable in the wound fluid samples.

Discussion

The aim of this study was to evaluate the efficacy of TCOT on nonhealing DFUs and identify potential pathways through which the healing may take place. Similar results were not found from other investigators performing human study. In a murine diabetic wound model,¹⁹ full-excisional dorsal skin wounds were treated for 10 weeks continuously with pure oxygen (>99.9%) at low flow rates (3 mL/hour). After 6 days, oxygen treatment resulted in a mean reduction of the original wound size by 60.2% as compared with only 45.2% in control mice. After 10 days, oxygen-treated wounds were 83.1% closed in size, compared with 71.2% in wounds on control mice. In a human study,²⁰ the application of TCOT was evaluated in nine patients with chronic wounds. After 4 weeks of treatment, mean wound surface area and wound infection checklist scores were significantly reduced. The current study authors' preliminary study²⁰ indicated, after 4 weeks, wound reduction was approximately 87% (55.7% to 100.0%) in the treatment and 46% (15% to 99%) in the control group, and the difference between treatment- and control-treated wounds was statistically significant at weeks 3 and 4.

Wound fluid analysis, including assessment of cytokines, proteinases, and growth factors in the wound fluid as well as the macrophage numbers, suggested TCOT affects macrophages, which may be a cellular marker of resolution of inflammation as well as restoration of a healthy tissue turnover.²⁰

Some of the cellular and humoral components of inflammation and wound healing were found to be affected by the treatment at different times, but the results were not consistent. To this end, not only the technology itself, but also the observed wound healing and biomarker dynamics in response to TO delivery may represent substantial breakthroughs. These biomarkers can be monitored in the wound fluid obtained during the treatment of DFUs through a non-invasive collection method. In addition, immunofluorescence was used to study the macrophage infiltration in tissues using the biopsies obtained during the excisional treatment of the wounds. This approach has several implications. One of the main implications is the identification of potential mechanisms in wound healing in DFUs. Taken together, these data may add to the understanding of how DFUs progress and which targets can be used for therapeutic intervention. Collectively, these methods and findings represent new avenues for further research and testing of new compounds.

The data support the notion that macrophages play a major role and are critical to the inflammatory response and tissue turnover in DFUs.^{22,23} In addition to the actual counts of the cells, cytokines such as the IL-6 provide substantial evidence that these cells are important in the resolution of inflammation and the transformation of the wound care into a healing phase.^{24,25} One interesting finding in this study was the demonstration that epithelial cells also may play a role and could be the mediators during wound healing in DFUs by their interaction with the fibroblasts. This is supported by the fact that MMP-2 and MMP-9, which are critical for the epithelial cell function and fibroblast-mediated wound healing, have been substantially suppressed at an early phase of wound healing when the treatment included TCOT. Because all of the DFUs were chronic, they are colonized with bacteria. It is likely the inflammatory markers (eg, MMP-2 and -9) could be a result of the "antibiotic effect" of oxygen on bacteria. In addition, chemotactic migration, mediated by increased IL-8, may be mediated by the epithelial cells, while bFGF, which is critical for the fibroblast function,^{2,8} all are impacted by the TCOT compared to the control treatment. In summary, these findings support that TCOT not only resolves the inflammation, but also helps restore tissue turnover.

Previous research on oxygen treatment for nonhealing DFUs has suggested this approach has value.^{12,13,26} On the other hand, previous studies failed to reach a consensus.¹⁷ Most likely, the lack of consistency is the result of various technologies used to deliver the oxygen. In this study, a continuous flow of oxygen was used. This aspect of the technology requires further testing in larger clinical trials, but based

on the evidence presented, this strategy supports that an incessant flow of oxygen would be key to predictable healing. The technology exposes the skin to oxygen using a very low flow rate, which prevents the tissues from drying and further helps hydrate the tissues during healing. Notably, TCOT is a noninvasive, topically applied treatment that can be initiated in any care setting, allowing the patient to be ambulatory. Wound dressings are usually changed every 3 to 7 days per care plan, presenting an easy-to-manage clinical protocol.

Limitations

An important limitation of this study is that treatment was limited to 4 weeks and wounds were not followed until healing, so time to healing is not known. In addition, as with similar studies, the amount of wound fluid that could be obtained was limited, especially during the healing phases of the DFUs. This limitation has been overcome by the use of novel technologies such as multiplex assays. Yet the availability of some of the major components of inflammation in DFU fluids such as the TNF- α is limited and below the detection limits. On the other hand, many other cytokines such as the IL-6²² can be tested, which may provide similar outcomes. Therefore, the technology is new and will provide new insights to the treatment of this serious clinical problem.

The small sample size in this study also limits the ability to discern additional information that may be helpful for a more complete understanding of the mechanism of this therapy. Also, the process and techniques for concurrently evaluating mechanism of action and wound healing is still in its infancy and will benefit by larger and more detailed work.

Conclusion

TCOT may facilitate healing of DFUs by reversing the inflammatory process through reduction in pro-inflammatory cytokines and tissue-degrading proteases. Further studies to identify and validate the diagnostic biomarkers predicting the development and prognosis of chronic wound treated by TCOT are warranted. ■

Acknowledgment

This study was supported by Ogenix Inc (Norwood, MA). The clinical trial, data collection, analysis collection, and the manuscript was the responsibility solely of the investigators/authors.

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