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 either 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 ($TcPO_2$) measurements, ulcers can be divided into two types: (1) nonhypoxic ($TcPO_2$ 30-40 mm Hg) and (2) hypoxic ($TcPO_2$ 0-<30 mm Hg).[1] The latter group, characterized by the presence of yellow necrotic tissue ($TcPO_2$ 13-30 mm Hg) or black gangrenous tissue ($TcPO_2$ 0-13 mm Hg),($TcPO_2$ from 0-<30 mm Hg), are recalcitrant and considered "unlikely to heal,"[2] resulting in high rates of major amputations or flap surgeries.[3] In recalcitrant wounds, vascular thrombi are observed (Heng MCY 2000; unpublished data); these wounds are recognized clinically by the recurrent formation of necrotic tissue at the air-wound interface, as the underlying presumably hypoxic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 injury[4–6] 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,[4,7] thus sequestering hydroperox-

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ides and rendering them harmless. This free-radical quenching property of hyperbaric oxygen[4,7] is essential in avoiding reperfusion injury in treatments attempting to promote angiogenesis in necrotic wounds. On the other hand, oxygen at the toxic range,[7–15] which may be as low as 1.04 atmospheres if treatment is prolonged,[8] is also capable of producing destruction of endothelial cells.[8] At the toxic range, oxygen has so much intrinsic energy that it allows the transfer of electrons from reduced nicotinamide adenine dinucleotide (NADH) to molecular oxygen without the need for the catalyst assistance of the cytochrome system. Bypassing the cytochrome system results in two detrimental events. First, the energy is released in a destructive burst within the thin-walled membranes of the cytoplasm, resulting in cell destruction rather than being dissipated harmlessly by the cytochrome system within the thick-walled mitochondria. Second, there is no adenosine triphosphate (ATP) generation from glucose metabolism because the generation of ATP from NADH requires involvement of the cytochrome system. Without ATP generation, wounds are unlikely to heal, even in the presence of growth factors.

Using oxygen pressures low enough to avoid toxicity is important in wound healing. This consideration is important because the susceptibility to oxygen toxicity is increased in previously damaged tissues,[16] thereby aggravating pre-existing vascular damage and thrombotic disease in necrotic wounds.[17] This is particularly true among diabetic patients,[18,19] accounting, in part, for the poor results seen when higher oxygen pressures are used in such patients.[20] Within the putative therapeutic range for wound healing, which avoids both reperfusion injury and oxygen toxicity, the formation and survival of new blood vessels would be expected to occur. By avoiding injury, the stimulus for scar tissue formation is decreased. Angiogenesis, with minimal scar tissue formation, is then the basis for healthy tissue growth and wound healing in hypoxic/necrotic wounds that would otherwise worsen despite therapy.

The authors have identified a range of pressures, 1.004 to 1.013 atmospheres, which appears to be capable of inducing angiogenesis in necrotic wounds (Heng MCY 1999, unpublished data). To validate these findings, this study evaluates angiogenesis and change in size over 4 weeks of necrotic/gangrenous wounds treated by topical hyperbaric oxygen at 1.004 to 1.013 atmospheres (THOT). The authors also determined collagen deposition, which compromises the density of blood vessels and cost savings, which also reflect wound healing.

Methods

Study population. This single center study was performed at the long-term care facility of the Veterans Affairs Greater Los Angeles Healthcare System (VAGLAHS in Sepulveda, Calif.). Inclusion criteria were (1) all nonambulatory residents of the long-term care facility at study onset (confined to bed or wheelchair), plus all new consecutive nonambulatory admissions within a stipulated 12-month period; and (2) presence of necrotic/gangrenous wounds. Exclusion criteria include life-threatening gangrene, uncontrolled diabetes, and untreated sepsis. The study was approved by the Institutional Review Board of VAGLAHS, Sepulveda.

Number of patients. From a preliminary study in a sample of 9 ulcers (6 patients) treated with THOT and 11 ulcers (6 patients) treated with SWC over a 2-month period, the investigators obtained significant differences in healing rate between ulcers treated by THOT and

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KEY POINTS

- Too little or too much oxygen may delay wound healing. The authors conducted a prospective, randomized, controlled clinical study to compare the effectiveness of topical hyperbaric oxygen with standard wound care to a control of standard wound care alone in 40 patients with 79 chronic wounds.
- At baseline, no significant differences with respect to patient or ulcer characteristics were observed. The proportion of healed ulcers in the treatment group was higher than in the control group, and significant differences were observed when comparing reduction in wound surface area for all wounds combined, when including only one ulcer per patient, and when comparing diabetic ulcers only.
- Histologically, wounds in the treatment group had a higher capillary and lower fibroblast density, and a decreased density of collagen deposition than the control group. Costs also were found to be lower in the treatment group.
- The limitations of this study (patients with multiple wounds, wounds of varying depth, and concerns about randomization) generally reflect the problems inherent in conducting chronic wound care research. Nevertheless, the results reported in this study warrant additional research to identify the therapeutic range of oxygen pressures for treating hypoxic wounds.

TABLE 1
DISTRIBUTION OF ULCERS IN THOT AND CONTROL GROUPS

Ulcer location	THOT Group*	SWC Group**
Sacral	6 (20.7%)	10 (20%)
Ischial	4 (13.8%)	8 (16%)
Trochanter/hip	3 (10.3%)	5 (10%)
Heel/foot	10 (34.5%)	15 (30%)
Others (leg, elbow, back)	6 (20.7%)	12 (24%)
	29	**50**

** 16 Stage II, 6 Stage III, 7 Stage IV*
***31 Stage II, 8 Stage III, 11 Stage IV*

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 scans[21] 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

Risk factors	THOT # Patients	Control # Patients	Fisher's test
Age	73.8 (SD = 6.4) (range 61–86)	75.5 (SD = 8.0) (range 60–97)	1.0000
Sex	13 M	26 M, 1 F	
Bed-/chair-ridden	10/13 (77%)		0.2984
Diabetes mellitus	7/13 (54%)	15/27 (56%)	0.1748
Int. malignancy	4/13 (31%)	8/27 (33%)	0.3995
CHD, PVD, CVA	10/13 (77%)	4/27 (15%)	1.0000
Systemic infection	9/13 (70%)	20/27 (74%)	0.3119
Osteomyelitis	3/13 (23%)	13/27 (48%)	1.0000
Renal dialysis	2/13 (15%)	5/27 (19%)	0.2421
Multiple sclerosis	2/13 (15%)	1/27 (4%)	0.2421
Smoking: current	0	1/27 (4%)	
Hemoglobin <12 G%	8/13 (62%)	0	0.7369
	8/13 (62%)	14/27 (52%)	0.7369
Ulcer Duration	**(before**	14/27 (52%)	
Hematocrit < 36%	7/29 (24%)		0.7380
# ulcers ≥ 2 weeks	22/29 (76%)	7/50 (14%)	0.3598
# ulcers < 2 weeks	1/13 (7.7%)	43/50 (86%)	

Standard wound care (SWC) protocol. The SWC protocol followed recommendations by the National Pressure Ulcer Advisory Board[23] including turning the patient every 2 hours and using tissue pressure reduction techniques and appropriate support surfaces (replacement mattresses for Stage II ulcers and low-air-loss beds [Kinair III, KCI, San Antonio, Tex.] for Stage III and IV ulcers). Patients with underlying osteomyelitis or persistence of necrotic tissue were treated with intravenous antibiotics (ceftriaxone 1 g to 2 g daily and/or oral ciprofloxacin 500 mg bid). Patients with sepsis were prescribed ceftriaxone (or vancomycin) and gentamycin for as long as indicated. Urinary tract infection was treated with sulfamethoxazole/trimethoprim or ciprofloxacin according to urinary culture sensitivities.

Surgical procedures. Sharp debridement was performed on necrotic tissue to produce active bleeding of the wound base and repeated as often as necessary to remove necrotic tissue. Except for gangrene, extensive debridement was not utilized because this procedure tends to convert hypoxic ulcers to nonhypoxic ones by excising to areas of adequate blood supply.[24] Patients with digital gangrene and/or life-threatening osteomyelitis received digital or forefoot amputation whenever appropriate, with subsequent treatment of the skin defect with either THOT or SWC.

Dressings. Wet-to-dry dressings were prescribed for necrotic or purulent wounds, and hydrocolloid dressings were prescribed for nonexudative wounds. The dressings were changed 1 to 3 times daily as needed.

Measurements.

Wound measurements. Ulcer size was determined by weekly ulcer outline tracings taken by nurses blinded to treatment assignments. From these tracings, surface area was calculated using a Filotecnic Planimeter (error < 1%). Reliability of surface area measurements, assessed by comparing measurement of 10 ulcers, showed a test-retest intrarater reliability of Pearson's r = 0.98, and an interrater reliability of r = 0.97.

Ulcer severity, determined by wound team consensus, used a modified version of severity staging of pressure ulcers[23] and diabetic ulcers.[25] In this study, Stage II ulcers were wounds with necrotic tissue, which after debridement, revealed a depth of up to 3 mm. Stage III ulcers were infected and/or undermined wounds, with necrotic tissue involving the subcutaneous tissue down to deep fascia. Stage IV ulcers were deep, infected, and undermined ulcers with necrotic tissue involving muscle, tendons, and/or bone.

Histological assessment. Biopsy specimens were obtained from the center of the wound base at the start and at 3 weeks after treatment. The specimens were processed for light microscopy, stained with hematoxylin and eosin, examined under a Leitz light microscope, and photographed at 400X magnification (3 fields/section) by a technician blinded to treatment assignments. The specimens were printed on 5" x 7" Kodak paper to a magnification of 500X. The histological data (ie, capillary density, fibroblast density, and density of new collagen deposition) were quantified by an experienced pathologist who also was blinded to patient assignment. Blood vessel, fibroblast, and collagen quantification showed a test-retest intrarater reliability Pearson's r of 0.93, 0.88, and 0.95 respectively.

Cost analysis. The following itemized costs were assessed: THOT ($120 per disposable unit); nursing ($45 per ulcer per day for turning patients and dressing ulcers); debridement ($150 per procedure); dressings ($3 to $5 per dressing change); antibiotic costs ($2 to $15 per day), pressure-relieving mattresses ($48 per day); and low-air-loss beds ($68 per day).

Data analysis. Differences between groups involving categorical data were evaluated by chi-square or by Fisher exact tests. Repeated measures analysis of variance (SSPS 7.5) was used to compare effects of treatment group and diabetes on ulcer size at 4 weeks (cm^2) and improvement per day (cm^2); data were reported as mean ± standard deviation (SD). Logistic regression was used to predict healing, with treatment group, diabetes, and ulcer stage entered as categorical variables. Planned contrast vectors for ulcer stage compared healing in Stage II to IV and Stage III to IV.

Results

The recruited patients fell into 2 groups: (1) THOT group (13 inpatients with 29 ulcers; 21 diabetic); and (2) SWC (control) group (27 inpatients with 50 ulcers; 16 diabetic).

Clinical findings. In the THOT group, growth of abundant bright-red granulation tissue was observed with no reformation of necrotic tissue after the first week of THOT in 25 of 29 wounds. No clinical scarring was observed in 28 of 29 wounds. In the THOT group, 26 of 29 ulcers healed after 2 to16 weeks, 16 of 16 Stage II

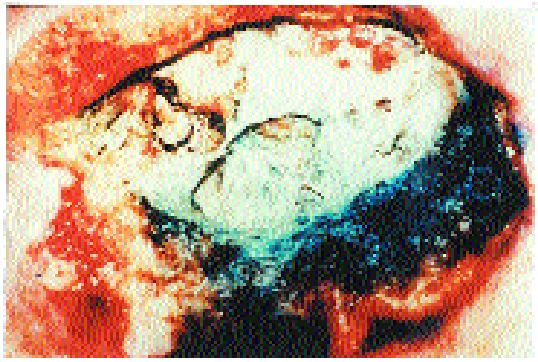

Figure 1a
Typical necrotic sacral ulcer with necrotic/gangrenous base.

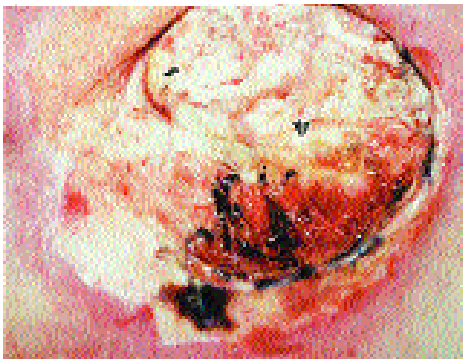

Figure 1b
Same ulcer, after debridement, showing extension down to bone (single arrow) and tendons (double arrows).

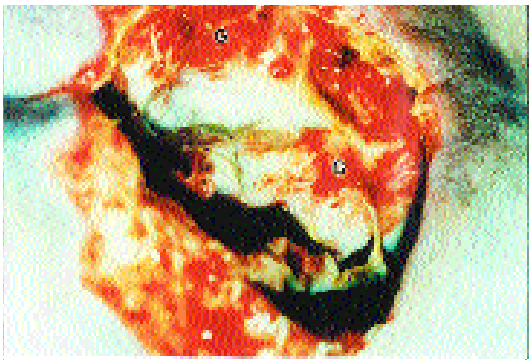

Figure 1c
Early granulation tissue (G) after 2.5 weeks of THOT with almost no recurrence of necrotic slough formation after only debridement.

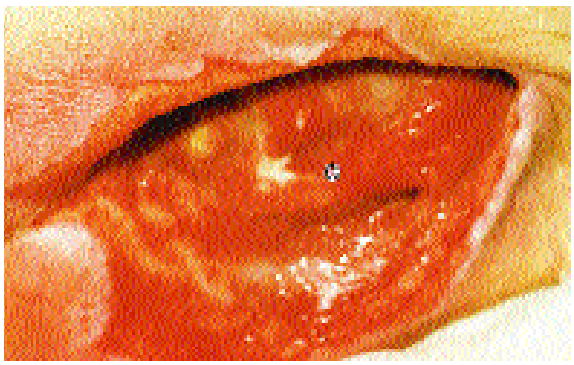

Figure 1d
Abundant granulation tissue (G) after 6 weeks of THOT.

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 cm^2 ± 2.8, $t = 0.48$, $p = 0.64$); size at 4 weeks (mean difference 0.64 cm^2 ± 4.2, $t = 0.15$, $P = 0.88$); or change per day (mean difference = 0.0004 cm^2 ± 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

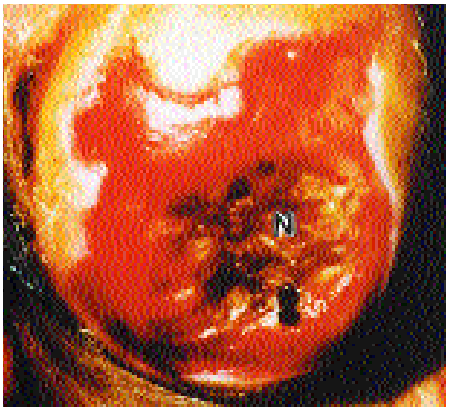

Figure 2a
Gangrenous Stage IV diabetic left heel ulcer (post-debridement) colonized by methicillin-resistant S. aureus in a diabetic patient, who also was in chronic renal failure. Note the recurrence of necrotic tissue (N) after debridement prior to THOT therapy.

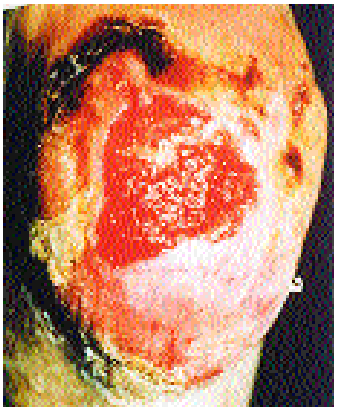

Figure 2b
Observe marked improvement of the wound after 10 weeks of THOT.

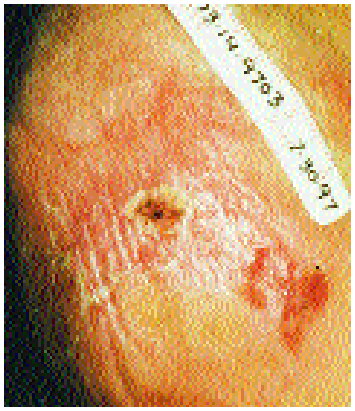

Figure 2c
Note lack of scarring and preservation of the ball of the heel. Also note sparing (apart from mild ecchymosis) of the left heel during a later episode of necrotizing fasciitis during which gangrenous ulcers developed on the sacrum and right heel.

patients had one ulcer, others had two or more. To permit statistical analyses, which assumed independence of observations (ulcers), the authors performed an alternative analysis. They randomly selected one ulcer per patient without regard to ulcer stage, treatment group, or treatment outcome. This resulted in 40 ulcers – 20 at Stage II, 9 at Stage III, and 11 at Stage IV. Among the 13 THOT patients, there was one statistical outlier who was eliminated on analyses of wound size and wound improvement but included in comparisons of ulcer stage. This patient had a very large Stage IV ulcer (193 cm^2 at baseline) that improved rapidly (72 cm^2 at 4 weeks). The final sample for the THOT group contained 12 patients, one ulcer per patient, and (by chance) four ulcers at each stage (II, III, and IV). To achieve randomization for the controls as well as the treatment group, we then took a stratified random sample of the 27 patients in the control group to balance the design with four patients (ulcers) at each stage. Randomization was done by lot, without regard to subject characteristics or ulcer size at enrollment or after 4 weeks. The final sample for analysis included a randomly selected ulcer in patients with multiple ulcers, and a balanced, stratified random sample of controls, with 12 ulcers per group and 4 ulcers at each stage. The results of the single ulcer randomized sample (1 ulcer per patient) are summarized in Table 4.

Assessment of healing based on ulcer size

Multiple ulcers per patient random sample (n = 58). Examination of selected random sample from the SWC group and the THOT group showed one outlier in the THOT group. This was the 193-cm^2 ulcer that improved to 72 cm^2 at 4 weeks, with improvement per day of more than 8 cm^2. It was a statistical outlier on all measures and was excluded from analyses of size and improvement per day. However, it was included in comparisons of ulcer stage. If this case were included, the mean size for the intervention group at enrollment would have been 17.2 ± 3.5 cm^2 and 5.42 ± 14.12 at 4 weeks. Tests for homogeneity of variance (Cochran's C, Bartlett-Box, and Boxes M) comparing variability between THOT and SWC groups were significant ($P < 0.001$). After excluding the outlier, these tests showed homogeneity of variance ($P > 0.94$), indicating that this assumption for analysis of variance was met. In both SWC and THOT groups, ulcers that were healed by 4 weeks were recorded as size = 0 and stage = 0. Because the distribution of ulcer sizes at enrollment was positively skewed in both groups, natural log transformation was done on ulcer size at enrollment and at 4 weeks; the log transformation data resulted in a normal distribution. The mean ulcer sizes (standard deviation) before and after natural log transformation are shown in Figure 4.

The groups did not differ at enrollment in log (ulcer size at baseline), $t = 1.64$, $P = 0.11$. Repeated measures analysis of variance on log (ulcer size) showed a significant group by time interaction, $F(1,55) = 68.2$, $P < 0.0001$. Size of ulcers was significantly smaller after 4 weeks with THOT, but were larger with SWC. Table 3a summarizes ulcer size at enrollment and at 4 weeks of THOT and

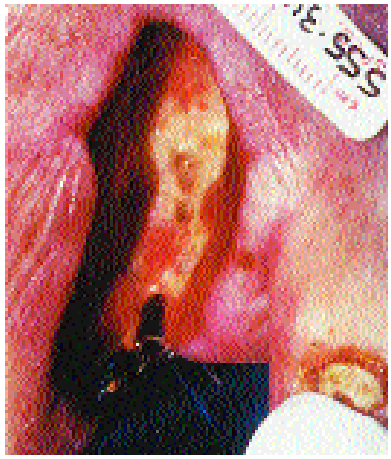

Figure 3a
The above illustrates a post-amputation ulcer in a diabetic patient with renal failure showing presence of necrotic slough in the wound base.

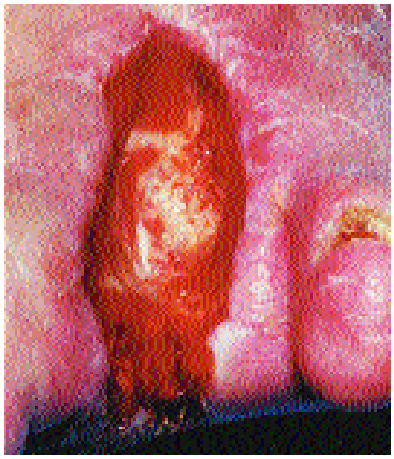

Figure 3b
Observe good growth of granulation tissue with THOT after less than 2 weeks, with no recurrence of necrotic tissue.

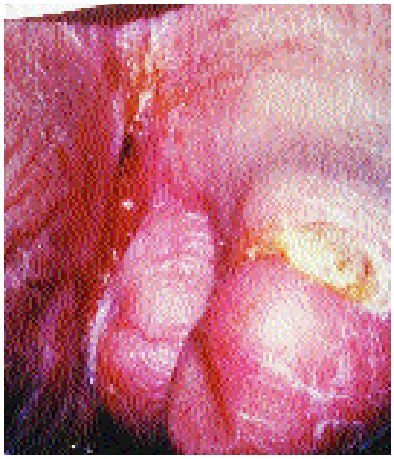

Figure 3c
No clinical scarring was observed in this patient after healing.

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 =	THOT Treatment (n =
Ulcer size at enrollment in cm²	7.8 (8.8)	10.9 (7.8)
Ulcer size at 4 weeks in cm²	11.8 (11.9)	3.0 (11.8)
Natural log (ulcer size at enrollment) in cm²	1.8 (0.9)	2.2 (0.8)
Natural log (ulcer size at 4 weeks) in cm²	2.1 (1.1)	0.7 (1.27)
Improvement per day in cm²	-0.121 (0.22) (worsened)	+0.731 (1.27)‡ (improved)
Percent change in ulcer size	4.46 (8.97) (worsened)	-8.99 (10.74)‡ (improved)

Results are expressed as mean (SD)
**Selected random sample containing one to multiple ulcer per patient (see text)*
† One outlier with very fast healing rate was excluded from THOT group analysis
‡ $P < 0.0001$

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

	SWC (n = 12)	THOT Treatment (n =
Ulcer size at enrollment in cm²	2.7 (9.9)	12.0 (10.7)
Ulcer size at 4 weeks in cm²	12.7 (10.7)	3.6 (5.7)
Natural log (ulcer size at enrollment) in cm²	1.9 (0.9)	2.2 (1.0)
Natural log (ulcer size at 4 weeks) in cm²	3.0 (0.8)	1.0 (1.1)
Improvement per day in cm²	-0.9 (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
‡ $P < 0.0001$

TABLE 5
CHANGE IN ULCER SIZE BY STAGE

	Mean Ulcer Size (SD) in Enrollmen	4	Wilcoxon *P* value
Stage II Ulcers			
THOT (n = 16)	7.13 (6.21)	0.04 (0.05)	0.0004 (improved)
Control† (n = 31)	5.68 (7.40)	8.68 (13.6)	0.3234 (ns)
	5.44 (5.99)	8.71 (7.49)	0.0970 (ns)
Control‡ (n = 20)	11.05 (6.90)	2.72 (3.59)	0.027 (improved)
Stage III Ulcers	7.78 (7.00)	10.65 (12.4)	0.499 (ns)
THOT (n = 6)	5.38 (3.59)	7.85 (7.57)	0.285 (ns)
Control† (n = 8)			
Control‡ (n = 4)	20.92 (12.00)	11.25 (8.86)	0.028 (improved)
Stage IV Ulcers	16.35 (12.82)	22.46 (14.90)	0.005 (worse)
THOT (n = 6)§	19.22 (13.15)	27.08 (18.17)	0.043 (worse)

ns = not significant
**Wilcoxon Matched-pairs Signed Ranks test*
† Results from all control subjects, n = 50 ulcers
‡ Results from random sample of 29 ulcers from control group
§ One outlier with rapid healing removed from Stage IV THOT group

SWC groups were significantly different at 4 weeks, χ^2 = 22.73, $P < 0.0001$.

Table 5 shows the mean change in ulcer size from 0 to 4 weeks in the THOT group and in controls. Wilcoxon matched-pairs signed-ranks tests were performed separately for each group and stage to see if changes in size were significant after 4 weeks. The nonparametric analysis was utilized because of the great differences in variability across stage and groups. In addition, analyses were performed on all 50 ulcers in the entire control group, as well as on the random sample of 29 ulcers. Significant improvement was seen in the THOT group with all three stages of ulcers. However, controls showed no significant change in Stage II or Stage III ulcers and a significant worsening in Stage IV ulcers. Analysis of both control samples showed the same results.

Healing also was assessed by the "improvement per day" parameter. Improvement per day (cm^2) was calculated by subtracting ulcer size at 4 weeks from initial size and dividing by number of days to healing, or by 28 days if not healed. A positive value reflects healing, while a negative value reflects worsening. One outlier (Stage IV ulcer) with a very fast rate of healing in the THOT group was excluded from data analysis. Two-way analysis of variance was performed in the randomized groups to test (1) whether improvement per day in ulcers in the THOT group was different from controls (main effect of THOT treatment), (2) whether improvement per day in ulcers in diabetics was different from ulcers in nondiabetics (main effect of diabetes), and (3) whether treatment effect in diabetic ulcers was different from treatment effect in nondiabetic ulcers (treatment by diabetes interaction). The improvement per day was significantly greater in the THOT group, with healing of +0.731 ± 1.27 (SD) cm^2, while the SWC group worsened (mean = -0.121 ± 0.22 (SD) cm^2 per day, F (1,53) = 7.08, $P < 0.01$); Mann-Whitney U tests comparing THOT to SWC groups were significant ($P < 0.0001$). The results are summarized in Table 3.

Single ulcer per patient random sample (n = 24). Because the distribution of ulcer sizes at enrollment was

TABLE 6
CHANGE IN SIZE OF DIABETIC ULCERS IN THOT AND SWC CONTROLS

	n	Locatio	Size of Ulcers (cm^2) Enrollmen	4
THOT				
Stage II	15	i: 2; t: 1; s: 3; h/f/l: 9	7.4 (6.3) (range 1–24)	0 (range 0)
Stage III	4	t: 1; s: 1; h/f: 2	10.2 (7.6) (range 2.3–19.5)	2.7 (4.6) (range 0–9.5)
Stage IV	2	h/f: 2	23.8 (4.4) (range 13.6–34)	13.0 (7.8) (range 7.4–18.5)
SWC				
Stage II	8	i: 2; s: 2; h/f/l:4	10.6 (12.9) (range 1–35)	15.5 (24.7) (range 0–23.7)
Stage III	4	t:2; h/f: 2	10.4 (9.7) (range 1.5–24)	16.5 (16.3) (range 2.6–38.5)
Stage IV	4		14.5 (14.5) (range 1–35)	20.9 (14.0) (range 8.8–39)

i = ischial; tr = t rochanteric; s = sacral; h = heel; f = foot; l = leg

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 ± 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 diabetic (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.

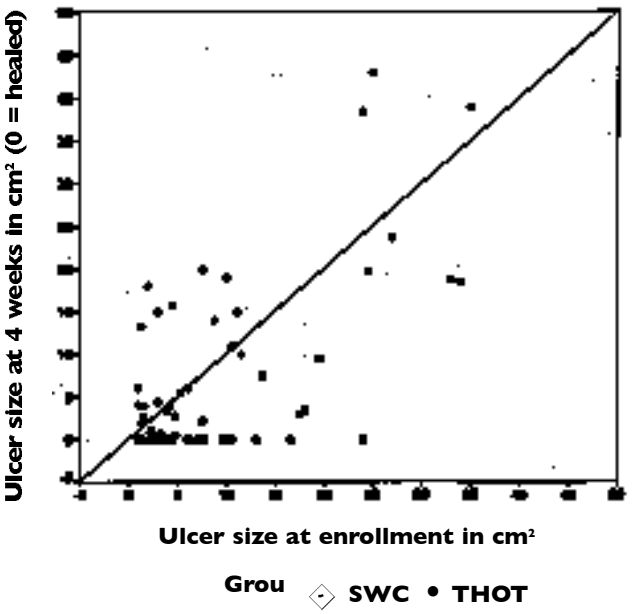

Figure 4
The above diagram shows the change in size of necrotic/gangrenous ulcers (cm^2) at 0 and 4 weeks of treatment in Stages II, III, and IV ulcers treated with both THOT and SWC.

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 7
LOGISTIC REGRESSION ANALYSIS OF HEALED ULCERS AT 4 WEEKS

Predicto	Bet	SE	Wald	df	*P*-value	Odds Ratio (CI)
THOT vs SWC	4.27	1.29	11.02	1	0.0009	71.3 (5.8–896)
Stage II vs Stage IV	3.69	1.55	5.65	1	0.0175	39.88 (1.92–835)
	0.43	1.48	0.09	1	0.769	1.54 (.054–16.18)
Stage III vs Stage		1.06	2.63	1	0.105	0.18 (.022–1.43)

predictors, these cases were retained for modeling. All three predictors entered the model as shown in Table 7. Although the model diagnostics were acceptable, the large odds ratios are due to a combination of factors: First, the model contains cells with zero cases (no SWC ulcers at Stage III or IV healed) and almost complete separation between groups. Only 3 of 50 SWC ulcers healed versus 18 of 29 THOT ulcers. Second, the inclusion of the three outliers based on Z residuals contributed to the large variability and thus large odds ratios and confidence intervals. Third, the number of cases is relatively small for logistic regression. Thus, the logistic regression is a preliminary indicator that THOT treatment and ulcer stage are predictive of healing, but will need replication in a larger sample.

The model predicted that THOT ulcers healed better than SWC ulcers ($P < 0.0005$); Stage II ulcers healed better than Stage IV ulcers ($P = 0.0175$); but Stage III ulcers did not heal better than Stage IV ulcers ($P = 0.7692$), and healing was not predicted on whether ulcers were diabetic ($P = 0.1049$).

Histological data from wound biopsies. Biopsy specimens were taken from all ulcers before treatment and 3 weeks after treatment from 29 of 29 ulcers in the THOT group and 35 of 50 ulcers in the SWC group. The remaining 15 of 50 SWC ulcers were not biopsied at 3 weeks because of the following: progressive gangrene (10 ulcers), bone involvement (3 ulcers), and patient refusal (2 ulcers).

Pretreatment biopsy specimens of necrotic tissue were identical in both THOT and SWC ulcers, consisting mainly of necrotic tissue with sparse or thrombosed blood vessels. Histological data was assessed by comparing biopsy specimens obtained at 3 weeks in the two treatment groups. Three histological criteria (ie, capillary density, fibroblast density, and density of new collagen) were assessed from the same photographic data in each patient. The density of fibroblasts was categorized as 1 (mild), 2 (moderate), and 3 (severe). Similarly, collagen density was categorized as 1 (0–25%), 2 (26–50%), 3 (51–75%), and 4 (76–100%).

The capillary density/hpf was higher in the THOT specimens (7.3 ± 1.3; range 5 - 9/hpf) compared to the SWC specimens (1.9 + 1.0; range 0–3/hpf; $P < 0.001$). Fibroblast density was decreased in the THOT specimens (1.3 ± 0.5; range 1–2) compared to the SWC specimens (2.8 ± 0.4; range 2–3; $P < 0.001$). Similarly, the density of collagen deposition was decreased in the THOT specimens (1.3 ± 0.5; range 1–2) compared to the SWC specimens (3.7 ± 0.4; range 3–4; $P < 0.001$).

Cost analysis. Cost analysis data is summarized in Table 8. The cost savings with THOT treatment compared to SWC controls at 4 weeks was 81.3% for Stage II ulcers, 37.9% for Stage III ulcers, and 36.1% for Stage IV ulcers.

Change in treatment assignment. Because of poor response in the SWC group, 6 of 11 worsening ulcers (3 Stage II, 4 Stage III, and 4 Stage IV) were treated with THOT at 4 weeks, with the following results: 3 of 3 Stage II ulcers healed by 4 weeks after change in treatment assignment, 3 of 4 Stage III ulcers healed by 6 weeks, and 1 of 4 Stage IV ulcers healed by 6 weeks. None of the THOT-treated wounds worsened or required change in treatment assignment.

Discussion

Non-necrotic, granulating wounds possess adequate blood supply and tend to heal with current therapeutic modalities.[24] On the other hand, necrotic wounds are characterized by inadequate vascularity and tend to worsen rather than heal despite therapy. Because the wounds in this study were of the latter variety, they possess an intrinsic tendency to do poorly. For this reason, and because of the fact that our Stage II and III ulcers were deeper than previously described wounds,[26] the healing rate in our control ulcers was worse than that observed by others.[26]

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

	Average Cost of Ulcer	
	THOT (necro t-	**SWC (necrot-**
Stage II Ulcers		
Nursing costs	$2.70	$40.50
Cost of dressings	$0.12	$10.10
Cost of debridement	$7.50	$15.00
Support surfaces	$0.00	$24.00
Cost of antibiotics	$4.50	$16.50
Cost of THOT	$5.00	--
Cost of Stage II ulcer per day	**$19.82**	**$106.10**
Stage III Ulcers	$32.00	
Nursing costs	$6.40	$45.00
Cost of dressings	$17.50	$17.76
Cost of debridement	$0.00	$52.50
Support surfaces	$8.50	$48.00
Cost of antibiotics	$53.60	$26.00
Cost of THOT	**$117.00**	--
Cost of Stage III ulcer per day		**$188.26**
Stage IV Ulcers	$37.35	
Nursing costs	$14.00	$48.00
Cost of dressings	$15.00	$32.40
Cost of debridement	$18.00	$70.00
Support surfaces	$16.50	$68.00
Cost of antibiotics	$62.50	$37.30

For the purpose of comparison, the national average reimbursement per ulcer per day for Stage II, III, and IV ulcers are $150, $300, and $500 respectively.[3] Total nursing times in turning patients is 60 minutes per day as is changing dressings.

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 ulcers[26] 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 $TcPO_2$, 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-β1 and TGF-β2 isoforms. The TGF-β1 gene is upregulated in tissue injury,[29] with TGF-β2 isoform implicated in wound healing with scarring. Neutralizing TGF-β by its antibody results in wound healing without scarring.[30] The TGF-β3 isoform down-regulates TGF-β1 and TGF-β2.[31] We have yet to determine whether the antiscarring properties of THOT are achieved directly through suppression of TGF-β1 and TGF-β2, by the upregulation of TGF-β3, 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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