Available online at www.sciencedirect.com
SCIENCE DIRECT®
Brain, Behavior, and Immunity 19 (2005) 217–222
BRAIN, BEHAVIOR, and IMMUNITY
www.elsevier.com/locate/ybrbi

Hyperbaric oxygen therapy ameliorates stress-impaired dermal wound healing

Praveen K. Gajendrareddy[a,d], Chandan K. Sen[b,c], Michael P. Horan[a], Phillip T. Marucha[d,*]

[a] Department of Oral Biology, College of Dentistry, The Ohio State University, Columbus, OH 43210, United States
[b] Departments of Surgery and Molecular and Cellular Biochemistry, The Ohio State University, Columbus, OH 43210, United States
[c] Davis Heart and Lung Research Institute, The Ohio State University, Columbus, OH 43210, United States
[d] Department of Periodontics, University of Illinois at Chicago, Chicago, IL 60612, United States

Received 2 June 2004; received in revised form 27 August 2004; accepted 22 September 2004
Available online 21 November 2004

Abstract

Psychological stress has been shown to dysregulate healing in both humans and animals. Studies indicate the possibility for decreased oxygen supply, and increased oxygen demand, in the wounds of the stressed animals. Oxygen is an important mediator of wound healing, and its availability can limit healing rate. Hence, in a mouse model of stress-impaired healing, the hypothesis that hyperbaric oxygen therapy would ameliorate the effect of stress on dermal wound healing was tested. Hyperbaric oxygen therapy (HBO) twice a day during early wound healing significantly ameliorated the effects of stress, bringing healing to near-control levels. There was no significant effect of HBO on the wounds of control animals. Wound inducible nitric oxide synthase (iNOS), modulated by psychological stress and oxygen balance, was studied for gene expression by real-time PCR. Expression of iNOS increased in stressed mice on days 1 (205%; $p < .0001$), 3 (96%; $p < .03$), and 5 (249%; $p < .03$), post-wounding. HBO treatment of the stressed animals decreased iNOS expression by 62.6% ($p < .02$) day 1 post-wounding. There was no significant effect of HBO on wound healing and iNOS expression in the control animals. Methods aimed at increasing tissue oxygenation, like HBO, have a high therapeutic potential. Their molecular mechanisms, implicated in wound healing, elude clarification due to the lack of appropriate animal models. Our current findings represent the first experimental evidence, demonstrating that HBO corrects stress-impaired dermal wound healing.

Keywords: Stress; Wound healing; Oxygen; Hypoxia; Hyperbaric oxygen therapy; Inducible nitric oxide synthase; Nitric oxide; Inflammation; Cutaneous healing

1. Introduction

Psychological stress impairs wound healing in humans and animals. The stress of academic examinations or caregiving for Alzheimer's patients has been shown to delay wound healing by 40 and 27%, respectively (Marucha et al., 1998; Kiecolt-Glaser et al., 1995). In a mouse model, restraint stress causes a 30% delay in wound healing (Padgett et al., 1998). This impairment of healing is associated with a stress-induced suppression of cytokine and growth factor expression (Mercado et al., 2002a,b), and an increase in the number of opportunistic bacteria in the wound (Rojas et al., 2002). The bacteria, as well as the anti-bacterial defense mechanisms, require oxygen. Oxygen consumption and superoxide production by phagocytic wound and blood cells were several-fold higher than their corresponding cells in resting state (Hohn and Hunt, 1975), increasing oxygen demand.

Psychological stress has been associated with impaired vascular function in conditions like Raynaud's

* Corresponding author. Fax: +1 312 996 0943.
E-mail address: marucha@uic.edu (P.T. Marucha).

0889-1591/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbi.2004.09.003

phenomenon (O'Connor, 2001). Stress-induced activation of the sympathetic nervous system results in the release of potent hormones such as catecholamines, which can alter blood flow. The sympathetic α-adrenergic receptors predominate in the skin and their activation evokes a potent vaso-constrictive response (Ahlquist, 1976). The peripheral vaso-constriction would restrict oxygen delivery to the healing tissues. Oxygen influences a plethora of healing processes including collagen synthesis, angiogenesis, epithelialization, and metabolic reactions for leukocyte bactericidal action (Whitney, 1989). Indications of increased oxygen demand and decreased oxygen supply in the wounds of the stressed animals led to the hypothesis that stress impairs healing by disrupting oxygen balance in the wounds.

Factors like warmth, oxygen, and sympathetic blockade have been shown to accelerate wound healing (Suh and Hunt, 1998). The rate of wound healing is limited by the availability of oxygen (Hunt and Dunphy, 1969). Therefore methods aimed at increasing tissue oxygenation, like hyperbaric oxygen therapy (HBO) have a high therapeutic potential, and oxygen therapy seems to hold promise for the treatment of exposed dermal wounds (Kalliainen et al., 2003; Sen et al., 2002c; Suh and Hunt, 1998). Currently, approved uses for HBO include chronic non-healing wounds, thermal burns, air embolism, decompression sickness, clostridial gas gangrene, refractory osteomyelitis, necrotizing soft tissue infections, blood loss anemia, carbon monoxide poisoning, cyanide poisoning, crush injuries, graft preparation, osteoradionecrosis, and refractory mycoses (Brown and Sands, 1995). Recent studies support that oxygen and reactive derivatives may support wound healing by facilitating key processes such as wound angiogenesis (Sen et al., 2002b,c). Though HBO is a widely accepted treatment modality, the molecular mechanisms implicated in wound healing remain unclear. This can be attributed to the lack of an animal model of dysregulated healing where an effect of HBO could be consistently demonstrated.

Studies by Palmer et al. (1998) have shown that hypoxia can increase the expression of genes encoding inducible nitric oxide synthase. Nitric oxide (NO) is an oxygen metabolite that plays an important role in wound healing (Lee et al., 2001; Schaffer et al., 1996; Thornton et al., 1998; Yamasaki et al., 1998). Oxygen balances modulate inducible nitric oxide synthase (iNOS) expression and activity (McCormick et al., 2000). Therefore, iNOS is a good target gene for studying hypoxia in stress-induced dysregulation of wound healing. Under the hypothesis that stress-induced impairment of healing is modulated by oxygen, the present study tests the ability of hyperbaric oxygen therapy to ameliorate the effect of stress on dermal wound healing. Furthermore, this model may provide the opportunity to test the mechanisms through which HBO modulates impaired dermal healing.

2. Materials and methods

2.1. Animals

Virus antibody free, female SKH-1 mice, 5–6 weeks of age, were obtained from Charles River (Wilmington, MA). The mice were housed 4–5/cage, maintained on 12 h light/dark cycles, and acclimated to the animal facility for at least one week.

2.2. Anesthesia

The mice were anesthetized by an intra-peritoneal injection of 0.25 ml of a solution of 7.8 mg/ml Ketaset (Aveco, Fort Dodge, IA) and 0.44 mg/ml Rompum (Haver–Lockhart, Shawnee, KS).

2.3. Restraint stress paradigm

The restraint paradigm used was as described previously by our laboratory (Padgett et al., 1998). Briefly, the mice were confined to loose fitting, well-ventilated 50 ml conical tubes for a period of 12–14 h during their active cycle. The mice were subject to restraint three cycles prior to wounding and five cycles after. The control groups were subject to food and water deprivation during the same period, to control for the inability of the stressed animals to access food and water during confinement.

2.4. Wounding and wound measurement

Using a biopsy punch, each mouse had two circular 3.5 mm cutaneous wounds placed just behind the shoulder blades to prevent them from licking their wounds. Photographs of the wounds were taken, using a Nikon Coolpix 990 digital camera, immediately after wounding and every 24 h thereafter, until healing was complete. The digital images of the wounds were then analyzed using an Apple iMAC 350 DV computer and Canvas 7 software. Measurements of the wound area were standardized to a 3.5 mm diameter circle, as well as to the wound area at the time of wounding. Entire wounds were harvested using a circular 6 mm diameter biopsy punch. Since each animal received two wounds, both wounds obtained from the same animal were pooled in 1 ml TRIzol (Life Technologies, Rockville, MA), frozen in liquid nitrogen, and stored at −80 °C until further usage.

2.5. Hyperbaric oxygen therapy

Hyperbaric oxygen therapy to the mice was achieved by subjecting the mice to HBO using 100% oxygen, at 2.5 atm pressure, for a 2 h period, twice a day. A specialized chamber designed for HBO use (Chandan K. Sen, The Ohio State University) was flushed with 100% oxygen for 10 min and then pressurized to 2.5 atm over

a 15 min period. After therapy, decompression was done over a 15 min period. The animals were provided with food and water while within the chamber. The therapy was provided for five days, from the day of wounding to day 4 post-wounding. On the day of wounding, the therapy was provided to the animals shortly after all the mice in the treatment groups had recovered from the anesthesia used in the wounding procedure.

2.6. Tissue processing

Frozen tissue in TRIzol reagent was homogenized using a tissue tearor and total RNA was extracted. mRNA selection was done using magnetic oligo(dt) beads and a magnetic particle concentrator (Dynal, Oslo, Norway) in accordance to the manufacturer's protocol. The mRNA was subjected to reverse transcription to synthesize cDNA and used to analyze gene expression by real-time PCR (ABI Prism 7700-sequence detection system). Primers and probe were designed using Primer Express software (Applied Biosystems, Foster City, CA). Primers and probe for iNOS were obtained from Applied Biosystems, Foster City, CA, and are as follows: forward primer, CAGCTGGGCTGTACAAACCTT; reverse primer, TGAATGTGATGTTTGCTTCGG; and probe, CGGGCAGCCTGTGAGACCTTTGA. Gene expression was standardized to G3PDH (Rodent GAPDH Control Reagents, Applied Biosystems, Foster City, CA) gene expression in the respective samples.

2.7. Statistical methods

Three-way ANOVA was done to determine the effects of time, stress, and HBO on wound healing and iNOS gene expression. Two-way ANOVA was used to determine the effects of stress and time on iNOS gene expression. Differences between individual groups were analyzed by Fisher's PLSD post hoc test, with the treatment as the effect and a significance level of 5%. Data were analyzed using statview 5.0.1. $p < .05$ determined the statistical significance of the differences.

3. Results

The experiments involving HBO consisted of four groups: controls, controls with HBO, stress, and stress with HBO. Results from Fig. 1 reconfirm previous data (Padgett et al., 1998) on stress-induced impairment in wound closure. HBO twice a day during the early phases of wound healing significantly restored wound healing in the stressed animals to near-control levels. This effect was seen from day 1 post-wounding until almost complete healing had occurred. These results show that HBO can ameliorate stress-impaired healing. HBO did not have any significant effect on wound closure in control animals.

Nitric oxide plays a multitude of roles in wound healing, as a cellular messenger molecule and an antibacterial agent (Schaffer et al., 1996; Thornton et al., 1998; Wetzler et al., 2000). Furthermore, studies have shown that hypoxia can increase the expression of genes encoding inducible nitric oxide synthase (Palmer et al., 1998). Analysis of the effects of stress on iNOS expression (Fig. 2) showed that iNOS expression increased in stressed mice on days 1 (205%; Mean diff.: −205.247, Crit. diff.: 81.36, $p < .0001$), 3 (96%; Mean diff.: −91.82, Crit. diff.: 81.360, $p < 0.03$), and 5 (249%; Mean diff.: −98.48, Crit. diff.: 84.46, $p < 0.03$), post-wounding. Gene expression studies in the

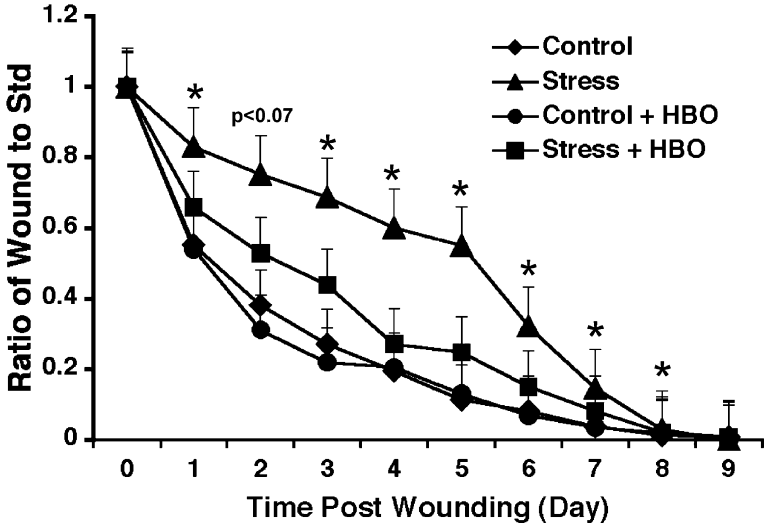

Fig. 1. HBO ameliorates the effect of stress on wound healing. Compiled data from two individual experiments. Areas of the wound and standard were obtained from digital photographs. The area of the wound was then standardized, by comparing it to the area of the standard. This was compared to the relative wound size on day zero. Data are represented as means ± SEM. Days 1–8 ($n = 6$–10 mice per group). *$p < .05$, for wounds of stressed animals as compared to those of stressed animals treated with HBO from the same day.

wounds of HBO treated animals (Fig. 3) showed that iNOS expression was decreased by 62.6% (Mean diff.: 378, Crit. diff.: 288.87, $p < .02$) in the HBO treated stressed animals on day 1. HBO did not have a significant effect on iNOS gene expression in the stressed animals on day 5 post-wounding. The HBO treatment had no significant effect iNOS gene expression in the control animals.

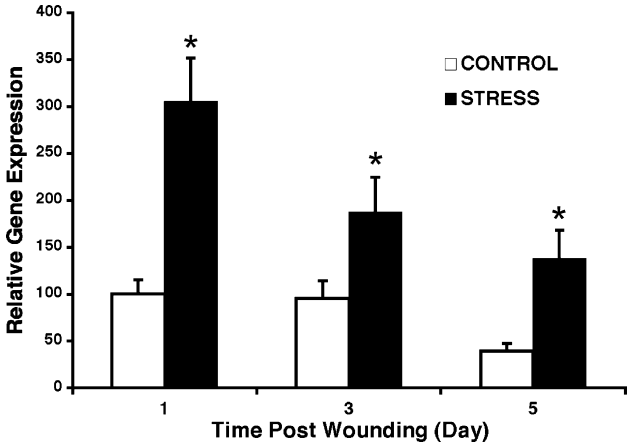

Fig. 2. iNOS gene expression in wounds is up-regulated by stress. Compiled data from three individual experiments are expressed as a percentage of the fold increase in gene expression as compared to day 1 control group gene expression. Gene expression was analyzed by real-time PCR on cDNA synthesized from wound tissues obtained on days 1, 3, and 5 post-wounding, and unwounded tissues obtained on the day of wounding. Bars represent means ± SEM. Days 1, 3, and 5 ($n = 19$–21 mice per group). *$p < .05$, compared to the control group from the same day.

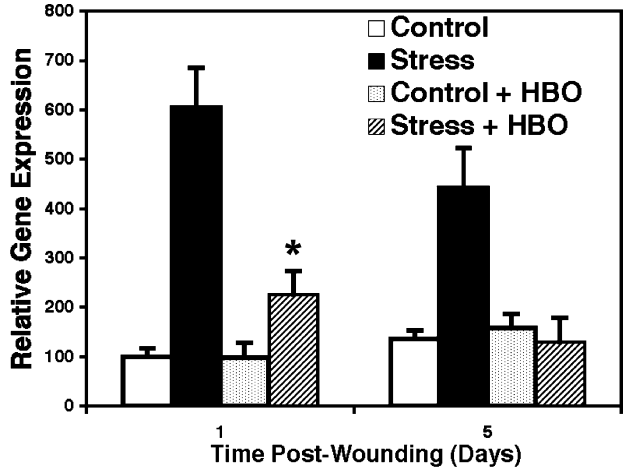

Fig. 3. HBO restores iNOS gene expression in wounds of stressed animals to those of controls. Compiled data from three individual experiments are expressed as a percentage of the fold increase in gene expression as compared to day 1 control group gene expression. Gene expression was analyzed by real-time PCR on cDNA synthesized from wound tissues obtained on days 1 and 5 post-wounding, and unwounded tissues obtained on the day of wounding. Bars represent means ± SEM. Days 1 and 5 ($n = 7$–9 mice per group). *$p < .05$, for wounds of stressed animals as compared to those of stressed animals treated with HBO from the same day.

4. Discussion

The current study was conducted to determine if oxygen modulates the effects of stress on wound healing. Wound healing is known to be a highly regulated process consisting of a complex set of sequential events (Schaffer and Nanney, 1996; Suh and Hunt, 1998). The disruption of any of these events by stress can lead to the impairment of the healing process. As shown previously (Padgett et al., 1998), stress significantly delayed wound healing in restrained animals. There was also a significant increase in iNOS expression in the stressed animals on days 1, 3, and 5 post-wounding. HBO ameliorated stress-impaired healing, suggesting an important role for oxygen in stress-impaired healing. Moreover, iNOS expression in the wounds of the HBO treated stressed animals showed no significant difference in comparison to control values.

Stress has the potential to alter both the delivery and the utilization of oxygen in the tissues. Stress activates the hypothalamic–adrenal–pituitary axis (HPA) and the sympathetic nervous system. This results in the release of potent hormones such as catecholamines and corticosteroids, which can alter blood flow and gene expression. Catecholamines, acting through sympathetic α-adrenergic receptors that predominate in the skin, evoke a potent vaso-constrictive response (Ahlquist, 1976). Thus, stress might restrict oxygen delivery to the healing tissues as a result of stimulation of α-adrenergic receptors, which results in peripheral vaso-constriction. Infusion of epinephrine has been shown to decrease wound oxygen by approximately 45% (Jensen et al., 1985). Hence, in the stressed animals, catecholamine mediated vascular changes may be a cause for dysregulated oxygenation.

Studies by Jonsson et al. (1988) in their experiments with musculocutaneous and random pattern flaps showed that lower tissue pO_2 values increased the susceptibility to infection, whereas increasing inspired oxygen enhanced resistance to infection. Previous studies in the stress model have shown that the stressed animals suffer from increased opportunistic bacterial infection (Rojas et al., 2002). Further, oxygen consumption and superoxide production by phagocytic wound and blood cells were seen to be several-fold higher than their corresponding cells in resting state (Hohn and Hunt, 1975), thus increasing oxygen demand and further decreasing available oxygen. Henceforth, we would expect that the pO_2 of infected wounds to be lower than that of the controls. Furthermore, social stress has been shown to impair tissue oxygen utilization (Manhold et al., 1971). During stress, impaired oxygen delivery and utilization could lead to impaired healing. The restoration of wound healing by HBO in the stressed mice signifies the potential role for oxygen as a mediator of stress-impaired healing.

Hypoxia, i.e., reduced tissue oxygenation, is a potent inducer of iNOS expression. Hypoxia can increase the expression inducible nitric oxide synthase, through the hypoxia-inducible factor (HIF) pathway (Palmer et al., 1998). HIF 1 is a heterodimer of HIF 1α and HIF 1β. HIF 1β is constitutively expressed while HIF 1α is regulated by oxygen. During hypoxia, HIF activation results in the activation of genes that decrease oxygen demand and increase oxygen supply. Significant among these genes is nitric oxide synthase-2 (inducible nitric oxide synthase) (McCormick et al., 2000; Wiesener and Maxwell, 2003). Hence, iNOS expression in the wounds of the stressed animals can be used as a marker for the hypoxic state of the wounds. HBO, which could counteract stress-induced hypoxia while restoring healing to control levels in the stressed mice, also restored iNOS expression to near-control levels. This restoration was significant on day 1 post-wounding, with a similar pattern on day 5 post-wounding. HBO did not have any effect on iNOS expression in the control animals, and this corroborates our results of the effect of HBO on wound healing. We establish here that stress-impaired healing is mediated by altered wound oxygenation, as evidenced by iNOS gene expression, which in turn is ameliorated by HBO. Hence, it can be inferred that iNOS expression is a marker of stress-impaired healing as a result of altered wound oxygenation, and that the amelioration of stress-impaired healing by HBO is through an oxygen-mediated pathway, although this does not implicate iNOS itself as a mediator of stress-impaired healing.

Systemic and topical HBO has been effective in the treatment of impaired healing. Niinikoski et al. (1972) reported enhanced healing in HBO when using 35–70% oxygen. They concluded that increasing oxygen increased the tissue pO_2. Beneficial effects of HBO therapy have also been reported by Fulton (2000) showing that HBO reduces erythema, edema, and enhanced collagen formation during healing. Benefits of HBO have been noted in conditions of impaired healing as in osteoradionecrosis (David et al., 2001), through the stimulation of angiogenesis, an oxygen-regulated process. Now, our study provides further conclusive evidence of the benefits of HBO in the treatment of dysregulated healing.

In summary, our current findings represent the first experimental animal model demonstrating that systemic HBO corrects stress-impaired dermal wound healing. Previous studies in the model suggest a role for glucocorticoids in stress-impaired healing (Padgett et al., 1998). These studies demonstrate a critical role for oxygen. This observation is consistent with the observation that correction of wound hypoxia will support molecular and reactive oxygen dependent processes in favor of healing (Sen et al., 2002a,c).

Acknowledgments

This work was supported by NIH-DE13749 and NIH-DE12792 to Phillip T. Marucha, and by NIH-GM27345 to Chandan K. Sen.

References

Ahlquist, R.P., 1976. Present state of alpha- and beta-adrenergic drugs I. The adrenergic receptor. Am. Heart J. 92, 661–664.

Brown, R.B., Sands, M., 1995. Infectious disease indications for hyperbaric oxygen therapy. Compr. Ther. 21, 663–667.

David, L.A., Sandor, G.K., Evans, A.W., Brown, D.H., 2001. Hyperbaric oxygen therapy and mandibular osteoradionecrosis: a retrospective study and analysis of treatment outcomes. J. Can. Dent. Assoc. 67, 384.

Fulton Jr., J.E., 2000. The use of hyperbaric oxygen (HBO) to accelerate wound healing. Dermatol. Surg. 26, 1170–1172.

Hohn, D.C., Hunt, T.K., 1975. Oxidative metabolism and microbicidal activity of rabbit phagocytes: cells from wounds and from peripheral blood. Surg. Forum. 26, 85–87.

Hunt, T.K., Dunphy, J.E., 1969. Effects of increasing oxygen supply to healing wounds. Br. J. Surg. 56, 705.

Jensen, J.A., Jonsson, K., Goodson III, W.H., Hunt, T.K., Roizen, M.F., 1985. Epinephrine lowers subcutaneous wound oxygen tension. Curr. Surg. 42, 472–474.

Jonsson, K., Hunt, T.K., Mathes, S.J., 1988. Oxygen as an isolated variable influences resistance to infection. Ann. Surg. 208, 783–787.

Kalliainen, L., Gordillo, G.M., Schlanger, R., Sen, C.K., 2003. Topical oxygen as an adjunct to wound healing: a clinical case series. Pathophysiology 9, 81–87.

Kiecolt-Glaser, J.K., Marucha, P.T., Malarkey, W.B., Mercado, A.M., Glaser, R., 1995. Slowing of wound healing by psychological stress. Lancet 346, 1194–1196.

Lee, R.H., Efron, D., Tantry, U., Barbul, A., 2001. Nitric oxide in the healing wound: a time-course study. J. Surg. Res. 101, 104–108.

Manhold, J.H., Doyle, J.L., Weisinger, E.H., 1971. Effects of social stress on oral and other bodily tissues. II. Results offering substance to a hypothesis for the mechanism of formation of periodontal pathology. J. Periodontol. 42, 109–111.

Marucha, P.T., Kiecolt-Glaser, J.K., Favagehi, M., 1998. Mucosal wound healing is impaired by examination stress. Psychosom. Med. 60, 362–365.

McCormick, C.C., Li, W.P., Calero, M., 2000. Oxygen tension limits nitric oxide synthesis by activated macrophages. Biochem. J. 350 (Pt 3), 709–716.

Mercado, A.M., Padgett, D.A., Sheridan, J.F., Marucha, P.T., 2002a. Altered kinetics of IL-1 alpha, IL-1 beta, and KGF-1 gene expression in early wounds of restrained mice. Brain Behav. Immun. 16, 150–162.

Mercado, A.M., Quan, N., Padgett, D.A., Sheridan, J.F., Marucha, P.T., 2002b. Restraint stress alters the expression of interleukin-1 and keratinocyte growth factor at the wound site: an in situ hybridization study. J. Neuroimmunol. 129, 74–83.

Niinikoski, J., Hunt, T.K., Dunphy, J.E., 1972. Oxygen supply in healing tissue. Am. J. Surg. 123, 247–252.

O'Connor, C.M., 2001. Raynaud's phenomenon. J. Vasc. Nurs. 19, 87–92 quiz 93–94.

Padgett, D.A., Marucha, P.T., Sheridan, J.F., 1998. Restraint stress slows cutaneous wound healing in mice. Brain Behav. Immun. 12, 64–73.

Palmer, L.A., Semenza, G.L., Stoler, M.H., Johns, R.A., 1998. Hypoxia induces type II NOS gene expression in pulmonary artery endothelial cells via HIF-1. Am. J. Physiol. 274, L212–L219.

Rojas, I.G., Padgett, D.A., Sheridan, J.F., Marucha, P.T., 2002. Stress-induced susceptibility to bacterial infection during cutaneous wound healing. Brain Behav. Immun. 16, 74–84.

Schaffer, C.J., Nanney, L.B., 1996. Cell biology of wound healing. Int. Rev. Cytol. 169, 151–181.

Schaffer, M.R., Tantry, U., Gross, S.S., Wasserburg, H.L., Barbul, A., 1996. Nitric oxide regulates wound healing. J. Surg. Res. 63, 237–240.

Sen, C.K., Khanna, S., Babior, B.M., Hunt, T.K., Ellison, E.C., Roy, S., 2002a. Oxidant induced vascular endothelial growth factor expression in human keratinocytes and cutaneous wound healing. J. Biol. Chem. 14, 14.

Sen, C.K., Khanna, S., Babior, B.M., Hunt, T.K., Ellison, E.C., Roy, S., 2002b. Oxidant-induced vascular endothelial growth factor expression in human keratinocytes and cutaneous wound healing. J. Biol. Chem. 277, 33284–33290.

Sen, C.K., Khanna, S., Gordillo, G., Bagchi, D., Bagchi, M., Roy, S., 2002c. Oxygen, oxidants, and antioxidants in wound healing: an emerging paradigm. Ann. N. Y. Acad. Sci. 957, 239–249.

Suh, D.Y., Hunt, T.K., 1998. Time line of wound healing. Clin. Podiatr. Med. Surg. 15, 1–9.

Thornton, F.J., Schaffer, M.R., Witte, M.B., Moldawer, L.L., MacKay, S.L., Abouhamze, A., Tannahill, C.L., Barbul, A., 1998. Enhanced collagen accumulation following direct transfection of the inducible nitric oxide synthase gene in cutaneous wounds. Biochem. Biophys. Res. Commun. 246, 654–659.

Wetzler, C., Kampfer, H., Pfeilschifter, J., Frank, S., 2000. Keratinocyte-derived chemotactic cytokines: expressional modulation by nitric oxide in vitro and during cutaneous wound repair in vivo. Biochem. Biophys. Res. Commun. 274, 689–696.

Whitney, J.D., 1989. Physiologic effects of tissue oxygenation on wound healing. Heart Lung 18, 466–474.

Wiesener, M.S., Maxwell, P.H., 2003. HIF and oxygen sensing; as important to life as the air we breathe?. Ann. Med. 35, 183–190.

Yamasaki, K., Edington, H.D., McClosky, C., Tzeng, E., Lizonova, A., Kovesdi, I., Steed, D.L., Billiar, T.R., 1998. Reversal of impaired wound repair in iNOS-deficient mice by topical adenoviral-mediated iNOS gene transfer. J. Clin. Invest. 101, 967–971.