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Topical Oxygen Therapy Induces VEGF Expression and Improves Closure of Clinically Presented Chronic Wounds

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Abstract

1. Chronic wounds, especially in diabetics, represent a serious threat to human health.
2. Correcting a compromised state of tissue oxygenation by the administration of supplemental O_2 is known to benefit wound healing. Beyond its role as a nutrient and antibiotic, O_2 supports wound healing by driving redox-signaling.
3. HBO (hyperbaric oxygen) therapy is widely used and approved by CMS to treat specific ulcerations. The current literature supports that approaches to topically oxygenate wounds may be productive.
4. Here, we present the results of two simultaneous studies testing the effects of HBO and portable topical oxygen (TO) therapies. These two therapeutic approaches have several contrasting features.
5. A total of 1854 patients were screened in outpatient wound clinics for non-randomized enrollments into the HBO (n=32, 31% diabetic) and TO (n=25, 52% diabetic) studies.
6. Under the conditions of the current study, HBO treatment seemed to benefit some wounds while not benefiting the others. Overall, HBO did not result in statistically significant improvements in wound size in the given population over the time monitored in this study.
7. TO significantly improved wound size. Among the three (VEGF, TGFβ1 and COL1A1) O_2-sensitive genes studied in wound-edge tissue biopsies, TO treatment was associated with higher VEGF165 expression in healing wounds. Expression of the other genes mentioned was not affected by TO. All of the genes studied did not significantly change in expression in patients of the HBO study. This work establishes a link between VEGF gene expression and healing outcome for TO therapy.
8. Taken together, this report presents evidence demonstrating that TO treatment benefits wound healing in patients suffering from chronic wounds. TO treatment is associated with induction in VEGF expression in the wound edge tissue and improvement in wound size.

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Keywords

redox; wound therapy; wound care; tissue repair; wound patient; topical therapeutics; skin; translational research

Introduction

Hypoxia, caused by disrupted vasculature as well as complicating vasculopathies and other systemic limitations, limits wound healing. Correcting a compromised state of tissue oxygenation by the administration of supplemental O_2 benefits wound healing in the peri-operative and outpatient settings [1]. Clinical trials have shown that keeping patients warm and administering supplemental O_2, both of which enhance wound oxygenation, decreases the rate of wound infection in surgical patients and shortens the average length of hospitalization [2,3]. Beyond its role as a nutrient and antibiotic, O_2 supports wound healing by driving redox-sensitive gene expression and signal transduction which influences a wide array of healing responses [4–6].

Clinical use of O_2 to promote wound healing began in the 1960's with administration of systemic HBO to treat wounds [7]. Today, HBO therapy is approved by the Center for Medicare and Medicaid Services in the United States to treat specific ulcerations. Our own laboratory has noted beneficial effects of HBO therapy in restoring wound healing that was impaired by psychological stress[8]. Encouragingly, recent reports have demonstrated that HBO therapy may mobilize bone marrow-derived endothelial progenitor cells which could benefit the healing of chronic wounds affected by diabetes and peripheral arterial disease [9]. On the down side, HBO poses the threat of oxygen toxicity in specific cases [10–12]. This risk may be managed by adopting a personalized approach for HBO therapy where the treatment specifically aims at addressing wound hypoxia on a case by case basis. Although HBO is a clearly promising mode of wound therapy, it requires extensive facilities which may not be available to all patients. Furthermore, a population of wound patients may simply not qualify or consent to receive HBO therapy. The approach to topically oxygenate wounds using a variety of approaches is distinct from the conventional HBO therapy in many ways. For example, topical approaches do not involve high pressure, are not systemic in nature and therefore do not pose the uncommon risk of systemic oxygen toxicity [13]. The hypothesis that wounds may benefit when oxygenated topically is supported by the current literature[13–16].

Among many known growth factors, VEGF is believed to be the most prevalent, efficacious and long-term signal that is known to stimulate angiogenesis in wounds [17]. This work represents the side-by-side presentation from the result of two simultaneous studies testing the effects of HBO therapy and topical oxygen (TO) therapy, respectively. The goals were to examine changes in wound closure outcomes and in the expression of oxygen-sensitive genes including VEGF in biopsies collected from the wound-edge tissue.

Materials and Methods

Study Design

This study was approved by the Institutional Review Board of The Ohio State University. Subjects were duly consented and enrolled at Ohio State University's Comprehensive Wound Center (CWC) outpatient sites. At total of 1854 patients that visited our wound clinics were screened to successfully enroll 57 patients in the HBO and TO studies. The inclusion criteria were: (a) age: 30–70 years; (b) the wound had been present for at least 4 weeks; (c) patients were not immunosuppressed or therapeutically anticoagulated; (d) patients were able to give their own consent. The selected age group represents >90% of the patients seen in our wound

clinics. Patients were excluded if they had previously received oxygen therapy for the wound being studied. The type of oxygen therapy administered was based upon the clinical decision of the physician managing the wound. Patients that did not qualify for HBO were asked if they wanted to enroll in the TO study. In order to qualify for HBO therapy, patients were required to meet CMS criteria which include standards that have been established for fourteen clinical diagnoses that are currently approved for HBO.

Wound-edge tissues were rapidly freed from blood by rinsing in saline and snap frozen in liquid nitrogen. For those subjects who consented, wound-edge biopsies were obtained three time points (T_0, T_1 and T_2) during the 14 week study period. Three mm punch biopsy was performed exactly on the perimeter of the wound which represented the wound edge. The tissue harvested was immediately freed of blood using ice-cold saline and placed in OCT and snap-frozen in liquid nitrogen. Wound dimension recording was performed at all of these time-points on all patients enrolled. The three time points were defined as follows:

β-actin	GTACCACTGGCATCGTGATGGACT CCGCTCATTGCCAATGGTGAT
$VEGF\text{-}A_{165}$	TGCCCACTGAGGAGTCCAACAT CACGTCTGCGGATCTTGTACAAACA

T_0, just before the first round of oxygen therapy

T_1, seven weeks into the study or upon meeting specific criteria set for the HBO and TO studies whichever came first. For the HBO study, completion of 50% of therapy was set as the criterion. This would refer to say completion of five out of a total of ten dives prescribed. For the TO study, 50% wound closure was set as the criterion.

T_2, fourteen weeks into the study or wound closure whichever came first.

Supplemental Oxygen Therapy

HBO was administered to patients that met specified CMS criteria for receiving HBO therapy. It was administered at the CWC outpatient clinics under the supervision of physicians according to the Undersea and Hyperbaric Medicine Society (UHMS) guidelines [18]. Sechrist model 3200 chambers (Sechrist Industries, Anaheim, CA) were used for the HBO treatments.

The first TO treatment for all patients was performed in outpatient sites of CWC so that pre-treatment wound-edge tissue biopsies could be obtained and patients could be instructed on the use of the device. Treatments, other than the T_0, T_1 and T_2 time-points during which biopsies were collected, were performed in the patients' homes. Treatments which followed the collection of wound-edge biopsies were performed in the clinic. The TO device (GWR Medical Inc., Chadds Ford, PA) is a single use disposable device that connects to a portable oxygen source as described previously [19]. The device inflates to approximately 1 atmosphere of pressure and has a release valve in the event of excess pressure build up. Representatives from the manufacturer of the TO devices provided patients with instruction on device usage. TO was administered to the wound for 90 minutes per day for 4 consecutive days in a week followed by three days of no oxygen supplementation. This cycle was repeated each week. TO therapy was discontinued at the discretion of the managing physician.

RNA Isolation

Total RNA was isolated from wound biopsy tissue material stored in liquid nitrogen using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA). Samples were placed in a 2 ml microcentrifuge tube with RLT buffer from the RNeasy Fibrous Tissue Mini Kit and 5 mm stainless steel beads. Tissue samples were disrupted and homogenized using a TissueLyser

equipment (Qiagen, Valencia, CA). Tissue disruption was carried out twice for 3 minutes each at 20–30 Hz.

mRNA Quantification

mRNA were quantified by real-time PCR assay using double-stranded DNA binding dye SYBR green-I as described previously [6,20,21]. Gene expression results were standardized relative to β-actin. The primer-set used for the individual genes were as follows:

Primer sets for TGFβ-1 (PPH00508A) and collagen1A1 (PPH01299E) were obtained from SuperArray Bioscience Corporation (Frederick, MD).

Statistical Analyses

Patient demographics were compared across the two studies (HBO and TO) in order to present a comparative account of the two study populations. Differences in normally distributed continuous variables were tested using the two-sample *t*-test while continuous variables that were not normally distributed were tested using the Wilcoxon rank-sum test. Differences in categorical variables were tested using Fisher's exact test. Wound volume, in cm^3, was calculated from the wound surface area and depth as recording during standard clinical practice. The wound volume data was transformed using a cube root for variance stabilization and normality assumptions [22–26]. The difference in the cubed root of the final minus the cubed root of the initial wound volume was regressed on oxygen treatment (HBO or TO) and adjusted for the cubed root of the initial wound volume. Covariate interactions with the treatment variable were considered significant if the p-value ≤ 0.05 and the scale of the cubed root of the initial volume was tested using fractional polynomials [27]. Gene expression data was collected from only those patients who consented to provide biopsies. Some patients provided biopsies for one time point but not the other. Patients from whom paired gene expression data was available for the two time points being compared statistically were included in the relevant analyses. For presentation of gene expression data, wound closure was dichotomized *a priori* as healing if the final wound volume was less than or equal to the initial wound volume and not healing if the final wound volume was greater than the initial would volume. Wound size that got bigger therefore fell into the non-healing category. This categorization was solely for the purposes of grouping and had nothing to do with the testing of efficacy (as in Fig. 3) where a ratio of initial:final wound volume of 1 was interpreted as no effect. Data from VEFG, TGFβ1, and collagen 1A1 gene expression measurements were natural log transformed and regressed on dichotomized wound healing and adjusted for other covariates. This linear regression was run separately for both studies *i.e.* HBO and TO. One-sample *t*-tests were used to compare at various time points the VEGF ratio (one time point : another time point as indicated in the respective illustrations) to 1.0 (reflecting no change) for only those subjects that were in the healing group. All analyses were conducted using Stata 10.0, Stata Corporation, College Station, Texas.

Results

A total of 1854 patients were screened in the outpatient clinics of CWC for enrollments into the HBO and TO studies. The demographics of the subject populations of the HBO and TO studies were comparable as illustrated in Table 1. A total of fifty-seven patients with chronic wounds were enrolled. Based on the assessment of the respective physicians, thirty-two patients qualified for HBO therapy. Twenty-five patients consented to receive TO therapy. The mean age of the subjects in the HBO and TO studies was 52.3 and 54.7 years, respectively. The subject population of the HBO study primarily consisted of men who represented 90.6% of the total population. The subject population in the TO study was more even balanced for gender-distribution. Fifty-two percent of the TO subject population were men while the balance of

48% were women. Wound site was confined to either the trunk or lower extremity and there were no significant differences between the subject populations of the HBO and TO studies. The fractions of known diabetics in the HBO and TO studies were 31% and 52%, respectively. The wound etiology and location for each study are illustrated in Figure 1. While enrollment into the two treatment modalities was not randomized thus making direct comparison of the findings between the two studies not possible, the study design enabled the determination of whether the two modalities share a common mechanism of action.

The wound volume data was transformed using a cube root for variance stabilization and normality assumptions [22–26]. The difference in the cubed root of the final minus the cubed root of the initial wound volume was regressed on oxygen treatment (HBO or TO) and adjusted for the cubed root of the initial wound volume. Analysis of the effects of HBO on wound closure was based on a model which included the cubed root of the initial volume as a covariate and the best fit was determined to be linear using fractional polynomials. The untransformed results are shown in Figure 2. The diagonally dashed line of reference represents no change in wound volume in response to treatment. Observations (black dots) on or above the dashed line represents no benefit in wound size in response to treatment. Observations plotted below the dashed line of reference represent that the treatment improved wound closure outcome. The solid line represents the linear regression model based on wound closure data as collected from the HBO study (Fig. 2). Covariate interactions with the treatment variable were considered significant if the p-value ≤ 0.05 and the scale of the cubed root of the initial volume was tested using fractional polynomials [27]. In the case of the HBO study, the p-value was observed to be 0.150 (R^2=0.068). Thus, under the conditions of the current study, HBO treatment did not result in significant improvements in wound closure (Fig. 2). The statistical approach to determine the efficacy of TO treatment on wound closure outcomes was exactly identical to the approach described above for the HBO study. The untransformed results related to changes in wound volume in response to TO treatment are illustrated in Figure 3. In the present study, TO treatment significantly improved wound closure by decreasing wound volume. For the TO study, the regression line shown in solid was significantly (p-value, 0.001, R^2=0.414) different from the reference dashed line (Fig. 3).

Next, we turned towards the examination of O_2-sensitive genes in the wound-edge tissue biopsies collected from consenting patients. Three genes, VEGF, TGFβ1 and collagen 1A1 (COL1A1) were selected based on their known sensitivity to oxygen and functional relevance of the gene products to wound healing. Each subject had a baseline level of gene expression determined by using the T_0 biopsy for real-time PCR measurements, since they were obtained prior to any exposure to oxygen therapy. All measured mRNA expression levels were standardized against β-actin mRNA expression. The effect of supplemental oxygen therapy was analyzed by measuring the relative change in target gene expression for each individual compared to their baseline (T_0). This allowed each patient to serve as their own control for these analyses. Relative change in gene expression was calculated dividing the rate of gene expression at a selected time-point by the observed baseline. All data were log transformed to perform statistical comparisons between ratios.

At the intermediate time-point T_1, results adjusted for age of each subject were analyzed. Both TGFβ1 as well as COL1A1 did not exhibit any statistically meaningful responsiveness to TO treatment (Table 2). In contrast, VEGF expression was significantly higher in TO treated healing wounds (Table 2). This finding led to our emphasis on the study of VEGF. The expression of VEGF was analyzed also for the final time-point T_2. Analysis of the VEGF expression data from the three time-points in TO treated healing wounds are illustrated in Table 3. Interestingly, when the initial time-point (T_0) was compared with the intermediate time-point (T_1), a trend in favor of TO-induced VEGF expression in the wound-edge tissue was noted (Table 3). However, the observation was not statistically significant (p-value = 0.07).

This effect became statistically significant if the initial time-point (T_0) was compared with the final time-point (T_2). Plotting of individual VEGF response data points against log-transformed wound closure (T_2:T_0) demonstrated the results presented in Table 3 on a individual basis (Fig. 4). Data points to the left of the vertical line (wound volume ratio < 1) indicate that the wound was smaller than the original size while observations to the right indicate the wound got bigger with time as represented in the Y-axis. Data points below the horizontal line (VEGF ratio < 1) indicate lowering of VEGF gene expression over the specified time period. Data points above the horizontal line (VEGF ratio > 1) indicate induction of VEGF gene expression. Of note, all the healing wounds in the TO treatment study showed VEGF induction (Fig. 4c), an effect that is listed to be statistically significant in Table 3.

The difference in gene expression over dichotomized (healing vs. non-healing) wound outcomes for the HBO study was not statistically significant for VEGF, TGFβ1, or collagen 1A1 for any of the time intervals compared (Table 4). Analysis of the VEGF expression data from the three time-points in HBO treated healing wounds are illustrated in Table 5. HBO did not cause a significant increase in VEGF expression at any interval (T_1:T_0 or T_2:T_1) or cumulatively over the course of treatment (T_2:T_0). In the contrast to the findings noted in the TO study, changes in VEGF expression in the wound-edge tissue of patients enrolled in this study were not statistically significant. However, a closer look at the scatter plot data reveals that in numerous subjects HBO treatment did markedly induce VEGF. However, there were some subjects did not respond. Therefore, taken together, the effect was not statistically significant (Fig. 5). It would be of interest to identify the conditions under which HBO is effective in inducing VEGF in the wound tissue of patients.

Discussion

Achieving closure in a chronic wound requires provision of adequate oxygen delivery to the tissue, adequate protein and other nutritional factors, a moist environment, an appropriate inflammatory *milieu*, debridement, appropriate management of infection, and correction of contributing medical diagnoses. Hypoxia is a limitation that is commonly noted in problem wounds. Achieving appropriate levels of tissue oxygenation to support healing represents a major requirement in the treatment of chronic wounds [28–30]. Systemic HBO represents a therapeutic modality that is widely utilized as a standard of care in numerous wound clinics. Adjunctive HBO therapy has been demonstrated to be clinically effective in several studies [31–34]. In the treatment of hypoxic and ischemic wounds, the most important effects of hyperbaric oxygenation are the stimulation of fibroblast proliferation and differentiation, increased collagen formation and cross-linking, augmented neovascularization, and the stimulation of leukocyte microbial killing. Ischemic soft tissues also benefit from hyperoxygenation through improved preservation of energy metabolism and reduction of edema [35]. In addition, HBO therapy may have important effects on the biology of cytokines and other mediators of inflammation [36]. In patients whose wounds were favorably affected by HBO therapy, increased levels of nitric oxide were observed in response to HBO treatment [37]. Therapeutic HBO can increase the mobilization of endothelial progenitor cells from the bone marrow into peripheral blood which has the clear potential of benefiting wound healing in patients affected by diabetes and peripheral arterial disease [9]. A randomized blind study examining the effects of HBO therapy on experimental wounds in humans noted that the HBO group significantly benefited from a 42% reduction in wound hyperemia, a 35% reduction in the size of the lesion, and a 22% reduction in wound exudation [38]. The favorable effects of HBO in clinical studies are supported by numerous experimental studies demonstrating that HBO therapy improves tissue oxygenation [39–44]. Our own studies in mice have demonstrated that impairments in wound closure caused by psychological stress can be corrected by HBO therapy [8].

Factors that have limited a wider acceptance of HBO therapy in mainstream wound care include inconsistent results in a clinical setting [45,46], arguable flaws in some study designs [47] and insufficient number of clinical trials [48,49]. The high cost of providing HBO has also raised concerns [47]. In that vein, the cost of not accepting a potentially productive treatment modality may be also counter-argued [50]. Our observation that HBO therapy did not favorably impact wound closure outcome in the total population studies is on one hand consistent with the previous literature reporting lack of efficacy of HBO under specific conditions. On the other hand, however, examination of individual outcomes revealed that the lack of efficacy of HBO on wound closure was not uniformly noted over the entire population of subjects studied. One may rationally argue that the HBO study included responders as well as non-responders. The mixed findings resulted in a lack of statistical significance under the conditions specified. It becomes increasingly important to identify the specific conditions under which HBO becomes effective in favorably affecting wound outcomes. Of note in that context is the fact that HBO superoxygenates tissues to levels multi-fold higher than their baseline pO_2 [51–53]. From a mechanistic standpoint, it is important to recognize that while correction of wound hypoxia is desirable, excessive oxygenation may pose the risk of oxygen toxicity and cell cycle arrest [11,54–56]. This notion is supported by findings of a mathematical model developed to assess the effect of wound tissue pO_2 on healing outcomes [57]. We posit that a personalized approach to utilize HBO therapy that is based on achieving a prescribed wound tissue oxygen tension as opposed to utilizing the same regimen for all patients will provide more consistent favorable beneficial effects of HBO therapy.

Topically applied oxygen gas is able to modestly raise the pO_2 of the superficial wound tissue [15]. Of note, a series of recent observations demonstrate that the topical route of wound oxygenation may be effective in benefiting wound healing [13–16]. Both HBO as well as TO devices are FDA approved. Recently, FDA has proposed to reclassify TO devices from the most stringent class III (pre-market approval) to a safer class II (http://www.fda.gov/cdrh/ode/guidance/1582.html). Encouraging results obtained from the use of TO in both clinical [13] as well as pre-clinical [15] settings warrant further interest addressing the significance of TO in treating problem wounds in a clinical setting. If proven to be effective, portable TO therapy has the added advantage of benefiting a much larger potential patient population especially under conditions of public disaster and in a field-setting where HBO therapy may not be applicable. In this context it is important to recognize that although HBO and TO both seek to oxygenate wounds, they are quite different treatment approaches. The current study examined the effects of TO in patients that were not selected for HBO therapy. For the first time, the effects of TO has been studied not only to examine closure outcomes in a clinical setting but to also gain mechanistic insight on how TO therapy may influence wound healing.

The state of tissue oxygenation is a key determinant of inducible VEGF expression and angiogenesis. While hypoxia can initiate neovascularization by inducing angiogenic factor expression, it cannot sustain it. A threshold level of oxygenation is required to support the metabolic needs of tissue remodeling. Acute hypoxia facilitates the angiogenic process [59] while chronic hypoxia impairs wound angiogenesis [60]. Sustained hypoxia causes death and dysfunction of tissue. VEGF is a major long-term angiogenic stimulus at the wound site. On one hand, hypoxia is a potent trigger of inducible VEGF expression [61]. On the other hand, hyperoxia induces VEGF as well [54,62–65]. This study provides the first evidence that supplemental TO treatment significantly induces VEGF expression in the wound-edge tissue of patients suffering from chronic wounds. This work is consistent with previous findings suggesting that TO treatment may induce wound angiogenesis [66].

Given the essential role of angiogenesis in wound healing, it is not surprising that there was a statistically significant correlation between VEGF and healing outcomes, as shown with TO

therapy. A similar effect was expected with HBO, but was not observed. Healing responses induced by HBO in this patient population occurred by means that were independent of VEGF expression. This hypothesis is consistent with the current literature [9,37]. One potential reason for this is the differences in the levels of wound tissue oxygenation achieved with each of these modalities. The levels of oxygenation achieved with 2 atmospheres of pressure in HBO therapy can range from 300–1200 mmHg, which far exceeds the pO_2 of healthy skin or that achieved in the wound tissue with TO [15,19]. This work establishes a link between VEGF gene expression and healing outcome for TO therapy. If validated in a larger population, this finding could help identify a biomarker to gauge response to TO treatment. Taken together, this study presents evidence supporting that TO treatment may benefit wound healing in patients suffering from chronic wounds. TO treatment is associated with induction in VEGF expression in the wound edge tissue and improvement in wound closure outcome. Approaches to topically oxygenate exposed dermal wound tissue warrant serious interest.

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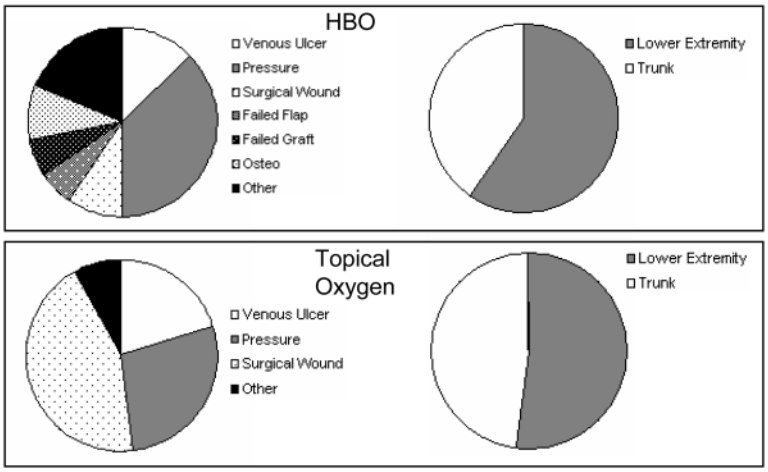

Figure 1.
Wound etiology and locations for patients enrolled in the HBO and topical oxygen studies.

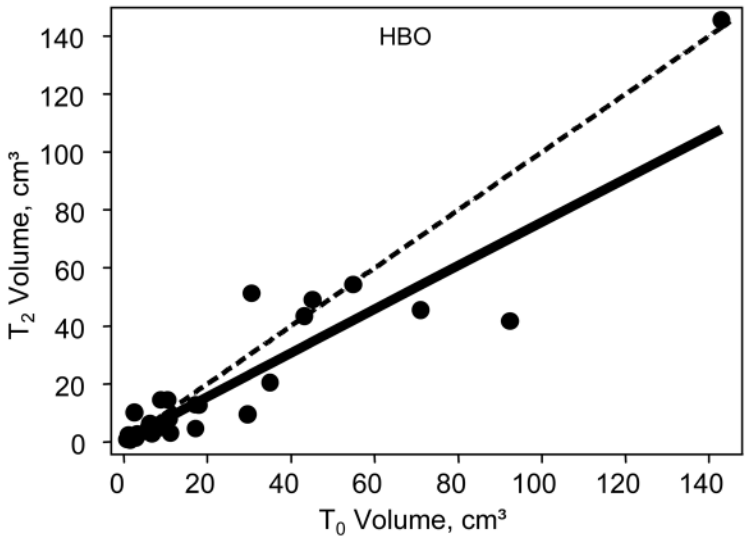

Figure 2. Wound closure in response to HBO treatment

Analysis of the effects of HBO on wound closure was based on a model which included the cubed root of the initial volume as a covariate and the best fit was determined to be linear using fractional polynomials. The untransformed results are shown here. The diagonally dashed line of reference represents no change in wound volume in response to treatment. Observations (black dots) on or above the dashed line represents no benefit in wound size in response to treatment. Observations plotted below the dashed line of reference represent that the treatment improved wound closure outcome. The solid line represents the linear regression model based on wound closure data. The solid line was tested to be statistically not significantly different from the reference dashed line (p-value = 0.150, R^2 = 0.068)

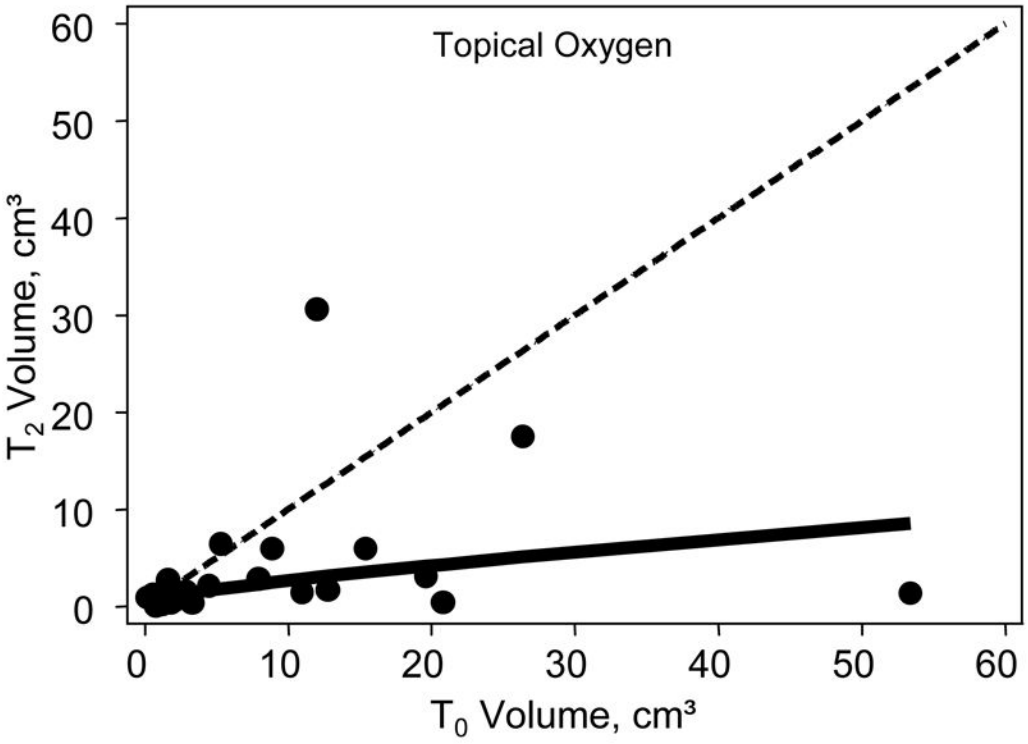

Figure 3. Wound closure in response to topical oxygen treatment

Analysis of the effects of topical oxygen on wound closure was conducted as described in the legend of Figure 2. The solid line represents the linear regression model based on wound closure data from the topical oxygen study. The solid line was tested to be significantly different from the reference dashed line (p-value < 0.001, $R^2 = 0.414$) indicating that topical oxygen treatment improved wound closure.

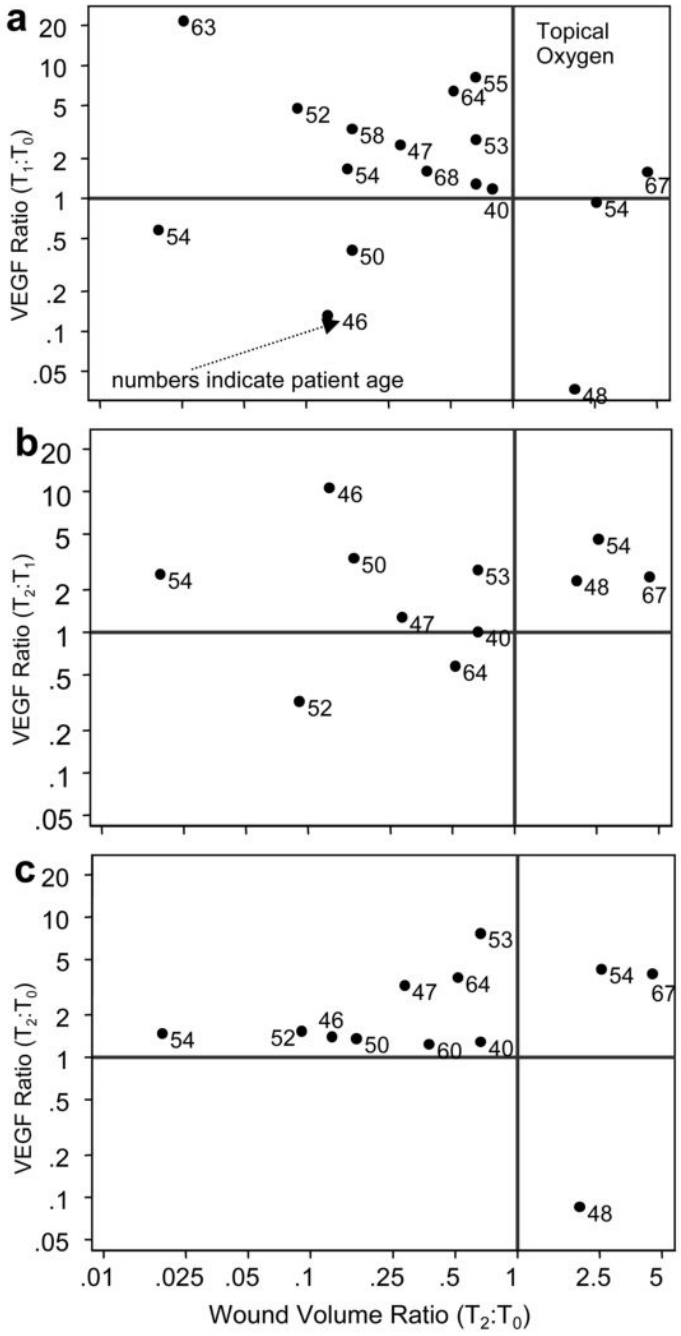

Figure 4. Scatter plot illustrating individual data points plotting topical oxygen induced changes in VEGF gene expression in the time period specified on the y-axis against changes in wound volume over the entire study period

Both the VEGF ratio and the wound volume ratio were log transformed. The number against each data point represent the age of the respective patient in years. a, VEGF changes during the time period T_0 (initial) to T_1 (interim); b, VEGF changes during the time period T_1 (interim) to T_2 (final); and c, VEGF changes during the time period T_0 (initial) to T_2 (final) *i.e* the entire study duration.

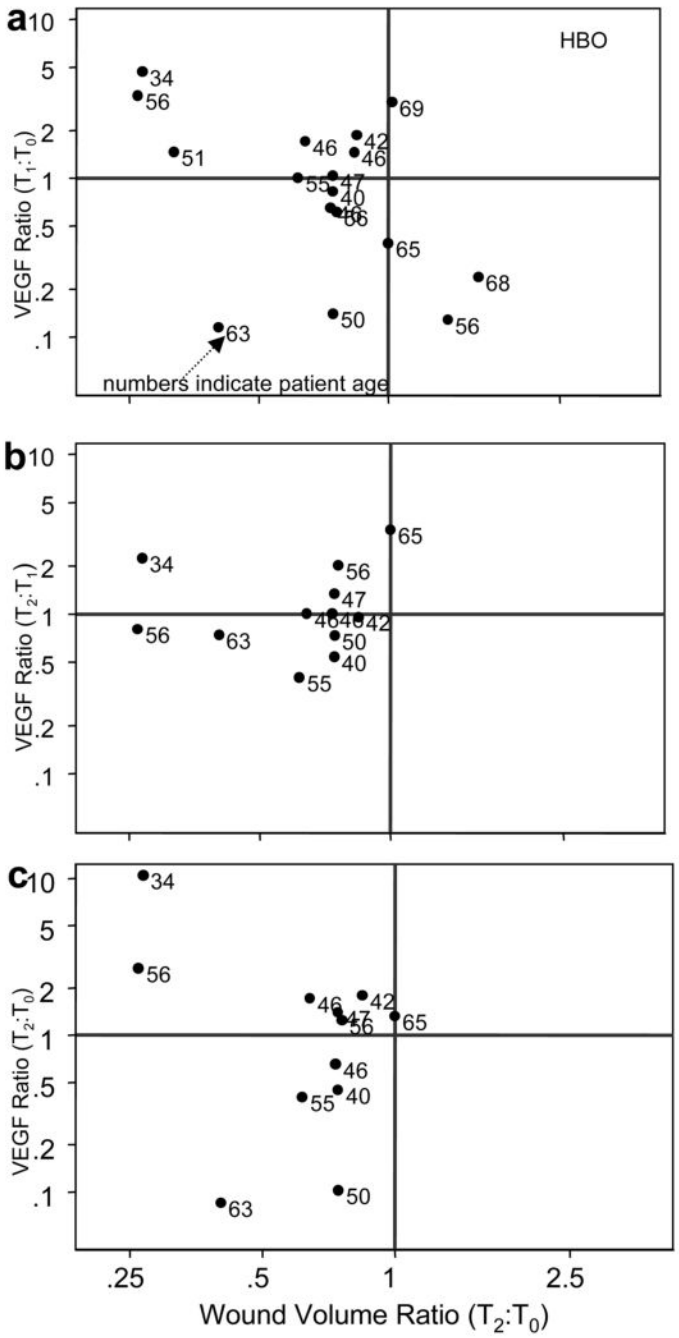

Figure 5. Scatter plot illustrating individual data points plotting changes in HBO-induced VEGF gene expression in the time period specified on the y-axis against changes in wound volume over the entire study period

Both the VEGF ratio and the wound volume ratio were log transformed. The number against each data point represent the age of the respective patient in years. a, VEGF changes during the time period T_0 (initial) to T_1 (interim); b, VEGF changes during the time period T_1 (interim) to T_2 (final); and c, VEGF changes during the time period T_0 (initial) to T_2 (final) *i.e* the entire study duration.

Table 1

Patient Demographics by Treatment Type

Attribute	HBO	Topical Oxygen
Count (% diabetic)	32 (31)	25 (52)
Age, mean (standard deviation)	52.3 (11.0)	54.7 (8.9)
Male, percent	90.6	52.0
Initial volume (cm^3), median (IQR)	9.4 (2.8 – 30.1)	3.3 (1.2 – 12.0)
Final volume (cm^3), median (IQR)	6.1 (2.5 – 17.5)	1.4 (0.6 – 2.8)

HBO: Hyperbaric Oxygen, IQR: Interquartile range

Table 2

Changes in Oxygen-Sensitive Genes in Wound-Edge of Patients Treated with TO

Gene	Oxygen Treatment	Count Healing : Not Healing	Expression, mean Wounds Not Healing	Wounds Healing	Wounds Healing to not healing Ratio	*p*- value[1]
VEGF	Topical Oxygen	14 : 3	0.85	4.02	4.73	0.031
TGFβ1	Topical Oxygen	15 : 3	0.62	2.52	4.06	0.347
COL1A1	Topical Oxygen	15 : 3	2.22	2.92	1.32	0.520

Results from T_0 *versus* T_1 shown.

[1] *p*-value is testing if the healing to not-healing wound volume is significantly greater than 1.0 as determined from linear regression of natural log transformed gene expression values from healing (yes/no) and is adjusted for the patient's age

Table 3

VEGF Expression for Healing Wounds Treated with HBO

Time Interval[1]	Oxygen Treatment	Number healing wounds	VEGF Ratio[2]	*p*-value[3]
T_2 to T_0	HBO	12	1.86	0.741
T_2 to T_1	HBO	12	1.27	0.767
T_1 to T_0	HBO	14	1.38	0.716

[1] T_0= baseline measurement; T_1 = midpoint of therapy; T_2 = 14 weeks or imminent closure

[2] VEGF ratio = log transformed ratio of VEGF/β-actin (T_X): VEGF/β-actin (T_Y) where T_X = first T in time interval and T_Y = second T in time interval

[3] *p*-value is testing if the healing VEGF ratio is significantly greater than 1.0 and is generated from a one–sample *t*-test that uses the natural logarithm of the VEGF ratio.

Table 4

Changes in Oxygen-Sensitive Genes in Wound-Edge of Patients Treated with HBO

Gene	Oxygen Treatment	Count Healing : Not Healing	Expression, mean Wounds Not Healing	Wounds Healing	Wounds Healing to not healing Ratio	*p*- value[1]
VEGF	HBO	14 : 3	1.12	1.38	1.23	0.995
TGFβ1	HBO	14 : 3	1.67	2.29	1.37	0.190
COL1A1	HBO	14 : 3	0.87	2.49	2.86	0.415

Results from T_0 *versus* T_1 shown.

[1] *p*-value is testing if the healing to not-healing wound volume is significantly greater than 1.0 as determined from linear regression of natural log transformed gene expression values from healing (yes/no) and is adjusted for the patient's age

Table 5

VEGF Expression for Healing Wounds Treated with TO

Time Interval[1]	Oxygen Treatment	Number healing wounds	VEGF Ratio[2]	*p*-value[3]
T_2 to T_0	Topical Oxygen	9	2.54	0.010
T_2 to T_1	Topical Oxygen	8	2.81	0.232
T_1 to T_0	Topical Oxygen	14	4.02	0.070

[1] T_0= baseline measurement; T_1 = midpoint of therapy; T_2 = 14 weeks or imminent closure

[2] VEGF ratio = log transformed ratio of VEGF/β-actin (T_X): VEGF/β-actin (T_Y) where T_X = first T in time interval and T_Y = second T in time interval

[3] *p*-value is testing if the healing VEGF ratio is significantly greater than 1.0 and is generated from a one–sample *t*-test that uses the natural logarithm of the VEGF ratio.