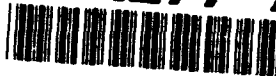


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LABORATORY

**COMPARISON OF THE EFFECTS OF HYPERBARIC
OXYGENATION AND TRANSFORMING GROWTH
FACTOR BETA ON WOUND HEALING IN RATS**

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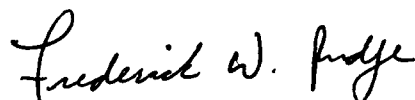
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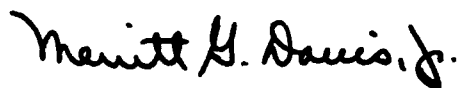
All animals were used and cared for in compliance with the Animal Welfare Act, DoD directive 3216.1 and AFR 169-2, "The Use of Animals in DoD Programs", and NIH publication 85-23, "Guide for the Care and Use of Laboratory Animals" (prepared by the Institute of Laboratory Animal Resources, National Research Council). Appropriate consideration was given to the policies, standards, and guidelines for the proper use, care, handling, and treatment of animals.

The Office of Public Affairs has reviewed this report, and it is releasable to the National Technical Information Service, where it will be available to the general public, including foreign nationals.

This report has been reviewed and is approved for publication.



FREDERICK W. RUDGE, Lt Col, USAF, MC, FS
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13. ABSTRACT (Maximum 200 words) A pilot investigation was conducted to determine the effects of hyperbaric oxygenation (HBO) and topical transforming growth factor beta (TGF-β1), alone and in combination, on the healing of standardized excisional wounds in normal rats. Excisional wounds were made on the dorsum of 96 adult male Sprague-Dawley rats, and rats were randomly allocated into groups receiving daily topical TGF-β1 or placebo solution. Groups were subdivided into animals treated with either ground level air, 2.0 ATA air, ground level 100% oxygen, or 2.0 ATA 100% oxygen. Each 4 days, wounds were measured, animals weighed, and 2 animals euthanized for histologic study to assess healing. The study found that TGF-β1 treated rats healed faster, with maximum effect 12 days after wounding. Breathing mix and depth had no significant effect on healing. Animals treated with oxygen gained significantly more weight than animals treated with air.					
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COMPARISON OF THE EFFECTS OF HYPERBARIC OXYGENATION AND TRANSFORMING GROWTH FACTOR BETA ON WOUND HEALING IN RATS

INTRODUCTION

The rate of wound healing is a major factor in recovery from trauma or surgery. In a military setting, the rate of wound healing can affect the length of time needed for return to duty after injury. Faster wound healing, and faster return to duty results in increased troop strength. Chronic nonhealing wounds result in increased morbidity and mortality, consume valuable medical resources, and affect the ability of the individual to return to gainful employment.

Both hyperbaric oxygen therapy and the application of topical growth factors have been used independently in the treatment of chronic wounds. A comparison of the effects of these two treatment modalities has not been previously performed to determine the relative merits of each of these forms of therapy, and no studies have been done to determine whether a combination of these therapies would result in further improvements in wound healing. Determination of the optimal therapy to promote wound healing will have a definite advantage in the treatment of combat related wounds and in the treatment of nonhealing wounds from both combat and noncombat causes. This study was undertaken with the following scientific objectives:

- A. Determine the effect of hyperbaric oxygenation (HBO) on healing of standardized wounds in normal rats.
- B. Determine the effect of application of transforming growth factor-beta (TGF- β 1) on healing of standardized wounds in normal rats.

C. Compare the effects of HBO and TGF- β 1 on wound healing in normal rats.

D. Determine the effects on wound healing in normal rats obtained by combining HBO with TGF- β 1.

TECHNICAL BACKGROUND

Chronic nonhealing wounds pose a significant clinical problem resulting in patient morbidity and mortality. Management of these types of wounds has been a source of exasperation for surgeons and other health care providers. Patients with a history of diabetes, prior radiation therapy, and arterial and venous insufficiency are prone to develop refractory wounds, resistant to many forms of standard therapy. Two recent developments in the treatment of problem wounds are hyperbaric oxygenation and platelet derived growth factors.

The use of hyperbaric oxygenation (HBO) as an adjunct in the management of problem wounds is increasing worldwide (1). HBO has been shown to enhance wound healing in diabetic wounds (2,3), wounds associated with radiation therapy (4), arterial insufficiency ulcers (5), and venous stasis ulcers (6). The utility of HBO in wound healing enhancement is related to the correction of local tissue hypoxia, leading to resumption of the normal healing process.

The second new significant modality in the treatment of chronic refractory wounds, application of platelet derived growth factors, such as transforming growth factor beta (TGF- β 1), is receiving increasing attention in the world literature. TGF- β 1 is a polypeptide present in a variety of tissues, most notably in platelets. This substance is released from platelets at the site of injury (7), and acts to regulate the wound repair process. It is known to be chemotactic

for inflammatory cells (8), and for fibroblasts (9), and stimulates angiogenesis (10) and formation of collagen (10, 11). In animal studies it has been shown to increase wound tensile strength (12), reverse the cytotoxic effects of doxorubicin (13), and accelerate the formation of granulation tissue in normal and diabetic rats (14, 15).

To date, although impressive results are reported from the individual use of HBO and TGF- β 1 to improve healing, no studies have been done to directly compare the results of each. Moreover, no studies have been done to determine the additive effects of using these two modalities of treatment in combination. This study compares the effects of these two forms of treatment and the effect of using them in combination.

EXPERIMENTAL METHOD/APPROACH

A. Preparation and surgery

Ninety-six adult male Sprague-Dawley CD-VAF/Plus rats (Charles River Laboratories, Wilmington, MA), male, 150-200 grams, were used for the study. (Eight groups of 12 animals.) All rats were of equivalent age. Rats were maintained on a diet of standard rat chow and water ad lib, and were individually housed in wire cages in an air-conditioned environment on 12-hour light-dark cycles. On the first day of the study, standardized wounds were made on the dorsum of each rat using aseptic technique. Each rat was anesthetized with a single intramuscular injection of Ketamine (10 mg/100 gm body weight) and Xylazine (2 mg.) and the dorsal surface shaved with clippers. A square wound, two centimeters on each side, was made on the dorsal surface using a standard grid as a guide, centered on the midline, and carried down to the panniculus carnosus. All animals were weighed immediately following wounding. Wounds were then photographed, using a fixed magnification with a macro lens. A solution consisting of 2%

methylcellulose gel was applied immediately to each wound. No additional dressing was applied. Methylcellulose gel has been shown in prior studies to have no effect on the healing of wounds (15). (Consideration was given to the application of a bio-occlusive dressing after each application of solution. This approach was abandoned when it was found, on a trial run prior to the experiment, that the animals were very adept at removing and consuming these dressings.) The animals were returned to individual cages, and divided randomly into 8 groups of 12 rats for the remainder of the study. A number was made at the base of each rat's tail with an indelible marker, to facilitate identification, and to prevent accidental placement into an incorrect group during the course of the study.

B. Conduct of study

Topical solutions were prepared for application to wounds, with 2% methylcellulose gel as a base. Solution A consisted of 1000 ml of 2% methylcellulose gel containing 1 mg of biosynthetic TGF- β 1 (Genentech, Inc., South San Francisco, CA). Solution B consisted of an equal quantity of 2% methylcellulose gel, containing buffered saline equal in volume to the amount of TGF- β 1 used to make Solution A. Prior to the study, these solutions were mixed and marked by an individual not involved otherwise with the project. The identity of the solution containing the TGF- β 1 was not revealed to the investigators until the project was complete and all data analyzed.

At the same time each day, wounds were irrigated with sterile saline to remove scabs and crusts of dried blood and methylcellulose. Loose crusts were carefully lifted off the wounds. No attempts were made to perform sharp debridement on the wounds. Solution A was applied to each rat in groups 5 - 8, and Solution B was applied to all rats in groups 1 - 4. As noted above, the investigator applying the salves was blinded regarding the contents of each solution. Approximately 2.0 ml of solution was applied to each wound daily. For the animals in the growth factor groups, 2000 ng of TGF- β 1 was applied to each wound daily. It was previously demonstrated that

the peak effect of TGF- β 1 occurs at a concentration of 1,000 to 10,000 ng/wound. (15)

Daily HBO treatments began on the first day after wounding and continued to completion of the study. Chamber depth, humidity, temperature, and O₂ and CO₂ concentrations were continuously monitored. CO₂ buildup was prevented by ventilating the containers with the treatment gas at 15 l/min. Groups 1 and 5 were treated with 21% oxygen at 1 ATA (room air control). To simulate a hyperbaric treatment, these animals were placed in a sealed container for 90 min daily which was vented with air at 15 l/minute to simulate a hyperbaric exposure. Animals in groups 2 and 6 received a daily 90-min exposure to 100% O₂ at 1 ATA. These animals were also placed in a sealed container which was vented with 100% O₂ for 90 minutes daily. Animals in groups 3 and 7 received a daily 90-minute exposure to 21% O₂ (air) at 2.0 ATA. Animals in groups 4 and 8 received a daily 90-min exposure to 100% O₂ at 2.0 ATA. The treatment depth of 2.0 ATA was chosen because it is a standard depth used in the treatment of problem wounds in a monoplace chamber.

The study, therefore, contained 8 groups of 12 animals each:

1. 21% O₂ at 1 ATA, No TGF- β 1
2. 100% O₂ at 1 ATA, No TGF- β 1
3. 21% O₂ at 2.0 ATA, No TGF- β 1
4. 100% O₂ at 2.0 ATA, No TGF- β 1
5. 21% O₂ at 1 ATA, + TGF- β 1
6. 100% O₂ at 1 ATA, + TGF- β 1
7. 21% O₂ at 2.0 ATA, + TGF- β 1
8. 100% O₂ at 2.0 ATA, + TGF- β 1

C. Data Collection

Each animal was weighed to the nearest gram on the day of wounding and on days 4, 8, 12, and 16. Weights were entered on a microcomputer for analysis. Wound surface area for each rat was measured from photographs of each wound on day 1 (baseline), and days 4, 8, 12, and 16 after wounding. Slide transparencies from each wound were projected onto a sheet of paper, and the outline of the wound traced. Two of the authors (F.R. and D.W.) traced the wounds independently, then each measured the areas of the tracings twice using a compensating polar planimeter. The four results were then averaged to obtain the area of each wound. Surface area was determined to the nearest square millimeter. The results were entered on a microcomputer for analysis.

Two rats randomly selected in each of the eight groups were euthanized by CO₂ overdose in a CO₂ chamber after being weighed and photographed, and the entire wound of each animal sharply excised and submitted for histologic study, with specimens provided to the pathologist coded to allow the written interpretation of each specimen to be made in blinded fashion. Each specimen was mounted on a sheet of cardboard immediately following excision to prevent contraction, and fixed in formalin. Specimens were embedded in paraffin, and later sectioned, with microscopic slides prepared using H and E and Masson's trichrome stains. Each specimen was examined for degree of inflammation, granulation tissue, edema, presence of mature collagen (Masson's positivity), and epithelialization. The degree of involvement for each of these areas was scored as follows:

None	0
Minimal (1 - 10%)	1
Mild (11 - 25%)	2
Moderate (26 - 50%)	3
Extensive (>50%)	4

Scores for each of the eight categories were entered on a microcomputer for analysis. Analysis of all data was performed on a Macintosh microcomputer, using StatView 4.0 (Abacus Concepts, Inc.)

D. Data Interpretation

Animal weights were tabulated for each rat on days 1, 4, 8, 12, and 16. For each of the groups, the mean and standard error of the mean (SEM) were calculated, and analysis of variance (ANOVA) used to determine significant differences between groups. Wound measurements were compared by wound size ratio using day one as the baseline. For each group the mean wound area and SEM were determined. ANOVA was performed, and, where differences existed, significance was determined by the Scheffe multiple comparison test; $p < 0.05$ considered significant.

Wounds from representative animals in each group from days 4, 8, 12, and 16 were scored histologically to determine differences between the groups.

RESULTS

Changes in wound areas, by group, are depicted in Figure 1. For each day of the study, wound area decreased for each group. There was no significant difference noted in the rate of decrease in wound size among the eight groups. When breathing gas (air vs. O₂) and treatment depth (1.0 vs. 2.0 ATA) were considered independently, there was no significant difference in healing. A significant effect was noted when wound solutions were considered independently. Animals treated with methycellulose containing TGF- β 1 healed significantly faster than methycellulose alone for each of the

measurement days. (Day 4: $F= 4.109$, $p= .046$; Day 8: $F= 4.118$, $p= .046$; Day 12: $F= 5.773$, $p= .020$; Day 16: $F= 5.085$, $p= .030$)

The mean weights of the animals in each group throughout the study are depicted in Figure 2. All animals showed a significant weight gain at every measurement. The differences in weight gain for the eight groups was not significant. However, when animals treated with air vs. 100% O₂ were considered without regard to treatment depth or topical solution used, a significant difference in weight gain was noted. When analyzed by ANOVA, animals treated with 100% O₂ gained significantly more weight at each measurement than those treated with air. (Day 1-4 gain: $F= 5.899$, $p= .02$; Day 4-8 gain: $F= 17.946$, $p< .0001$; Day 8-12 gain: $F= 20.271$, $p< .001$; Day 12-16 gain: $F= 8.877$, $p= .005$) When solution and treatment depth were considered separately, or when solution, treatment depth, and gas mixture were considered in combination, no significant differences in weight gain were noted.

Histologic evaluation of the wounds are depicted in Figures 3 - 6. Changes in wound vascularity/ granulation tissue for each of the groups are shown in Figure 3. For each group, wound granulation tissue increased at each measurement throughout the study, except day 12, when a decrease was noted in the TGF- β 1, 1 ATA air group. A significant increase in vascularity and granulation tissue was seen on day 16 in animals treated with 100% O₂ as compared to the air groups ($p= 0.001$). Neither depth nor solution had an effect on vascularity and granulation tissue.

Changes in the amount of collagen in each group are shown in Figure 4. Collagen content also increased in each group for each day of measurement, except day 16, when there was a decrease in the no-TGF- β 1, 1 ATA air group. Groups treated with 100% O₂ had a significantly increased amount of collagen over the air groups only on day 16 ($p= 0.03$).

Epithelialization is depicted in Figure 5. The amount of epithelialization in each group increased for each measurement day, except day 12, when there was a decrease from day 8 in the group treated with TGF- β 1 and O₂ at 1 ATA, and day 16, when there was a decrease from day 12 in two groups (TGF- β 1, 1 ATA air, and TGF- β 1, 2 ATA O₂).

The degree of inflammation is shown in Figure 6. Unlike the other histologic parameters, no clear pattern is noted with degree of inflammation among the different groups throughout the study. However, when breathing mixture was considered separately, significant differences were noted. Animals treated with 100% O₂ had a significant decrease in inflammation over the groups treated on air for day 8 ($p = 0.01$), and day 16 ($p = 0.03$).

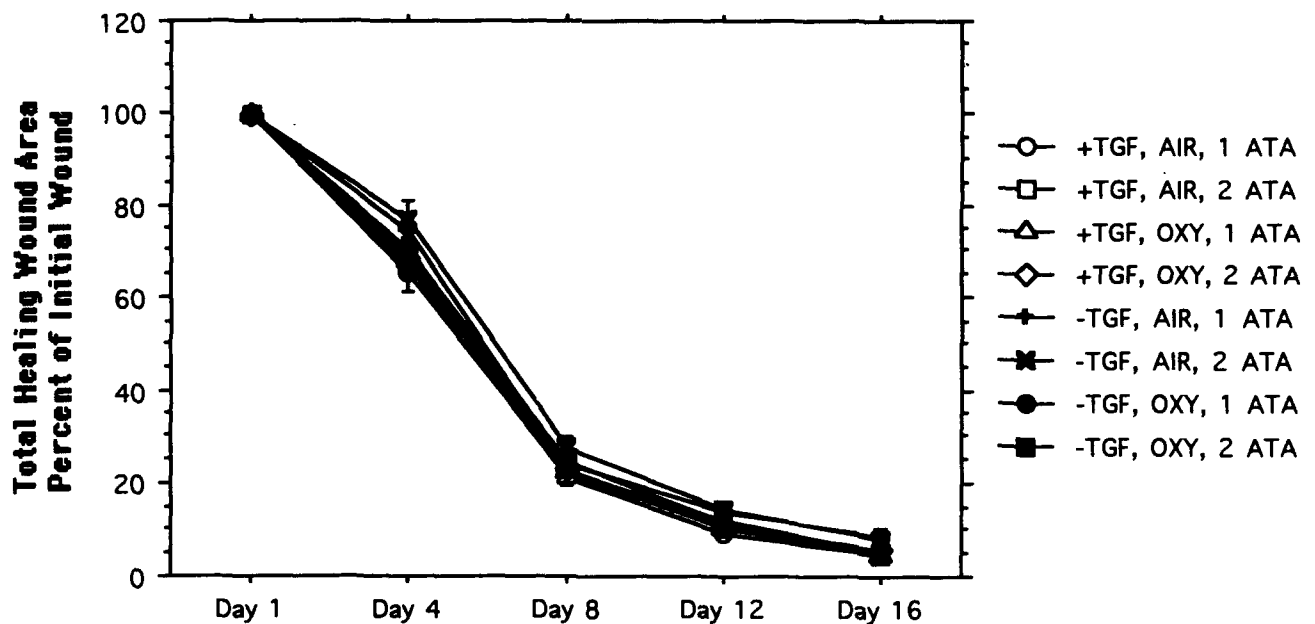


Figure 1. Change in wound area from day 1 baseline for each group.

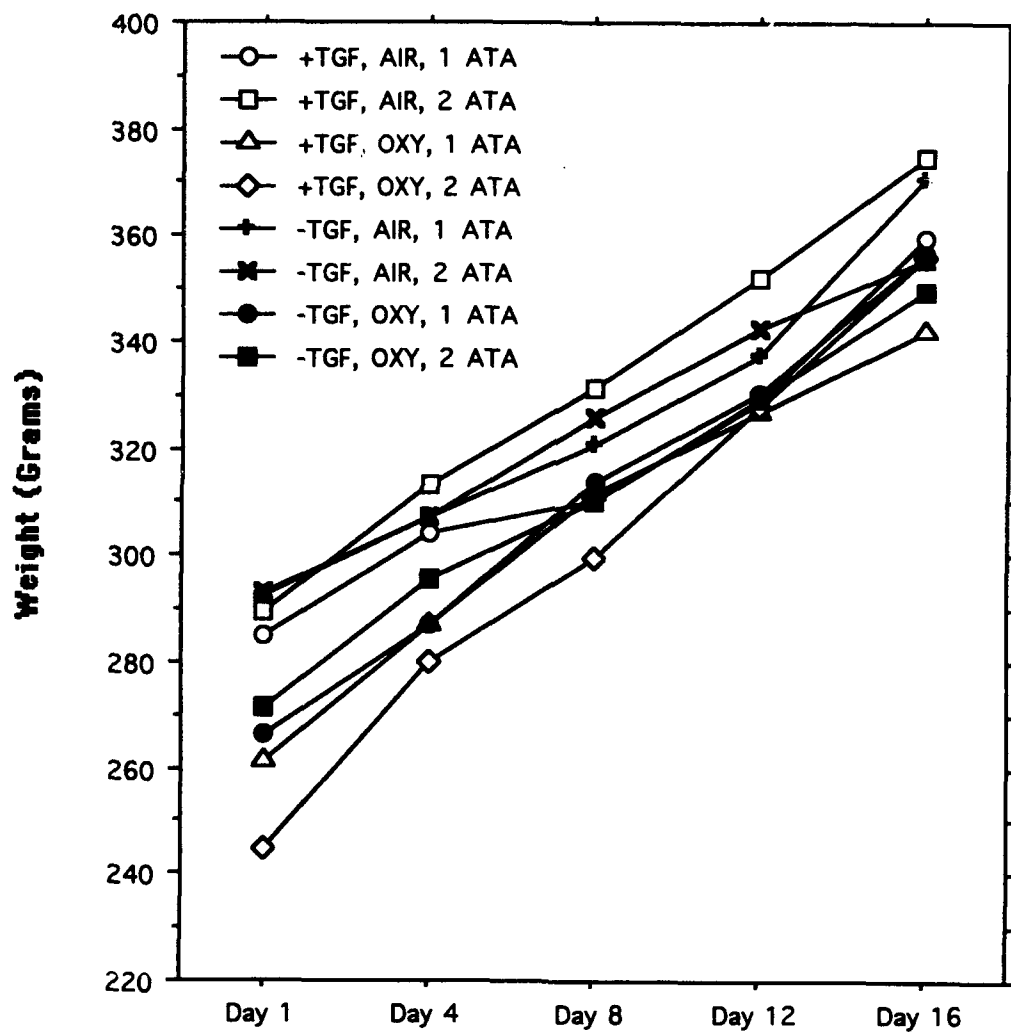


Figure 2. Mean animal weight (grams) for each group.

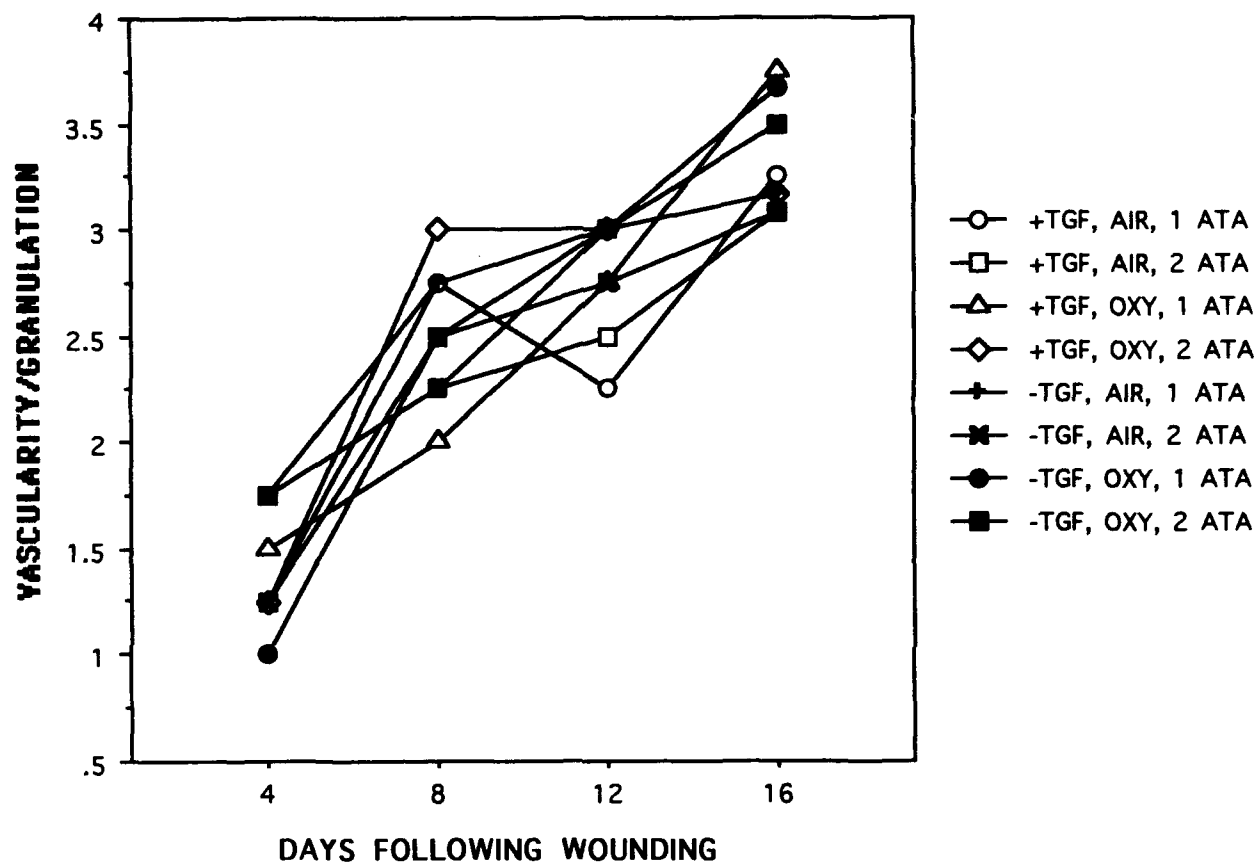


Figure 3. Degree of vascularity and granulation in wounds for each group (see rating scale on page 6).

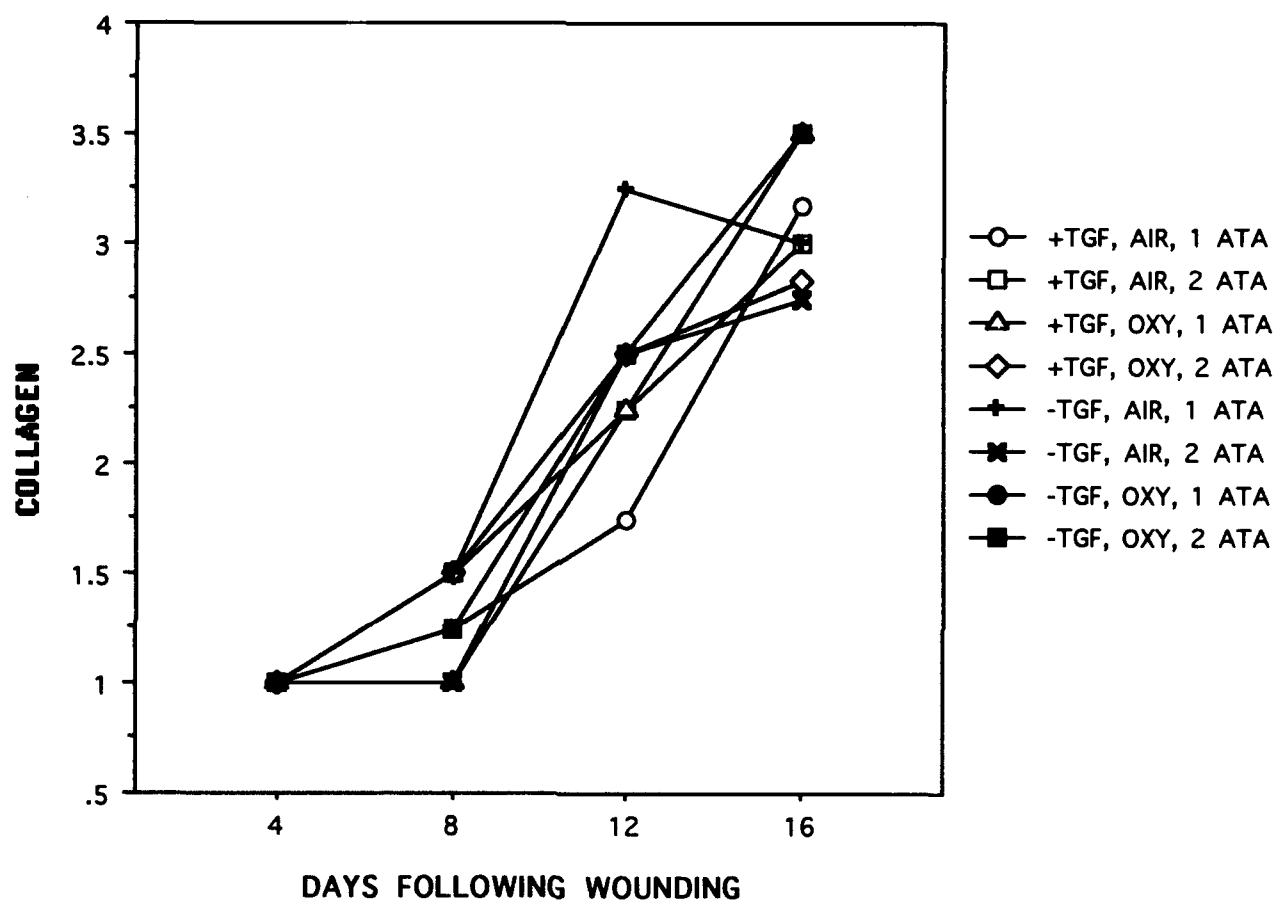


Figure 4. Collagen content for wounds in each group (see rating scale on page 6).

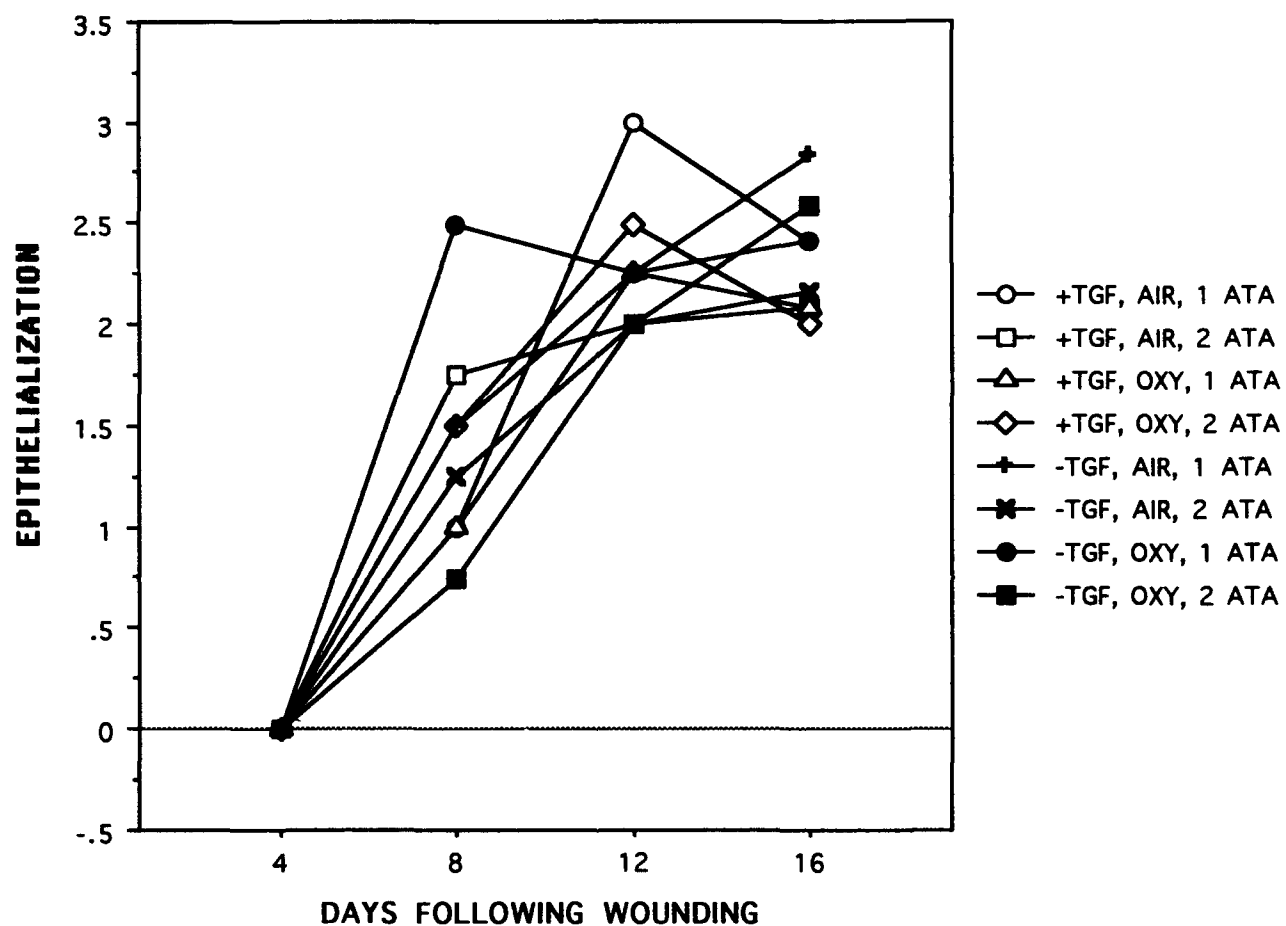


Figure 5. Degree of epithelialization for wounds in each group (see rating scale on page 6).

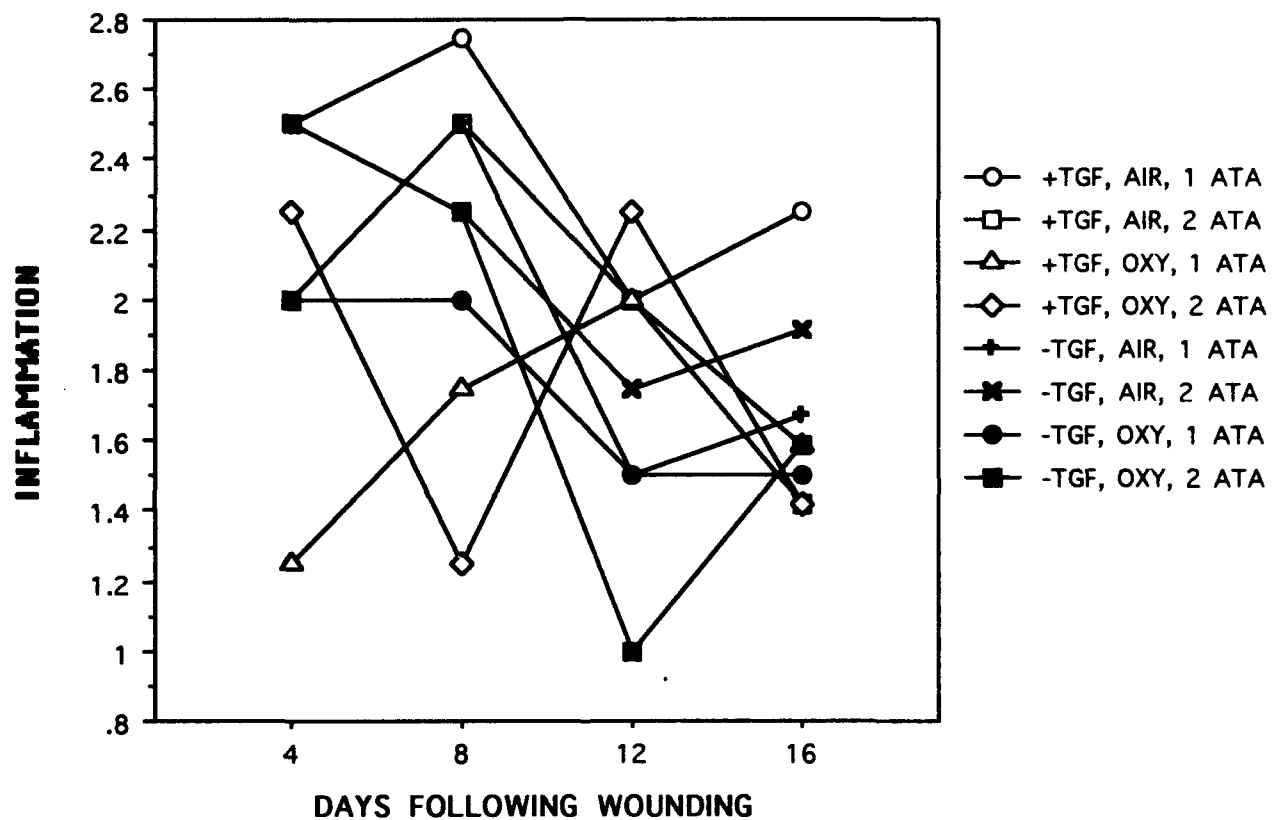


Figure 6. Degree of inflammation for wounds in each group (see rating scale on page 6).

DISCUSSION

In this study we compared the effects of two treatments which have gained increasing popularity for the treatment of wounds: hyperbaric oxygenation, and application of a topical growth factor, TGF- β 1. Many centers have opened in the United States in the past decade which specialize in the healing of problem wounds, most notably diabetic lower extremity ulcers. These centers increasingly use topical substances to stimulate wound healing in these wounds. In clinical trials, Knighton et al., have promoted healing in chronic cutaneous ulcers with the application of platelet-derived wound healing formula(16). This substance is an autologous blood product which contains at least five locally acting growth factors: platelet-derived growth factor (PDGF), platelet-derived angiogenesis factor (PDAF), platelet-derived epidermal growth factor (EGF), platelet factor 4 (PF-4), and TGF- β 1 (16, 17). Previous studies on wound healing in animal models have demonstrated that TGF- β 1, in collagen as a delivery vehicle, is a potent stimulant of wound healing in normal wounds (11, 18). Previous studies have also shown HBO therapy to be beneficial in wound healing (1, 3). Some wound healing centers have begun using both topical wound healing factors and HBO therapy together in the treatment of nonhealing wounds.

In this study, daily treatment with different concentrations of oxygen (air vs. 100% O₂) and atmospheric pressure (1 ATA vs. 2 ATA) appeared to have no effect on the rate of wound healing, although breathing mixture had a significant effect on different parameters of healing (granulation, inflammation) on some of the days. For each day of the study, breathing mixture and treatment depth did not significantly affect wound area. This finding is at odds with some previous studies, which found HBO to be of benefit in the healing of normal, nonhypoxic wounds (19, 20). Other studies, however, have also noted the failure of HBO to increase the rate of healing in normal wounds (21). In contrast, in wounds in which

healing is impaired by hypoxia (compromised skin flaps, radiated wounds, etc.), HBO has been repeatedly shown to be of benefit in increasing the rate of healing (22, 23).

In this study, all wounds were nonhypoxic, noncompromised wounds, which may account for the lack of effect of HBO on wound healing. Additional studies with a similar animal model, but with compromised wounds (e.g., wounds created in previously radiated or diabetic animals, wounds compromised by cytotoxic agents) might prove worthwhile in better defining the role of HBO therapy when it is used in conjunction with topical wound-healing substances.

Animals treated with 100% O₂ gained more weight than those treated with air. The reason for this is unknown. Animals received food and water ad lib throughout the study. No attempt was made to measure food intake, and no studies were done to measure metabolic rate or other physiologic parameters. A literature review failed to find prior mention of this phenomenon.

CONCLUSIONS

This study was performed to determine the effects of HBO and TGF- β 1, alone and combined, on the healing of uncompromised wounds in normal rats. Findings of this study are summarized as follows:

1. Wounds treated with methylcellulose containing TGF- β 1 healed faster than wounds treated with methylcellulose alone. The effect was most significant on day 12 after wounding.
2. Breathing mixture and treatment depth had no significant effect on the rate of wound healing.
3. The addition of HBO therapy to TGF- β 1 therapy did not result in further enhancement of healing over TGF- β 1 therapy alone.
4. Animals treated with O₂ gained more weight than animals treated with air. The mechanism for this phenomenon is unknown.

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