

Effect of Hyperoxia on Vascular Endothelial Growth Factor Levels in a Wound Model

Ahmad Y. Sheikh, AB; Jeffrey J. Gibson, MD; Mark D. Rollins, MD, PhD; Harriet W. Hopf, MD; Zamirul Hussain, PhD; Thomas K. Hunt, MD

Hypothesis: Hyperbaric oxygen (HBO) therapy increases vascular endothelial growth factor (VEGF) levels in wounds.

Design: Wounds were monitored for oxygen delivery during HBO treatment, and wound fluids were analyzed for VEGF and lactate on days 2, 5, and 10 following wounding.

Setting: Experimental animal model.

Interventions: Rats were randomized to HBO therapy and control groups. The HBO therapy was administered for 90 minutes, twice daily with 100% oxygen at 2.1 atmospheres absolute. Treatment was administered for 7 days following wounding.

Main Outcome Measures: Vascular endothelial growth

factor, PO₂, and lactate levels in wound fluid were measured on days 2, 5, and 10.

Results: Wound oxygen rises with HBO from nearly 0 mm Hg to as high as 600 mm Hg. The peak level occurs at the end of the 90-minute treatment, and hyperoxia of lessening degree persists for approximately 1 hour. The VEGF levels significantly increase with HBO by approximately 40% 5 days following wounding and decrease to control levels 3 days after exposures are stopped. Wound lactate levels remain unchanged with HBO treatment (range, 2.0-10.5 mmol/L).

Conclusions: Increased VEGF production seems to explain in part the angiogenic action of HBO. This supports other data that hypoxia is not necessarily a requirement for wound VEGF production.

Arch Surg. 2000;135:1293-1297

From the Departments of Surgery (Drs Hopf and Hunt), Anesthesia and Perioperative Care (Dr Hopf), and Restorative Dentistry (Dr Hussain), University of California Medical School (Mr Sheikh), San Francisco; the Department of Surgery (Dr Gibson), University of California, Davis; and the Department of Plastic Surgery (Dr Rollins), University of Pittsburgh, Pittsburgh, Pa.

VASCULAR endothelial growth factor (VEGF) is the most specific known growth factor for neovascularization.^{1,2} In vitro, wound macrophages produce VEGF in response to low oxygen tensions.^{3,4} Since wound cells are characteristically hypoxic,^{3,5} it is easy to assume that hypoxia is the principal inducer of VEGF expression in physiological settings such as cancer and wounds. However, further examination of VEGF in wounds challenges this conventional assumption.

Clinical and experimental experience demonstrate that increasing the oxygen concentration in severely ischemic and/or hypoxic wounds results in accelerated healing in the form of increased blood vessel growth.⁶⁻¹¹ The mechanism by which hyperoxic therapy might induce blood vessel growth (in more normal circumstances), however, is not well understood. In particular, the effect of elevations of PO₂ on VEGF levels has not been documented. Recent in vitro studies suggest that

hyperoxic exposure leads to upregulation of platelet-derived growth factor receptors and that oxidants such as hydrogen peroxide stimulate endothelial cells and keratinocytes to release VEGF.^{12,13} In addition, macrophages and endothelial cells that are exposed to hyperoxia up-regulate IL-8, transforming growth factor β, and VEGF messenger RNA.¹⁴⁻¹⁶ The possibility arises, therefore, that hyperoxia may actually induce VEGF production in wounds.

Lactate, a known instigator of VEGF production that is present in wounds, might also be affected by hyperoxia.^{17,18} In this study, we evaluated whether hyperoxia, as achieved by hyperbaric oxygen (HBO) treatment, influences wound fluid VEGF, PO₂, and lactate.

RESULTS

WOUND CYLINDER OXYGEN

The mean wound oxygen tensions during day 2 of hyperoxic exposure are shown in **Figure 1**. The **Table** gives baseline, peak,

MATERIALS AND METHODS

GENERAL EXPERIMENTAL DESIGN

Sixty-three male Sprague-Dawley rats were used. Protocols were approved by the University of California, San Francisco Committee on Animal Research. Four wire mesh wound cylinders were implanted underneath the dorsal skin of the rats. These rats were randomized to 2 groups: hyperoxic and control. Wound fluid was collected from the cylinders and assayed for PO₂, lactate, and VEGF. Plasma VEGF levels were measured in 8 animals in each group.

WOUND CYLINDER IMPLANTATION AND SAMPLING

Wound cylinders (N=242) were manufactured with 0.25 mm stainless steel wire mesh and capped with silicone disks on each end. Cylinders measured 3.5 cm in length and 1 cm in diameter. Animals were anesthetized with 3% halothane. Two longitudinal dorsal midline incisions were made. Blunt dissection created a space for cylinder implantation. Each incision was used to place 2 cylinders, one on each side, a total of 4 cylinders per animal. Wounds were closed with 4 to 6 superficial interrupted 3-0 silk sutures. Cylinders were aspirated to complete evacuation, yielding 200 to 1200 µL of fluid depending on the day sampled. Aspiration was carried out under 3% halothane anesthesia using a 20 gauge needle and syringe on day 2, 5, or 10 following wounding. Needles were introduced through the silicon cap to avoid bleeding. Each cylinder was sampled only once.

OXYGEN TREATMENT

Rats in the hyperoxic group (n=32) were treated with 100% oxygen at 2.1 (atmospheres absolute) ATA for 90 minutes, twice a day for a total of 7 days. Treatments were administered in a hyperbaric chamber designed for small animals (Model 100; Western Hyperbaric Services, Union City, Calif). Compression and decompression was completed at a rate of 1 ATA per minute and monitored during dives and ascents. The control animals (n=31) remained at 21% oxygen at 1 ATA for the duration of the study.

WOUND CYLINDER OXYGEN MEASUREMENT

Polarographic oxygen electrodes (Licox; Medical Systems Corp, Greenvale, NY) were used to measure wound oxygen tension before, during, and after hyperoxia, as described

and postexposure oxygen tensions in wounds on days 2, 5, and 10 of treatment. Baseline wound PO₂ levels were hypoxic (range, [7.4±3.9]-[13.7±16.9] mm Hg) on all days, and increased significantly during hyperoxic treatment (range, [373.2±118.1]-[537.4±72.0] mm Hg). Levels decreased rapidly on return to 1 ATA but remained significantly greater than baseline 1 hour after HBO was discontinued ([45.6±14.2]-[150.5±132.0] mm Hg).

WOUND FLUID ANALYSIS

In no case was a measurable level of VEGF found in plasma in either group. On the other hand, VEGF was measur-

previously.¹⁹ Animals (n=3 on day 2; n=5 on days 5 and 10) were sedated with 3% halothane, followed by pentobarbital (35 mg/kg), buprenorphine hydrochloride (0.025 mg/kg), and atropine (0.8 mg/kg). Probes were calibrated in room air prior to use and threaded up 18 gauge hubless spinal needles inserted subcutaneously into the cylinder. The spinal needles were removed prior to HBO treatment, and probes were centered in the cylinder. Oxygen tension was measured immediately before hyperoxic exposure and continuously monitored during treatment until 1 hour after oxygen exposure ceased. The monitor automatically corrected for changes in tissue temperature, which were measured continuously.

VEGF ANALYSIS

Wound fluid sampling was timed halfway between the twice-daily 90-minute treatments that began at 8:00 AM and 5:00 PM. Plasma VEGF was measured on the same schedule.

Fluid was transferred into 1.5 mL Eppendorf tubes immediately after aspiration and centrifuged at 5000g for 10 minutes at 4°C. The cell-free supernatant was then aspirated and frozen at -30°C. Thawed samples were analyzed by murine VEGF enzyme immunosorbent assay per manufacturer's instructions (R&D Systems, Minneapolis, Minn). Fifty milliliters of wound fluid (diluted 1:6 in calibrator diluent) was incubated with 50 µL of assay diluent for 2 hours at room temperature in a 96-well plate coated with polyclonal antibody to murine VEGF. After 5 washing steps, a solution of antibody against mouse VEGF conjugated to horseradish peroxidase was added. The mixture was incubated at room temperature for 2 hours. After addition of hydrogen peroxide and tetramethylbenzidine, optical density was determined using a microtiter plate reader set at 490 nm. Samples were compared with serial dilutions of a murine recombinant VEGF standard. Lactate levels were determined using a lactate analyzer (model 2700; Yellow Springs Instruments, Yellow Springs, Ohio).

STATISTICS

Statistical significance between baseline, peak, and post-exposure PO₂ was determined by 2-tailed paired *t* test, with significance defined as *P*<.01. Data are expressed as mean±SD. The VEGF and lactate levels exhibited a non-uniform distribution. Nonparametric statistical analyses were used to determine significance. The Kruskal-Wallis statistic was used to compare differences between days, and the Mann-Whitney rank sum test was used to evaluate differences on specific days.

able as early as day 2 in wound fluid (HBO medians, control group: 407.0, 347.1 pg/mL, respectively) and remained measurable through day 10 (**Figure 2**). Peak values occurred on day 5 at which time the HBO group was significantly elevated over the controls by 42% (HBO medians, control group: 1333.5, 935.0 pg/mL, respectively; *P*≤.007). Values decreased somewhat on day 10, both groups becoming similar (HBO medians, control group: 658.4, 500.1 pg/mL, respectively), but both remained significantly elevated compared with day 2 (*P*≤.01).

Plasma lactate in rats is approximately 1 mmol/L. Wound fluid levels were significantly higher (**Figure 3**). Lactate increased significantly in all wounds between days

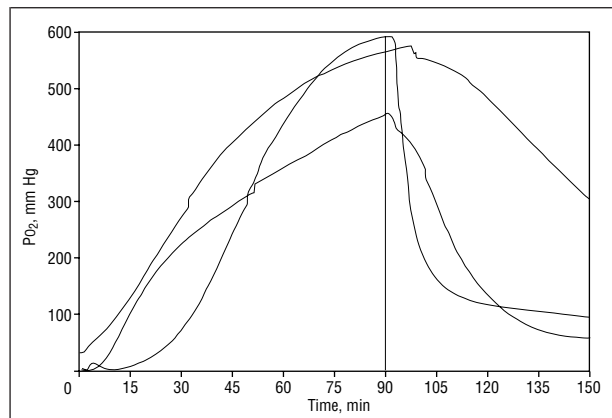


Figure 1. Curves depicting oxygen tensions within the wound cylinder during day 2 of hyperoxic treatment ($n=3$). Rollins et al¹⁹ have shown a slight diminution of peak PO_2 levels as therapy continues through day 7. Vertical line represents cessation of hyperoxic exposure.

Mean Oxygen Values Measured Within Wound Cylinders During Hyperoxic Treatment on Days 2, 5, and 10*

Day	Initial	Peak†	1-h Postimplantation‡
2 ($n=3$)	13.7 ± 16.9	537.4 ± 72.0	150.5 ± 132.0
5‡ ($n=8$)	13.4 ± 13.9	511.1 ± 138.4	78.4 ± 44.7
10‡ ($n=7$)	7.4 ± 3.9	373.2 ± 118.1	45.6 ± 14.2

*All values given for wound PO_2 as millimeters of mercury, mean ± SD.
 †Values significantly elevated compared with baseline ($P \leq .01$).
 ‡Also reported by Rollins et al.¹⁹

2 and 5, remaining elevated through day 10 (median control values: 4.20, 5.96, and 6.0 mmol/L, respectively) as previously reported.²⁰ However, the hyperoxic and control groups did not differ ($1-\beta \geq .90$), and no distinct trends were apparent.

COMMENT

In clinical experience, HBO therapy often stimulates the production of red granulation tissue when administered as treatment for unhealed, ischemic wounds. Since “granulation tissue” consists mainly of new blood vessels and their requisite, supporting collagenous matrix, this is likely due to an increase in both. Increased angiogenesis in response to hyperoxia has been observed in animal models by several investigators.^{6,11} Deposition of collagen in cell preparations and human and animal wounds has long been known to depend on PO_2 .²¹⁻²³ The current study explores the mechanism by demonstrating that periodic hyperoxia raises levels of VEGF in wound fluid.

Current literature shows, beyond a doubt, that hypoxia induces VEGF production by many cell types. It is easy to assume, therefore, that the characteristic hypoxic environment of wounds is the major stimulant for subsequent repair. If this were true, raising oxygen concentration would be expected to diminish VEGF and angiogenesis. However, our studies demonstrate that hypoxia is not necessary to sustain the release of VEGF in wounds. This concurs with studies by Howdeshiel et al²⁴ who demonstrate in a clever model that wound VEGF

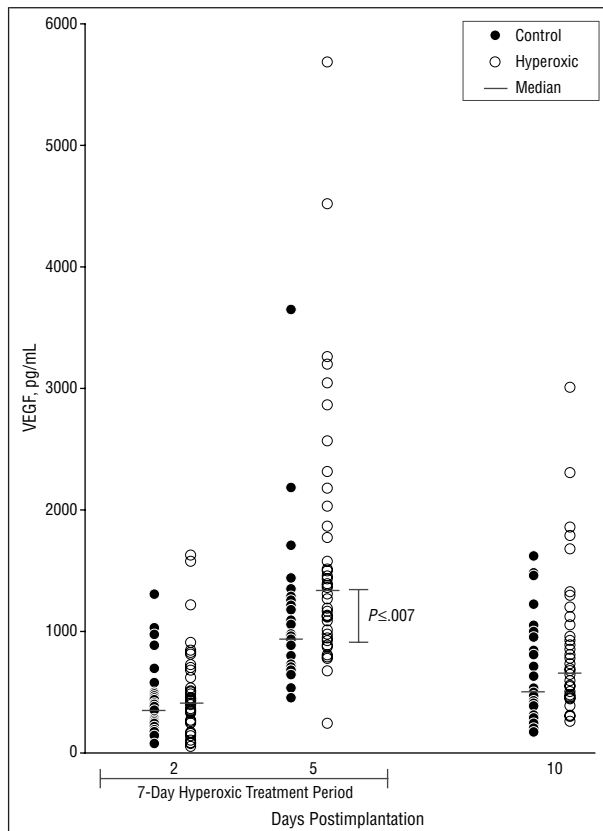


Figure 2. Vascular endothelial growth factor (VEGF) was measured in wound fluid aspirated on days 2, 5, or 10 following implantation. Animals were treated for 90 minutes, twice daily at 2.5 atm, 100% oxygen, for 7 days and compared with controls maintained at 1 atm, 21% oxygen. Both control and hyperoxic groups on days 5 and 10 differed from day 2, $P \leq .01$. The VEGF levels returned to control levels by 3 days following cessation of oxygen exposure. Plasma VEGF levels were below the detectable threshold.

levels remain notably high despite the fact that PO_2 in the wounds continuously approached that of arterial blood.

The question of how hyperoxia influences VEGF levels and angiogenesis is not resolved by these data. Macrophages are the major source of VEGF in wounds.^{3,17} Peak wound macrophage infiltration occurs by day 5 following wounding.¹⁷ This coincides with the VEGF peak observed in the control group. Previous studies have shown increased wound cell migration in response to hyperoxia, which may partially explain the increased levels of VEGF seen in the hyperoxic group.²⁵ Despite this presumptive increased cellularity, however, VEGF levels fell to control levels once the hyperoxic stimulus was removed. Hence, there are likely to be other mechanisms. Oxidants have been shown to stimulate VEGF production by some cells, including macrophages.^{13,26} Present data seem to support increased production as at least 1 mechanism.

Leukocytes, plentiful in wounds, make large amounts of oxidants, and the production rate is proportional to PO_2 within the range of 0 mm Hg to as much as 500 mm Hg. Some oxidants (hydrogen peroxide and nitric oxide) are measurable in wounds.^{27,28} Brauchle et al¹³ have shown that VEGF production by keratinocytes and bronchoalveolar cells in vitro is responsive to oxidant exposure at low concentrations.¹⁵ Cho et al²⁶ report a similar

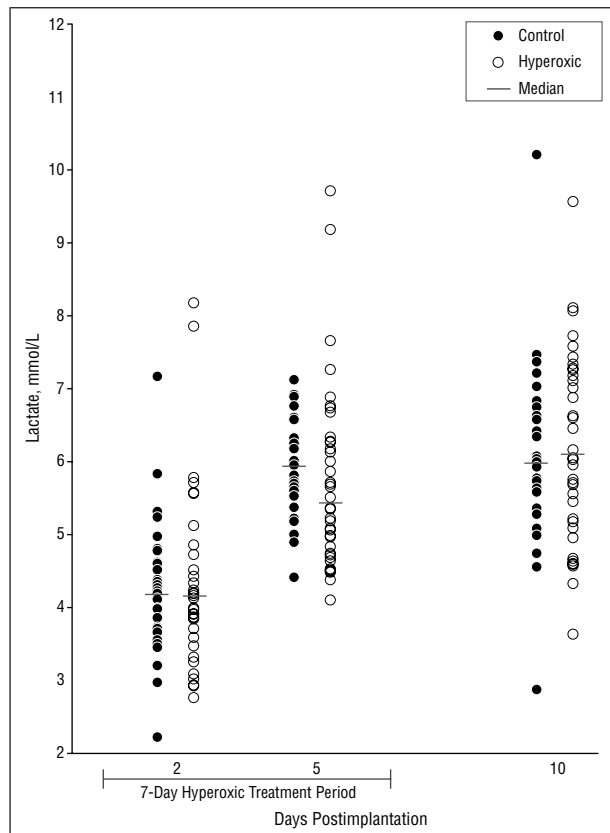


Figure 3. Lactate levels were measured in wound fluid aspirated on days 2, 5, or 10 following implantation. $P \leq .01$ for between days 2, 5, and 10.

finding for macrophages. Leukocytes are continuously present in wounds for many weeks. On this basis, increased oxygen would be expected to enhance oxidant production in wounds and, therefore, increase VEGF.

Oxidants in physiological concentrations also enhance collagen production by fibroblasts.²⁹ Furthermore, collagen synthesis is proportional to the concentration of molecular oxygen (PO_2) in the range of 0 mm Hg to 200 mm Hg. The mechanism involves procollagen hydroxylation, which requires molecular oxygen. Hence, collagen deposition, which serves to reinforce growing capillaries on exposure to blood pressure, is enhanced by both PO_2 and oxidant exposure.

The understanding of oxidant chemistry and related pathways in wounds is relatively young. Until recently, oxidant levels had not been measured. Some data now exists for nitric oxide and hydrogen peroxide.^{27,28,30} Addition of antioxidants and oxidant scavengers might be expected to mitigate the oxidant-induced VEGF response. However, data from introduction of such molecules into wound models have proven difficult to interpret.

Lactate was measured in these studies because it also enhances VEGF production at the concentrations found in wounds.^{5,18} The sustained lactate level (Figure 3) despite HBO indicates that an important stimulus for VEGF production persists between oxygen exposures and after their cessation at 7 days. Similarly, the hypoxia that recurs between HBO exposures (Figure 3) must also provide some stimulus for VEGF production. In this light, hyperoxia must furnish an intermittent stimulus for VEGF

production. Furthermore, this stimulus is either greater than that of hypoxia or, in some way, adds to residuals of the hypoxic signal. Since the properties of this stimulus are unknown, the optimal frequency and duration of exposures to hyperoxia have yet to be determined.

The data confirm again that increasing oxygenation of wounds does not lower lactate levels as one might expect.²⁰ This is because oxidant production by leukocytes is dependent on PO_2 up to levels of 600 mm Hg. The conversion of oxygen to superoxide (O_2^-) is responsible for approximately 98% of oxygen consumed in activated cells.³⁰ Therefore, the energy for the conversion must be linked to substrate concentration and must come from aerobic glycolysis, as proven experimentally.³⁰ Increased oxygenation, therefore, increases net production of lactate.^{17,31} The increased lactate production by activated leukocytes apparently offsets the reduction of lactate production, which occurs when oxygenation of fibroblasts increases.

The question arises as to whether the hyperoxia-induced fraction of VEGF is biologically active. In this model, such a quantification is technically difficult. However, supportive evidence of hyperoxia-induced, biologically active VEGF has been demonstrated (J.J.G., unpublished data, 2000), showing that hyperoxia-induced angiogenesis is abrogated in the presence of anti-VEGF antibody in a matrigel-murine wound model. Notably, collagenases such as matrix metalloproteinases, also a requisite for angiogenesis, are activated in oxidizing conditions.

Various VEGF-stimulating growth factors may be involved in regulating wound VEGF. Cell culture data suggest that hyperoxia may well change the concentration and receptor density of other wound-active growth factors.^{12,14-16} If VEGF concentration directly responds to hyperoxia, it is possible that other growth factors respond similarly and potentiate angiogenesis. Undoubtedly, more aspects of hyperoxia remain to be discovered.

These findings, taken with existing information, indicate the following: (1) VEGF production in wounds may be mediated by oxidants; (2) hypoxia may be a less important stimulus for neovascularization of wounds than previously thought; and (3) the benefits of HBO therapy for ischemic wound failure relate, in part, to increased VEGF concentration.

The authors wish to thank Heinz Scheuenstuhl, AB, for assistance with the described lactate assay.

Funding provided by the Genetech Foundation, South San Francisco, Calif, and National Institutes of Health, Bethesda, Md, grants GM27345 (Drs Hopf, Hussain, and Hunt) and GM08258 (Dr Gibson).

Reprints: Thomas K. Hunt, MD, Department of Surgery, University of California, San Francisco, 513 Parnassus Ave, Box 0522, HSW 1652, San Francisco, CA 94143-0522.

REFERENCES

1. Ferrara N. Vascular endothelial growth factor. *Trends Cardiovasc Med.* 1993;3:244-250.
2. Corral CJ, Siddiqui A, Wu L, Farrell CL, Lyons D, Mustoe TA. Vascular endothelial growth factor is more important than basic fibroblastic growth factor during ischemic wound healing. *Arch Surg.* 1999;134:200-205.

3. Knighton DR, Hunt TK, Scheuenstuhl H, Halliday BJ, Werb Z, Banda MJ. Oxygen tension regulates the expression of angiogenesis factor by macrophages. *Science*. 1983;221:1283-1285.
4. Shweiki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature*. 1992;359:843-845.
5. Jensen JA, Hunt TK, Scheuenstuhl H, Banda MJ. Effect of lactate, pyruvate, and pH on secretion of angiogenesis and mitogenesis factors by macrophages. *Lab Invest*. 1986;54:574-578.
6. Gibson JJ, Angeles AP, Hunt TK. Increased oxygen tension potentiates angiogenesis. *Surg Forum*. 1997;48:696-699.
7. Hunt TK, Pai MP. The effect of varying ambient oxygen tensions on wound metabolism and collagen synthesis. *Surg Gynecol Obstet*. 1972;135:561-567.
8. Hunt TK. The physiology of wound healing. *Ann Emerg Med*. 1988;17:1265-1273.
9. Hammarlund C. The physiological effects of hyperbaric oxygen. In: Kindwall EP, ed. *Hyperbaric Medicine Practice*. Phoenix, Ariz: Best Publishing Company; 1994: 17-32.
10. Cianci P HT. Adjunctive hyperbaric oxygen therapy in treatment of diabetic foot wounds. In: Levin ME, O'Neal LW, Bowker JH, eds. *The Diabetic Foot*. 5th ed. St Louis, Mo: Mosby-Year Book Inc; 1993:305-320.
11. Marx RE, Ehler WJ, Tayapongsak P, Pierce LW. Relationship of oxygen dose to angiogenesis induction in irradiated tissue. *Am J Surg*. 1990;160:519-524.
12. Bonomo SR, Davidson JD, Yu Y, Xia Y, Lin X, Mustoe TA. Hyperbaric oxygen as a signal transducer: upregulation of platelet derived growth factor-beta receptor in the presence of HBO₂ and PDGF. *Undersea Hyperbaric Med*. 1998;25:211-216.
13. Brauchle M, Funk JO, Kind P, Werner S. Ultraviolet B and H₂O₂ are potent inducers of vascular endothelial growth factor expression in cultured keratinocytes. *J Biol Chem*. 1996;271:21793-21797.
14. Darrington RS, Godden DJ, Park MS, Ralston SH, Wallace HM. The effect of hyperoxia on the expression of cytokine mRNA in endothelial cells [abstract]. *Biochem Soc Trans*. 1997;25:292S.
15. Maniscalco WM, Finkelstein RH, Finkelstein JN, Campbell MH. Vascular endothelial growth factor mRNA increases in alveolar epithelial cells during recovery from oxygen injury. *Am J Respir Cell Mol Biol*. 1995;13:377-386.
16. Deaton PR, McKellar CT, Culbreth R, Veal CF, Cooper JA Jr. Hyperoxia stimulates interleukin-8 release from alveolar macrophages and U937 cells: attenuation by dexamethasone. *Am J Physiol*. 1994;267:L187-L192.
17. Feng JH, Hussain MZ, Constant J, Hunt TK. Angiogenesis in wound healing. *Am J Surg Pathol*. 1998;3:1-8.
18. Zabel DD, Feng JJ, Scheuenstuhl H, Hunt TK, Hussain MZ. Lactate stimulation of macrophage-derived angiogenic activity is associated with inhibition of Poly (ADP-ribose) synthesis. *Lab Invest*. 1996;74:644-649.
19. Rollins MD, Gimbel ML, Gibson JJ, Hunt TK, Hopf HW. Oxygen levels during hyperbaric oxygen treatment in healing wounds. *Adv Exp Med Biol*. In press.
20. Hunt TK, Conolly WB, Aronson SB, Goldstein P. Anaerobic metabolism and wound healing: an hypothesis for the initiation and cessation of collagen synthesis in wounds. *Am J Surg*. 1978;135:328-332.
21. Jonsson K, Jensen JA, Goodson WH, et al. Tissue oxygenation, anemia, and perfusion in relation to wound healing in surgical patients. *Ann Surg*. 1991;214: 605-613.
22. Hunt TK, Zederfeldt B, Goldstick TK. Oxygen and healing. *Am J Surg*. 1969;118: 521-525.
23. Kurz A, Sessler DI, Lenhardt R. Perioperative normothermia to reduce the incidence of surgical-wound infection and shorten hospitalization: study of Wound Infection and Temperature Group. *N Engl J Med*. 1996;334:1209-1215.
24. Howdieshell TR, Riegner C, Gupta V, et al. Normoxic wound fluid contains high levels of vascular endothelial growth factor. *Ann Surg*. 1998;228:707-715.
25. Tompach PC, Lew D, Stoll JL. Cell response to hyperbaric oxygen treatment. *Int J Oral Maxillofac Surg*. 1997;26:82-86.
26. Cho M, Hunt TK, Hussain MZ. Hydrogen peroxide stimulates macrophage VEGF release. *Am J Physiol*. In press.
27. Schaffer MR, Tantry U, Gross SS, Wasserburg HL, Barbul A. Nitric oxide regulates wound healing. *J Surg Res*. 1996;63:237-240.
28. Schäffer MR, Tantry U, van Wesep RA, Barbul A. Nitric oxide metabolism in wounds. *J Surg Res*. 1997;71:25-31.
29. Kirkeby L, Ghani QP, Enriquez B, Hussain MZ, Hunt TK. Stimulation of collagen synthesis in fibroblasts by hydrogen peroxide. *Mol Biol Cell*. 1995;6:44.
30. Allen DB, Maguire JJ, Mahdavian M, et al. Wound hypoxia and acidosis limit neutrophil bacterial killing mechanisms. *Arch Surg*. 1997;132:991-996.
31. Newsholme P, Costa Rosa LF, Newsholme EA, Curi R. The importance of fuel metabolism to macrophage function. *Cell Biochem Funct*. 1996;14:1-10.