

Enhancement of Wound Healing by Hyperbaric Oxygen and Transforming Growth Factor β_3 in a New Chronic Wound Model in Aged Rabbits

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Hypothesis: Although hyperbaric oxygen (HBO) has been used clinically for 3 decades, there have been few controlled clinical trials. Animal models have not been adequate to test the efficacy of HBO in the treatment of chronic wounds, either by itself or in combination with growth factors. We hypothesize that HBO is as efficacious as a prototype growth factor in improving wound healing in a new animal model of ischemic chronic wounds.

Design: Twenty-five aged rabbits and 3 young rabbits had their ears rendered chronically ischemic and ulcers were created down to the level of cartilage. These ulcers were treated in 1 of 3 ways: with HBO, 90 minutes per day, Monday through Friday, for 4 weeks; with transforming growth factor β_3 at 1 $\mu\text{g}/\text{cm}^2$; or with both modalities combined. Controls were treated with vehicle or hyperbaric room air or both.

Results: This model created an aged/ischemic wound that failed to heal spontaneously up to 26 days after wounding (88% reduction compared with aged/nonischemic controls). Hyperbaric oxygen alone and transforming growth factor β_3 alone both improved healing rate (only 38% reduction in healing compared with aged/nonischemic controls). Combined therapy produced no additional improvement over either modality by itself.

Conclusions: In aged animals, HBO and transforming growth factor β_3 were equally effective in improving wound healing. Our data suggest that HBO alone may be more effective in the chronic wound than in the acute wound. There was no additive benefit to combining modalities as has been reported in the same wound model in young rabbits.

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CHRONIC, nonhealing wounds are very common and are difficult to treat. They cause significant morbidity to those afflicted, and are a considerable financial burden on the medical system.¹⁻³ Many underlying conditions, including diabetes, peripheral vascular disease, radiation injury, pressure injury, and venous stasis, are responsible for creating and perpetuating chronic wounds. Although this diverse group of conditions affects regional tissue beds in a variety of ways, inadequate tissue oxygenation, a known impediment to normal wound healing, is the common feature in each of these conditions.⁴

A clinically relevant animal model of a chronic wound has not been described heretofore. A standardized ischemic dermal ulcer model in rabbits, which we have used previously, has been a very useful experimental tool⁵⁻⁹; however, it lacks the chronicity that is present in its human counterpart. The vast majority of chronic nonhealing wounds occur in elderly persons, and the combination of age plus ischemia has been shown to act synergistically to impair

wound healing.¹⁰ We have developed a new animal model of a chronic ischemic wound in aged animals, which we believe more closely approximates the human chronic wound than any other animal model available. This combination of advanced age and prolonged ischemia allows us to produce a wound that exhibits only minimal healing 26 days after it is created.

Hyperbaric oxygen (HBO) and growth factors are the only treatment options available for clinical use that actually enhance the rate of wound healing in chronic wounds. Most animal studies show a beneficial effect from growth factors.^{8,9,11,12} Even though studies on humans have been somewhat less promising, enthusiasm for their use remains strong.^{13,14} Hyperbaric oxygen has been used for more than 30 years.^{15,16} Its clinical use for chronic wounds has been supported by case reports and a few clinical trials, but controlled testing on animals has been hampered by lack of a good model of a chronic wound.¹⁷⁻²² This study was designed to evaluate the effect of a conventional human treatment protocol of HBO, a multiple-dose treatment with transforming growth factor β_3 (TGF- β_3),

MATERIALS AND METHODS

Twenty-five aged (36-40 months) and 3 young (3-6 months) female New Zealand white rabbits were used in this study.²³

RABBIT EAR WOUNDING METHOD

After anesthetizing the animals with intramuscular injections of 60-mg/kg ketamine hydrochloride and 5-mg/kg xylazine hydrochloride, the ears of 10 aged rabbits (weight, 3.0-3.5 kg) were made ischemic as described for the standard ischemic dermal ulcer model.²⁴ Under sterile conditions, the central and rostral nutrient arteries were divided at the base of the ear to render the ear ischemic, while preserving all 3 veins and the caudal artery. The skin and soft tissues were divided circumferentially down to the level of bare cartilage, thus interrupting the entire dermal circulation. Three 6-mm dermal ulcers were created on each ear to the depth of bare cartilage using a 6-mm dermal punch, under a binocular microscope, magnification $\times 10$ (**Figure 1**). A fourth ulcer was placed over the remaining vascular pedicle to serve as a nonischemic control. Each wound was then treated with TGF- β_3 or a control vehicle according to the protocol described below, and then covered with a polyurethane occlusive dressing. To maintain the ischemia in the tissue bed, the vascular pedicles were rewounded on days 5, 10, 17, and 21. This involved disrupting any reconstitution of blood flow through the dermal circulation in the vascular pedicle.

All animals were killed and the wounds were harvested on the 26th day after wounding. Animals used in this study were given food and water ad libitum and housed and maintained in a facility approved by the American Association for Accreditation of Laboratory Animal Care in accordance with established guidelines.

TGF- β_3 TREATMENT

Transforming growth factor β_3 (0.3 μg per wound) was delivered in a methylcellulose gel vehicle directly into the wound bed. This represents a TGF- β_3 dose of 1 $\mu\text{g}/\text{cm}^2$ in a methylcellulose gel volume of 0.1 cc administered to these 6-mm-diameter wounds. Control wounds were treated with the methylcellulose gel vehicle alone. Wounds were then covered with a polyurethane occlusive dressing. The growth factor was administered at the time of the initial wounding and again on days 5, 10, 17, and 21, at which time the vascular pedicle was rewounded.

HBO TREATMENT

After recovering from anesthesia, the rabbits were placed inside an animal HBO chamber (B11-22; Reimers Engineering, Alexandria, Va). Then they were exposed to either 100% oxygen (test) or 21% oxygen (control) at 2.4 atmospheres of absolute pressure for 90 minutes once daily (Monday through Friday) for 4 weeks. Compression and decompression was done at a rate of 0.07 atmospheres of absolute pressure per minute. During the period of oxygen treatment and decompression, all animals were observed closely for evidence of ill health, and the ears were examined daily for alteration in color, temperature, edema, and necrosis.

HARVEST METHODS AND HISTOLOGIC ANALYSIS

A variety of wound healing parameters were obtained, including new granulation tissue (NGT), epithelial growth (EG), ingrowth (IG), peak height (PH), and collagen deposition. These parameters have been successfully employed previously to assess wound healing in this rabbit dermal ulcer model.¹⁸ The wounds were excised, bisected, and then 1 half of each ulcer was fixed in 10% formalin. The tissue samples were stained with hematoxylin-eosin and sirius red before examination. All slides were examined with the $\times 10$ objective and a CFW $\times 10$ eyepiece. Sirius red-stained slides were viewed with a polarizing filter. This technique allowed us to distinguish immature collagen, which appeared green or yellow, from mature collagen, which appeared red. The area of the wound containing NGT was measured using a square eyepiece micrometer disc. The NGT was calculated by estimating, to the nearest 1/4 square, the area of interest that partially covered the square. Calibration of the eyepiece disc allowed the setting of each square to equal 0.01 mm^2 . The EG, IG, and PH were measured with a microruler, and expressed as distance in micrometers. Sirius red slides were graded from 0% to 100% staining of new collagen in the new granulation tissue.

Two independent, blinded observers examined all slides. The scores from the 2 examiners were then averaged for each sample. If the scores for the 2 observers differed by more than 25%, a third blinded observer examined the slide(s). The analysis of variance was used to evaluate statistical significance for groups of 3 or more. A *t* test was used to evaluate statistical significance between 2 independent groups.

and combination treatment with both HBO and TGF- β_3 on the healing of a new model of a chronic wound. The TGF- β family of growth factors is effective in healing wounds in our model as well as in other models.⁸ Transforming growth factor β_3 is a potent growth factor found in wounds and is important in augmenting wound healing.

RESULTS

To determine the validity of our new chronic wound model, we compared the aged/ischemic wounds with aged/nonischemic wounds placed over the vascular pedicle, and with ischemic wounds in young animals.

Both the aged/nonischemic wounds and the young/ischemic wounds were completely healed at day 26 with no therapeutic interventions (**Figure 2**). New granulation tissue for the aged/nonischemic wounds showed a mean of 222 $\mu\text{m}^2 \pm 35 \mu\text{m}^2$ (n=10), whereas the NGT for the young/ischemic wounds (n=12) showed a mean of 255 $\mu\text{m}^2 \pm 17.5 \mu\text{m}^2$. The aged/ischemic wounds exhibited a mean NGT of only 25.5 $\mu\text{m}^2 \pm 20 \mu\text{m}^2$ (n=14). This represents a 90% decrease ($P < .001$) compared with young/ischemic controls, and an 88% decrease ($P < .001$) compared with aged/nonischemic controls. Epithelial growth was 600 μm (completely healed) for both types of control wounds. A mean of 120 $\mu\text{m} \pm 36 \mu\text{m}$ was calculated

for the chronic (aged/ischemic) wound. This represents an 80% decrease in epithelialization ($P < .001$) (**Figure 3**).

Prior to histologic comparison of treated wounds with nontreated wounds, it was determined that NGT, EG, IG, and PH did not significantly vary due to wound position on the ischemic ear. There was a trend, however, toward less healing in the more distal wounds. All ischemic wounds in all treatment groups were significantly altered compared with their nonischemic and young controls. Experiments were then performed to test the effects of HBO, TGF- β_3 , and the combination of HBO/TGF- β_3 on healing in the chronic wounds. New granulation tissue for HBO-treated chronic wounds ($n=14$) was measured at $102 \mu\text{m}^2 \pm 40 \mu\text{m}^2$. Using the nonischemic controls (stated above as $222 \mu\text{m}^2 \pm 35 \mu\text{m}^2$) as our healing goal (or 100% increase), and the chronic nontreated wounds as a baseline ($25.5 \mu\text{m}^2 \pm 20 \mu\text{m}^2$), HBO treatment produced an increase of 37% in healing in chronic wounds ($P < .001$). Wounds treated with TGF- β_3 ($n=15$) had a mean value of $100.25 \mu\text{m}^2 \pm 27 \mu\text{m}^2$, a 36% increase in healing ($P < .001$). The combined HBO/TGF- β_3 treatment ($n=13$) produced NGT with a mean of $109 \mu\text{m}^2 \pm 42 \mu\text{m}^2$, an increase of 42% over the chronic wound ($P < .001$). The difference between the HBO group and the HBO plus TGF- β_3 group was not significant ($P = .6$). Hyperbaric oxygen-treated aged/nonischemic wounds ($n=10$) showed an

NGT of $277.5 \mu\text{m}^2 \pm 70 \mu\text{m}^2$ compared with nontreated aged/nonischemic wounds ($n=9$) ($222 \mu\text{m}^2 \pm 70 \mu\text{m}^2$). This represented a 25% increase in healing ($P < .01$). Transforming growth factor β_3 -treated aged/nonischemic wounds ($n=10$) showed an NGT of $275.2 \mu\text{m}^2 \pm 65 \mu\text{m}^2$. This also represented a 25% increase in healing ($P < .01$) over nontreated aged/nonischemic wounds. Combination therapy with HBO and TGF- β_3 -treated aged/nonischemic wounds ($n=5$) exhibited a mean NGT of $313 \mu\text{m}^2 \pm 80 \mu\text{m}^2$, a 41% difference ($P < .001$) (**Figure 4**).

All nonischemic wounds and all wounds from young animals were completely epithelialized at the time of harvest on day 26. Each of these groups had an epithelial growth of $600 \mu\text{m}$ (100% closure). Nontreated aged/ischemic wounds ($n=14$) had a mean EG of $120 \mu\text{m} \pm 36 \mu\text{m}$, an 80% decrease compared with control nonischemic wounds ($P < .001$). Chronic wounds treated with HBO ($n=14$) showed a mean EG of $391 \mu\text{m} \pm 130 \mu\text{m}$, a 56% improvement over the nontreated chronic wounds ($P < .001$). Chronic wounds treated with TGF- β_3 ($n=14$) had a mean EG of $380 \mu\text{m} \pm 104 \mu\text{m}$, a 54% increase in EG over nontreated chronic wounds ($P < .001$). Chronic wounds treated with both HBO and TGF- β_3 ($n=15$) had a mean EG of $409 \mu\text{m} \pm 150 \mu\text{m}$, a 60% improvement over nontreated chronic wounds ($P < .001$). The difference between EG for HBO-treated or TGF- β_3 -treated chronic wounds and HBO plus TGF- β_3 -treated chronic wounds was not significant ($P > .75$) (**Figure 5**).

All nonischemic wounds were completely healed by harvest on day 26 and had a resulting IG distance of $600 \mu\text{m}$. Nontreated chronic wounds displayed an average IG of $90 \mu\text{m} \pm 30 \mu\text{m}$. Hyperbaric oxygen-treated chronic wounds showed an average IG of $230 \mu\text{m} \pm 58 \mu\text{m}$, a 54% improvement over nontreated wounds ($P < .001$). Transforming growth factor β_3 -treated chronic wounds showed an average IG of $307 \mu\text{m} \pm 150 \mu\text{m}$, a 40% increase over nontreated chronic wounds ($P < .01$). Wounds treated with HBO plus TGF- β_3 showed a mean IG of $276 \mu\text{m} \pm 70 \mu\text{m}$, a 44% increase in healing ($P < .001$). Again, combining TGF- β_3 with HBO treatment did not significantly enhance healing of chronic wounds over that seen with HBO or TGF- β_3 treatment alone (**Figure 6**).

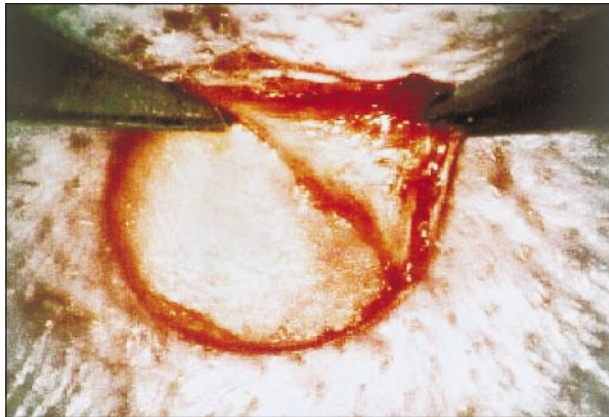


Figure 1. Surgical creation of a 6-mm ulcer on the ventral side of a rabbit ear.

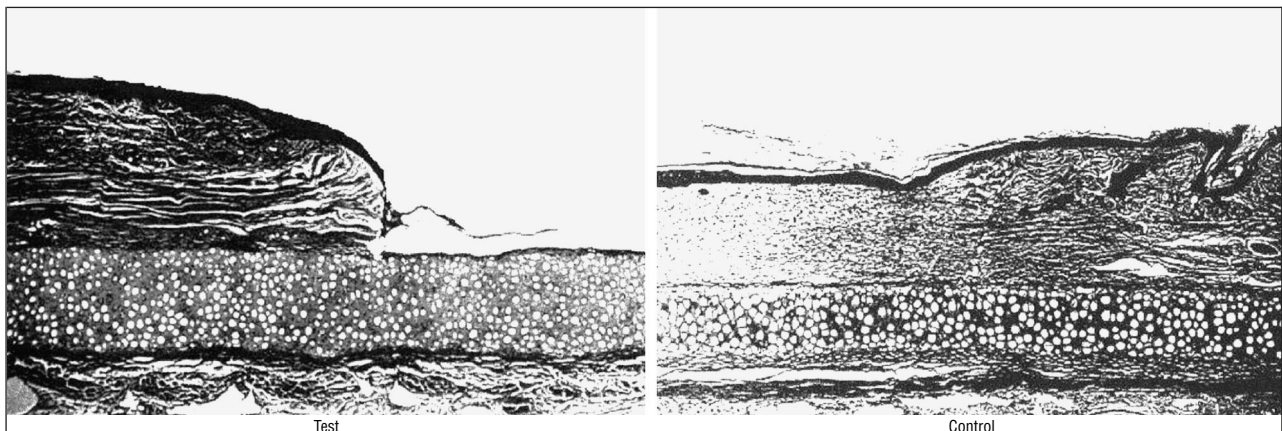


Figure 2. Histologic cross section of rabbit ulcers. Left, Chronic (aged/ischemic) ulcer. Right, Aged control ulcer.

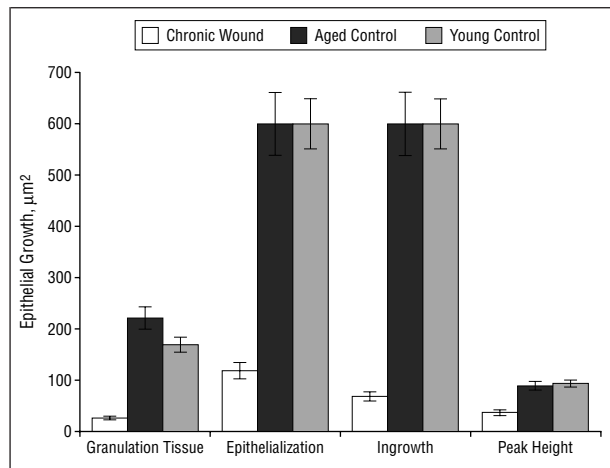


Figure 3. Parameters of wound-healing measurements in the chronic wound model.

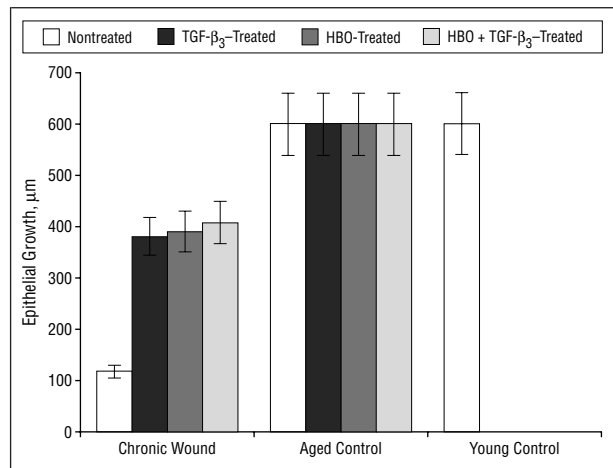


Figure 5. Epithelial growth. TGF-β₃ indicates transforming growth factor β₃; HBO, hyperbaric oxygen.

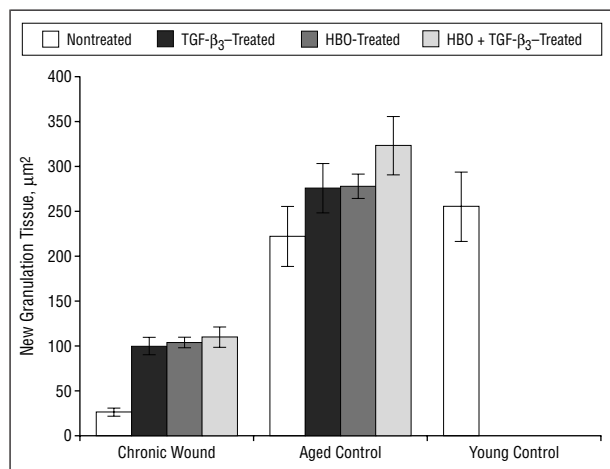


Figure 4. New granulation tissue formation. TGF-β₃ indicates transforming growth factor β₃; HBO, hyperbaric oxygen.

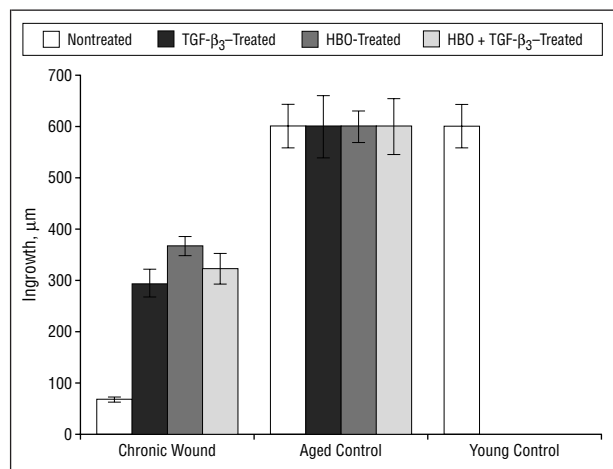


Figure 6. Ingrowth. TGF-β₃ indicates transforming growth factor β₃; HBO, hyperbaric oxygen.

Peak height, another indicator of granulation tissue, was evaluated in all wounds. The average PH in the various groups was tightly clustered between 45 μm and 55 μm, except for the TGF-β₃-treated aged/non-ischemic wound, which showed a PH of 80 μm ± 20 μm. This group is the only group to show a significant difference compared with the control group ($P < .01$). This result is consonant with our previous studies using TGF-β1, and reflects its influence on the wound architecture (**Figure 7**).

Sirius red-stained slides revealed significantly increased collagen deposition in treated wounds and controls. Collagen deposition was increased significantly by TGF-β₃ in the chronic wound model (21%, $P < .01$). In the aged/non-ischemic controls, the increase was not significant (12%, $P > .05$). Similarly, HBO also increased collagen deposition in the chronic wound model (22%, $P < .01$). However, in the HBO-treated aged/non-ischemic controls, the increase was not significant (11%, $P > .05$). Combination treatment also increased collagen deposition compared with controls in the chronic wound model (22.5%, $P < .01$), and again the increase in the aged/non-ischemic controls was not significant (10%, $P > .05$). There was no significant difference between the

treated groups. Combination treatment again showed no significant increase over either treatment modality alone in either the chronic wound model or the aged controls (**Figure 8**).

COMMENT

Chronic wounds are common, difficult to treat, and extremely costly both in dollars and in human misery.^{3,25,26} Although the provenance of chronic wounds is found in a disparate group of clinical conditions, the most common underlying factor in chronic wounds is inadequate tissue oxygenation. Even pressure ulcers are the result of repeated, intermittent ischemic insults. In the past, much has been learned about wound healing from the use of the standardized ischemic wound model previously described.^{5-9,24} Though this model is reproducible, predictably ischemic, and noncontractile, it is nevertheless an acute wound. We set out to create a model of a chronic wound as it is seen in clinical practice by adding the factor of advanced age, which is known to act synergistically with hypoxia to impede wound healing. Using techniques that create and perpetuate an ischemic tissue bed in an animal of advanced age, we have

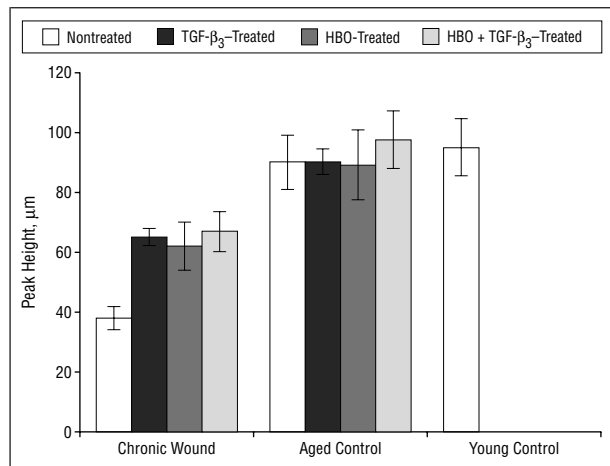


Figure 7. Peak height. TGF-β₃ indicates transforming growth factor β₃; HBO, hyperbaric oxygen.

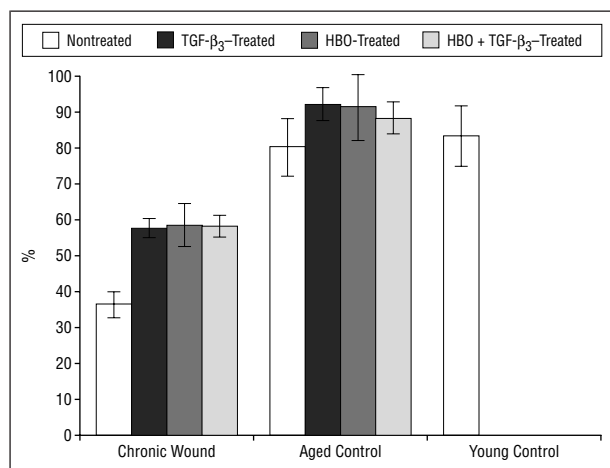


Figure 8. Collagen deposition. TGF-β₃ indicates transforming growth factor β₃; HBO, hyperbaric oxygen.

modified the standard rabbit ear ulcer model and have developed a new animal model of a chronic wound that more closely approximates the human chronic wound than any other animal model available. The ulcers created with the standard rabbit model healed in approximately 7 to 10 days, which precludes the study of long-term treatment protocols that are used in humans. The combination of advanced age and prolonged ischemia produced a wound that showed only minimal healing 26 days after creation of the wound. This allowed assessment of the typical 4-week course of HBO as it is commonly used in the treatment of human chronic wounds in current clinical practice.

It has been known for more than 3 decades that hypoxia is a major impediment to wound healing.^{15,16} Initially, clinicians used HBO to treat chronic wounds, assuming that oxygen was needed by hypoxic cells as the metabolite for cellular respiration and energy production. The beneficial effects of this treatment in selected cases seemed to reinforce this hypothesis. The short period of oxygen sufficiency followed by a prolonged period of hypoxia was believed to be a normal and inevitable part of healing in acute wounds secondary to small

vessel thrombosis, which was necessary to prevent bleeding from open wounds. It is counterintuitive, however, to believe that tissue that is dysfunctional due to a lack of oxygen would benefit substantially if it were “allowed to breathe” only 8% of the time. Recent studies suggest that intermittent, relatively short periods of supraphysiologic levels of oxygen act as an intracellular signal transducer that can up-regulate production of growth factor receptors.

Clinical studies using HBO have been promising, but the treatment protocol used in patients involves multiple treatments during a period of 4 to 8 weeks.^{17,22,25-29} Heretofore there has not been an animal model that could be used to test a typical clinical treatment course of HBO before the wounds would heal spontaneously. Our new model provides both the control of variables possible with animal studies, and the ability to observe the effects of HBO on a truly chronic wound.

The use of growth factors is another option in the treatment of wounds that has shown promise in animal models, yet proven to be only moderately effective in human clinical trials.^{13,14,18,30} This disparity may be due to the lack of an appropriate animal model that truly emulates the human chronic wound. It is not surprising that both HBO and TGF-β₃ were able to completely reverse the combined wound healing deficits of age and ischemia. This indicates that multiple-agent treatment will probably turn out to be the most successful therapeutic strategy. Platelet-derived growth factor is the only growth factor currently available for clinical use. However, all members of the TGF-β family have also been shown to benefit wound healing.³¹⁻³³ These and other studies with growth factors in animals and humans have shown increases in wound healing. However, no growth factor has been shown to be able to completely reverse the deficit in wound healing caused by ischemia or diabetes. Zhao et al¹⁸ have shown that the combination of platelet-derived growth factor and TGF-β₃ plus HBO completely reverses the wound healing deficit caused by ischemia in the standard young rabbit dermal ulcer model. This effect seemed to be additive. The findings in our current study suggest a different response in the chronic ischemic wound in aged animals.

Studies that compare the effect of HBO with the effect of a growth factor on chronic wounds have not been done in humans. In experimental animal studies involving acute wounds, growth factors seemed to be more efficacious than HBO in healing.¹⁸ However, in our study, HBO was equally as efficacious as TGF-β₃ in this model of a chronic wound. In addition, although the combination of HBO with the growth factor showed a trend toward an additive effect, it did not significantly improve wound healing over either modality alone. This pattern of results was seen throughout all of the parameters measured. This corroborates the clinical observation that in the right setting, HBO clearly enhances the rate of healing of chronic wounds. This may represent an intrinsic difference in the wound milieu of chronic wounds and their responses to therapeutic agents. It is possible that truly chronic wounds respond better to HBO treatment than do acute wounds. The latter may also reflect the fact that the maximum benefit of HBO requires multiple treat-

ments over an extended period of time. This type of response could not be substantiated using previous available animal models.

The results of these studies reinforce the idea that a chronic wound is not simply an acute wound that has lasted a long time. Rather, the results support the hypothesis that the biochemical makeup of a chronic wound is fundamentally different from that of an acute wound and that these differences need to be distinguished and treated appropriately.

In summary, we described a new animal model that more closely approximates a human chronic wound, and used it to examine the effects of HBO, TGF- β_3 , and the combination of these 2 therapies on the healing of chronic ischemic wounds in aged animals. The findings in these studies demonstrate the beneficial effects of both HBO and TGF- β_3 . Each is equally effective in this chronic wound model, with no significant additive effects. This is in contrast to the additive benefit of combination therapy as seen in the acute experimental wound. The results of this study also suggest that HBO as a single therapeutic agent is more efficacious in the chronic wound than in the acute wound.

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