

Angiogenesis in Necrotic Ulcers Treated with Hyperbaric Oxygen

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ABSTRACT

Necrotic/gangrenous wounds lack adequate blood supply and develop further vascular damage from tissue reperfusion injury or oxygen toxicity when exposed to oxygen at the wrong pressures. A prospective randomized study was performed to confirm the efficacy of topical hyperbaric oxygen at 1.004 to 1.013 atmospheres (THOT) in stimulating angiogenesis and healing of necrotic/gangrenous wounds. Participants included 40 inpatients (79 ulcers) recruited over 12 months who were assigned to treatment by either THOT or standard wound care (SWC). The results showed that 90% of the wounds healed in the THOT group compared to 22% in the SWC controls. Repeated measures ANOVA on log (ulcer size at 4 weeks) showed a significant group by time interaction, $F(1,55) = 68.2$, $P < 0.0001$. The size of ulcers (at 4 weeks) was significantly smaller with THOT, but larger with SWC. Capillary density/hpf (high power field) was significantly higher in THOT wounds than in SWC wounds ($P < 0.001$). It was concluded that THOT is effective in stimulating angiogenesis with enhanced healing of necrotic wounds.

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Based on transcutaneous oxygen partial pressure (TcPO₂) measurements, ulcers can be divided into two types: (1) nonhypoxic (TcPO₂ 30-40 mm Hg) and (2) hypoxic (TcPO₂ 0-<30 mm Hg).¹ The latter group, characterized by the presence of yellow

necrotic tissue (TcPO₂ 13-30 mm Hg) or black gangrenous tissue (TcPO₂ 0-15 mm Hg), (TcPO₂ from 0-<30 mm Hg), are recalcitrant and considered "unlikely to heal," resulting in high rates of major amputations or flap surgeries.² In recalcitrant wounds, vascular thrombi are observed (Heng MCY 2000; unpublished data); these wounds are recognized clinically by the recurrent formation of necrotic tissue at the air-wound interface, as the underlying presumably hypoxic tissues undergo necrosis from reperfusion injury³⁻⁶ when exposed to and reperfused by oxygen in the air.

Unopposed oxygen free radicals or reactive oxygen species (ROS) form the basis of reperfusion injury.⁴⁻⁶ ROS are a normal byproduct of oxidative phosphorylation. In the presence of an adequate blood supply, ROS are quenched by adequate supplies of free radical quenchers (ie, superoxide dismutase, catalase, and reduced glutathione).⁴ In wounds without adequate blood supply, however, the presence of unopposed ROS results in endothelial cell destruction and tissue necrosis, leading to worsening of these wounds.

Oxygen at greater than 1 atmosphere (hyperbaric) appears to have higher intrinsic energy than oxygen at 1 atmosphere (normobaric) or below 1 atmosphere (hypobaric). The intrinsic energy conferred on hyperbaric oxygen at specific pressures allows specific chemical reactions to take place at air temperature that would not otherwise occur under normobaric conditions. One desirable reaction consists of cross-linking of hydroperoxyl radicals to monounsaturated lipids,⁴⁷ thus sequestering hydroperox-

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**TABLE 1
DISTRIBUTION OF ULCERS IN
THOT AND CONTROL GROUPS**

	THOT	SWC	P
Diabetes	10/25 (40%)	10/25 (40%)	1.0000
Peripheral vascular disease	10/25 (40%)	10/25 (40%)	1.0000
Arteriosclerosis	10/25 (40%)	10/25 (40%)	1.0000
Other (hypertension, smoking)	10/25 (40%)	10/25 (40%)	1.0000
Total	40/100 (40%)	40/100 (40%)	

SWC ($p < 0.004$; power calculation 0.887). The study was, therefore, designed to treat at least 10 patients (20 wounds) with either THOT or SWC.

Study protocol. The study was prospective and used nonselected controls. Patients with necrotic wounds who met the inclusion criteria were recruited by referral to one of the authors. The protocol included complete medical history, physical examination, and laboratory evaluation for all patients, including a chest radiograph, bone radiographs, and ^{111}In leukocyte scans²¹ whenever indicated. The inpatients received 4 weeks of treatment by either THOT or SWC, with the option for crossover after 4 weeks if the ulcers worsened with either treatment.

Forty patients with 79 necrotic/gangrenous ulcers, who met the inclusion criteria, were recruited into the study within the 12-month stipulated period. Twenty-six inpatients (54 ulcers) were randomly assigned (by drawing lots) to treatment by either THOT or SWC. Because only two patients could be treated with THOT at any one time, 14 "overflow" inpatients (ie, patients admitted during the period when more than two patients were being treated with THOT) with 25 ulcers also were included in the SWC group. Exclusion criteria excluded 2 patients - one who

refused THOT and the other with unstable progressive gangrene-associated uncontrolled diabetes and infection. Ulcer location and baseline characteristics of the inpatient group are provided in Table 1 and Table 2. No significant differences were found between the two groups (see Table 2).

Study Procedures

Topical hyperbaric oxygen therapy at the therapeutic range (THOT). Following sharp debridement (see below), patients were given THOT. This is an improvement from the topical hyperbaric oxygen technique (1.03-1.04 atmospheres) previously reported.²² Oxygen is administered via an 84-in x 48-in pleated, polyethylene bag. The open end is taped around the chest at the level of the nipple, allowing multiple ulcers to be treated simultaneously. Using pressures validated by instruments specially designed for measuring low pressures (Sandia, National Labs, Albuquerque, New Mexico), intrabag pressures were maintained within a narrow range (1.004 to 1.013 atmospheres) at all times, ensuring a 15 L/min flow rate. The wounds were treated for 4 hours per day, 4 days per week for 4 weeks (or less if healed earlier).

**TABLE 2
CATEGORICAL DATA IN THOT AND
CONTROL ULCERS**

	THOT (n=26) (54 ulcers)	SWC (n=26) (79 ulcers)	P
Diabetes	10/26 (38%)	10/26 (38%)	1.0000
Peripheral vascular disease	10/26 (38%)	10/26 (38%)	1.0000
Arteriosclerosis	10/26 (38%)	10/26 (38%)	1.0000
Other (hypertension, smoking)	10/26 (38%)	10/26 (38%)	1.0000
Ulcer Duration (before treatment)			
#Ulcers \geq 2 weeks	7/26 (27%)	7/50 (14%)	0.7380
#Ulcers < 2 weeks	22/26 (76%)	43/50 (86%)	0.3598
Previous leg surgery	11/26 (42%)	17/27 (63%)	



Figure 1a
Typical necrotic lateral ulcer with necrotic/gangrenous base.



Figure 1b
Same ulcer after debridement, showing excision down to bone (single arrow) and tendons (double arrows).



Figure 1c
Early granulation tissue (G) after 2.5 weeks of THOT with almost no recurrence of necrotic slough formation after only debridements.



Figure 1d
Abundant granulation tissue (G) after 6 weeks of THOT.

ulcers by 2 to 6 weeks, 6 of 6 Stage III ulcers by 4 to 10 weeks, and 4 of 7 Stage IV wounds by 4 to 16 weeks. The three unhealed Stage IV ulcers (1 sacral, 2 heel) were complicated by osteomyelitis, with 2 of 3 patients dying from comorbid disease (disseminated prostate malignancy in 1 patient and aspiration pneumonia in 1 patient) before wound healing occurred. Figures 1, 2, and 3 depict the typical behavior of wounds treated by THOT.

In the SWC group, granulation tissue was slow to form with recurrence of necrotic tissue requiring repeated debridement in 43 of 50 wounds. Some degree of clinical scarring was observed in 49 of 50 wounds. In the SWC group, 11 of 50 (22%) wounds eventually healed (8 of 31 Stage II ulcers by 7 months, and 3 of 8 Stage III ulcers by 15 months). No (0 of 11) Stage IV ulcer was healed by the end of the study.

Wound measurements

Multiple ulcers per patient random sample (n = 58). All patients who received THOT treatment were correctly randomized to that condition. Some control group patients were also correctly randomized, but others were simply included in that group because they were admitted at a time when no additional THOT patients could be treated by the available trained staff. The initial plan was to randomize all admissions during a 12-month period. The THOT group contained 13 patients with 29 wounds, while the SWC group contained 27 patients with 50 wounds. One way to obtain strictly comparable groups is to randomly select 29 wounds from the 50 ulcers in the control group to compare to the THOT group. Randomization was performed on the SWC group by the SSPS computer algorithm without regard to any subject/ulcer characteristics at enrollment or after 4 weeks. Successful randomization was demonstrated by the fact that the random sample of 29 SWC ulcers was not significantly different from the 21 remaining unselected SWC ulcers in any of the following measures: size at enrollment (mean difference = $1.32 \text{ cm}^2 \pm 2.8$, $t = 0.48$, $p = 0.64$); size at 4 weeks (mean difference $0.64 \text{ cm}^2 \pm 4.2$, $t = 0.15$, $P = 0.88$); or change per day (mean difference = $0.0004 \text{ cm}^2 \pm 0.08$, $t = 0.055$, $P = 0.96$). Pearson chi-squared tests comparing the SWC-selected random sample with the SWC unselected ulcers showed no significant differences in frequency of diabetic ulcers ($\chi^2 = 1.12$, $p = 0.29$), frequency of ulcer stages at enrollment ($\chi^2 = 1.46$, $P = 0.48$) or at 4 weeks ($\chi^2 = 0.83$, $P = 0.84$). Successful randomization was demonstrated for the above relevant measures at baseline enrollment and at 4 weeks (see Table 3).

Single ulcer per patient random sample (n = 24). The sample initially included 79 ulcers among 40 cases. The THOT group contained 13 patients with 29 wounds, while the SWC group contained 27 patients with 50 wounds. Although most



Figure 3a
The above illustrates a post-amputation ulcer in a diabetic patient with renal failure showing presence of necrotic sloughs in the wound base.



Figure 3b
Observe good growth of granulation tissue with THOT after less than 2 weeks, with no recurrence of necrotic tissue.



Figure 3c
No clinical scarring was observed in this patient after healing.

SWC (multiple-ulcer random sample) groups.

At enrollment, the THOT group had 16 ulcers at Stage II, 6 at Stage III, and 7 at Stage IV, while the SWC group had 20, 4, and 5 at Stages II, III, and IV, respectively. The THOT group tended to have slightly more severe wounds than the SWC group, although this difference was not significant, $\chi^2 = 1.18$, $P = 0.55$. At 4 weeks, the THOT group had 18 healed ulcers, while SWC had healed only 2 of these necrotic/gangrenous ulcers. Of the unhealed ulcers at 4 weeks, the THOT group had 5 Stage II, 4 Stage III, and 2 Stage IV ulcers; the SWC group had 13 Stage II, 3 Stage III, and 11 Stage IV ulcers. THOT and

TABLE 3
COMPARISON BETWEEN SWC AND THOT GROUPS
(MULTIPLE ULCER RANDOM SAMPLE)

	SWC (n = 27)	THOT Treatment (n = 27)
Ulcer size at enrollment in cm ²	2.8 (0.8)	10.7 (7.8)
Ulcer size at 4 weeks in cm ²	11.5 (1.9)	3.6 (1.8)
Natural log (ulcer size at enrollment) in cm ²	1.3 (0.9)	2.2 (1.0)
Natural log (ulcer size at 4 weeks) in cm ²	2.3 (1.1)	0.2 (1.0)
Improvement per day in cm ²	-0.28 (0.22) (worsened)	+0.38 (0.27) (improved)
Percent change in ulcer size	4.6 (8.9) (worsened)	-8.9 (16.7) (improved)

TABLE 4
COMPARISON BETWEEN SWC AND THOT GROUPS
(SINGLE ULCER RANDOM SAMPLE)

	SWC (n = 12)	THOT Treatment (n = 12)
Ulcer size at enrollment in cm ²	2.7 (0.9)	12.0 (10.7)
Ulcer size at 4 weeks in cm ²	12.7 (10.7)	3.6 (5.7)
Natural log (ulcer size at enrollment) in cm ²	1.3 (0.9)	2.2 (1.0)
Natural log (ulcer size at 4 weeks) in cm ²	3.0 (0.8)	1.0 (1.1)
Improvement per day in cm ²	-0.3 (0.16) (worsened)	+0.69 (1.3) (improved)
Percent change in ulcer size	2.50 (5.26) (worsened)	-8.98 (14.04) (improved)

Results are expressed as mean (SD).
 * Selected randomized sample (see text)
 † One outlier with very fast healing rate was excluded from THOT group analysis
 ‡ $P < 0.0001$

positively skewed in both groups, natural log transformation was done on ulcer size at enrollment and at 4 weeks. The log-transformed data resulted in a normal distribution. The mean ulcer sizes (standard deviation) before and after natural log transformation are shown in Table 4. The groups did not differ at enrollment in log (ulcer size at baseline), $t = 0.69$, $P = 0.498$. A two-way repeated measures analysis of variance was performed comparing ulcer size (log transformed) across treatment groups (THOT and SWC) and ulcer Stage (II, III, IV) at baseline and at 4 weeks. Diagnostic tests showed equality of covariance matrices, $F = 1.38$, $P = 0.15$, and homogeneity of variance at baseline ($P = 0.645$) and 4 weeks ($P = 0.331$), and no outliers.

There was a significant treatment group by time interaction, ($F(1,18) = 28.43$, $P < 0.0001$). The size of the ulcers significantly decreased after 4 weeks with THOT, but increased with standard wound care. The THOT group improved by $+0.69 \pm 1.31$ (SD); (range $+0.09$ to $+4.80$) cm^2 per day, and SWC group worsened (-0.09 ± 0.16 (SD); range $+0.05$ to -0.48) cm^2 per day. There was a significant effect of stage, as expected, $F(2,18) = 3.66$, $P = 0.046$. Tukey's Honestly Significant Difference post-hoc comparisons showed Stage IV larger than Stage II at 4 weeks ($P = 0.044$) and marginally so at baseline ($P = 0.082$). However, there were no significant interactions with stage. In particular, the nonsignificant group by stage by time ($P = 0.410$) interaction showed that the group by time effect (improvement from baseline to 4 weeks in the THOT group) was equivalent across ulcer stages. Using the "improvement per day" parameter, the single-ulcer random sample ($n = 24$), THOT ulcers improved by a mean of 8.97% (SD 14.04%, range 1.63%–50.0%) per day, while SWC ulcers worsened/enlarged by -2.50% (SD 5.26, range -17.86% – +0.89%) per day. Mann-Whitney U tests comparing THOT to SWC groups was significant ($P < 0.0001$). Table 4 summarizes the healing parameters in the single-ulcer random sample ($n = 24$).

Healing of diabetic ulcers. Twenty-one of 28 ulcers (1 THOT outlier excluded) were diabetic in the THOT group, while 11 of 29 ulcers in the selected random SWC sample were diabetic. Pearson's chi-squared comparing the frequency of diabetic ulcers in the THOT and SWC groups showed significantly higher frequency of diabetic ulcers in the THOT group than in the SWC group ($P = 0.005$). There was no difference between dia-

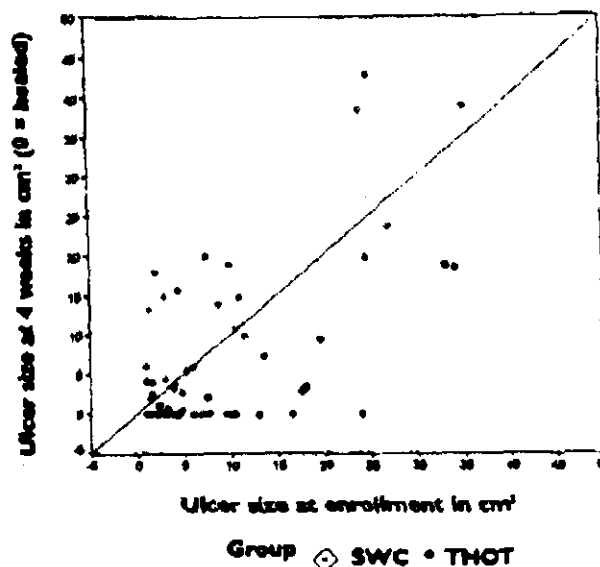


Figure 4
The above diagram shows the change in size of necrotic/gangrenous ulcers (cm^2) at 0 and 4 weeks of treatment in Stages II, III, and IV ulcers treated with both THOT and SWC.

betic (mean = $+0.554$, SD = 1.247) and nondiabetic (mean = -0.030 , SD = 0.303) necrotic ulcers in improvement per day; $F(1,53) = 1.668$, $P = 0.203$. The treatment by diabetes interaction was also not significant; $F(1,53) = 0.84$, $P = 0.364$. Thus, the treatment effect was similar in diabetic and nondiabetic patients. Details of diabetic ulcers are summarized in Table 6.

Logistic regression analysis. Logistic regression analysis was performed using treatment group (THOT, SWC), ulcer stage (II, III, IV), and diabetic diagnosis (yes, no) as categorical predictors of healing (at 4 weeks). Contrast vectors were set up to compare healing in Stage II versus Stage IV, and Stage III versus Stage IV ulcers. Hosmer-Lemeshow Goodness-of-fit test = 5.08, $df = 6$, $P = 0.53$ indicated a good fit, with expected values not significantly different from observed values. Cox & Snell estimated regression R^2 was moderate, 0.53. The model correctly classified 89.5% (51 of 57 ulcers), with a cutpoint of 0.5 predicted probability. Model diagnostics showed three outliers based on Z residuals. Scatterplots of leverage showed only one of these cases with a large leverage (0.3). These cases included two Stage IV ulcers and one Stage II ulcer in a nondiabetic patient who showed rapid healing, contrary to model predictions (all three were treated with THOT). Because eliminating the case with the large leverage did not change the significance of the

TABLE 8
COST OF WOUND CARE AT 4 WEEKS
IN THOT AND SWC GROUPS

Stage	Item	Average Cost of Items per Day (4 weeks)	
		THOT (n=29)	SWC (n=50)
Stage II	Wound care	\$2.70	\$40.50
	Cost of dressings	\$9.12	\$10.18
	Cost of medications	\$7.89	\$15.00
	Supply of antibiotics	\$0.00	\$24.00
	Cost of anesthesia	\$0.00	\$14.00
	Cost of surgery	\$0.00	\$0.00
Cost of Stage II necrotic ulcers		\$19.71	\$124.18
Stage III	Wound care	\$2.70	\$5.00
	Cost of dressings	\$9.12	\$17.70
	Cost of medications	\$7.89	\$87.50
	Supply of antibiotics	\$0.00	\$48.00
	Cost of anesthesia	\$0.00	\$7.00
	Cost of surgery	\$0.00	\$0.00
Cost of Stage III necrotic ulcers		\$19.71	\$167.20
Stage IV	Wound care	\$2.70	\$0.00
	Cost of dressings	\$9.12	\$0.00
	Cost of medications	\$7.89	\$0.00
	Supply of antibiotics	\$0.00	\$0.00
	Cost of anesthesia	\$0.00	\$0.00
	Cost of surgery	\$0.00	\$0.00
Cost of Stage IV necrotic ulcers		\$19.71	\$0.00

Note: For each patient, the cost of THOT was \$1.00 per day for 28 days, and the cost of SWC was \$2.00 per day for 28 days.

Despite the relatively small number of ulcers (79 ulcers), with 29 ulcers treated with THOT, this study is, nevertheless, important. These numbers reflect the problem ulcers treated at one medical center per fiscal year, providing invaluable data for potentially high economic costs should these wounds be allowed to remain unhealed or result in major amputations or flap surgeries.

Effect on wound healing. Repeated measures analysis of variance on natural log-transformed data on ulcer size, improvement-per-day data, stage improvement, number of ulcers healed, and logistic regression analysis of the categorical predictors of wound healing support increased wound healing of necrotic ulcers by THOT. All Stage II

and III wounds in the THOT group healed within 6 to 10 weeks, and 4 of 7 Stage IV ulcers within 16 weeks, which compares well with the poor healing rate of diabetic ulcers (25) and non-necrotic pressure ulcers²⁴ reported by others.

Effect on angiogenesis. Analysis of wound biopsy data also shows significantly increased formation of new blood vessels, associated with decreased collagen deposition, in wounds treated by THOT compared to controls. The ability of THOT to stimulate angiogenesis in necrotic wounds may be important in the ability of this technique to heal large and deep wounds in both diabetics and nondiabetics.

Rationale for the putative therapeutic range in wound healing. The presence of neovascularization observed in THOT biopsy specimens is an indication that 1.004 to 1.013 atmospheres used by THOT may well fit the criteria for the putative "therapeutic range" of oxygen pressures capable of stimulating wound healing in necrotic wounds. Within the "therapeutic range," the newly formed blood vessels are expected to survive both reperfusion injury and oxygen toxicity. In addition, topical application of oxygen at this range has been shown not to elevate TcPO₂, and consequently, is expected not to blunt the hypoxic stimulus for angiogenesis. Because oxygen applied topically at this range falls well below capillary pressures (1.025 atmospheres), THOT, by utilizing extremely low oxygen pressures, may also benefit by not compromising blood flow in the capillaries.

Side effects. No toxic or untoward effects were observed during this study with THOT. In particular, there were no episodes of cross-infection due to the disposability of the equipment.

Study limitations. The lack of a double-blind design is a limitation in this study. To ensure a double-blind design would have involved using hyperbaric air, which was contraindicated in view of our preliminary observations that treatment with hyperbaric air at similar pressures led to worsening of necrotic ulcers, presumably from reperfusion injury (Heng MCY 1999, unpublished data). The authors did, however, ensure that SWC patients were treated in the most comprehensive manner, including (1) turning the patient every 2 hours by establishing a charting regimen, (2) intravenous antibiotics for persistence of necrotic tissue, (3) adequate pressure-relieving devices and low-air-loss beds whenever indicated, and (4) establishing the same quality of nursing in that the

same wound nurses dressed the wounds in the same way.

The unequal sample size in the randomized groups may be another limitation of this study. However, because we compared THOT ulcers (omitting an outlier with very fast healing) with a selected random sample of SWC group, which did not differ from the nonselected SWC wounds, the results support valid conclusions of significantly greater healing with the THOT treatment compared to nonselected SWC controls. Moreover, because the ulcers in the THOT group tended to be larger and deeper, with a greater proportion of Stage III and IV ulcers than the SWC controls, the authors did not feel that the SWC group was necessarily compromised.

It has been thought that changing the hydrocolloid dressings too frequently may be detrimental to wound healing. The role of dressings is to maintain a moist environment without enhancing increased neutrophil-induced proteolytic activity in the exudate. In wounds that are not infected and granulating well (ie, wounds with adequate blood supply) neutrophils are few and proteolytic activity and exudate are limited. Such wounds do well with hydrocolloid dressings, which only have to be changed infrequently. Wounds with gangrenous eschar are nearly always infected because of lack of adequate blood supply. In such wounds, bacteria and neutrophil-secreted proteolytic enzymes abound in the inflammatory exudate. In such wounds, hydrocolloid dressings promote bacterial proliferation, and if used, should be changed frequently. For this reason, wet-to-dry saline dressings were preferred, particularly in wounds with recurrent necrotic tissue and abundant exudate. The saline lessened the viscosity of the exudate and promoted "mopping-up" of the proteolytic enzymes in the exudate by the gauze. The dressings were changed as needed, depending on the amount of exudate. The authors avoided calcium alginate dressings in infected wounds because the iodine content of the seaweed dressing aggravated the neutrophilic response induced by the bacterial infection. The investigators did not use wet-to-dry saline dressings in wounds with minimal exudate and without necrotic tissue.

Pathophysiology of scar tissue formation. Although the pathophysiology of scar tissue formation is unclear, inflammatory cytokines are implicated. Fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) are both mitogenic to endothelial cells and fibroblasts.^{27,28} These cytokines are regulated by a family of cytokines previously isolated from platelets, namely transforming

growth factors, particularly the TGF- β 1 and TGF- β 2 isoforms. The TGF- β 1 gene is upregulated in tissue injury,²⁹ with TGF- β 2 isoform implicated in wound healing with scarring. Neutralizing TGF- β by its antibody results in wound healing without scarring.³⁰ The TGF- β 3 isoform down-regulates TGF- β 1 and TGF- β 2.³¹ We have yet to determine whether the antiscarring properties of THOT are achieved directly through suppression of TGF- β 1 and TGF- β 2, by the upregulation of TGF- β 3, or by entirely different mechanisms.

It is uncertain whether angiogenesis is stimulated by THOT, or whether it is just an unblunted angiogenic response to hypoxia, in which blood vessels are allowed to survive and grow because of unhindered capillary blood-flow and additional protection provided by the scavenging of the oxygen free radicals. Although these are preliminary data, the concept of using angiogenesis (and decreased collagen deposition) to identify the therapeutic range of oxygen pressures for treating hypoxic wounds is an intriguing concept and one that contributes to better understanding of basic mechanisms in wound healing. - OWM

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