Oxygen, Oxidants, and Antioxidants in Wound Healing

An Emerging Paradigm

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ABSTRACT: Disrupted vasculature and high energy-demand by regenerating tissue results in wound hypoxia. Wound repair may be facilitated by oxygen therapy. Evidence supporting the mode of action of hyperbaric oxygen in promoting wound healing is sketchy, however. Topical oxygen therapy involves local administration of pure oxygen. The advantages of topical oxygen therapy include low cost, the lack of systemic oxygen toxicity, and possibility of home treatment. While this modality of wound care is of outstanding interest, it clearly lacks the support of mechanism-oriented studies. The search for mechanisms by which oxygen supports wound healing has now taken another step. Respiratory burst–derived oxidants support healing. Oxidants serve as cellular messengers to promote healing. Although this information is of outstanding significance to the practice of oxygen therapy, it remains largely unexplored. The search for "natural remedies" has drawn attention to herbals. Proanthocyanidins or condensed tannins are a group of biologically active polyphenolic bioflavonoids that are synthesized by many plants. Proanthocyanidins and other tannins facilitate wound healing. A combination of grape seed proanthocyanidin extract and resveratrol facilitates inducible VEGF expression, a key element supporting wound angiogenesis. Strategies to manipulate the redox environment in the wound are likely to be of outstanding significance in wound healing.

KEYWORDS: wound healing; antioxidants; polyphenolic bioflavonoids; oxygen therapy; oxidants

INTRODUCTION

Wound-healing abnormalities cause great physical and psychological stress to affected patients and are extremely expensive. Disrupted vasculature and high demand for energy to support processing and regeneration of wounded tissue are typical characteristics of a wound site. Low oxygen supply and high demand results in hypoxia. Oxygen delivery is a critical element for the healing of wounds.[1–3] In the

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presence of poor blood flow, the availability of oxygen to the wound site is thought to be a rate-limiting step in early wound repair. Indeed, transcutaneous oxygen ($TcPO_2$) alone is able to reliably estimate probability of healing in an ischemic extremity.[4] The time line of wound healing is altered by various local conditions, such as inflammation and neuropathy; however, the most important factor regulating the regional time line of healing is blood flow. Factors that can increase oxygen delivery to the regional tissue, such as supplemental oxygen, warmth, and sympathetic blockade, can speed healing.[5,6] Intermittent oxygen therapy has been shown to promote collagen synthesis and is beneficial for producing the extracellular matrices that support wound healing.[7]

HYPERBARIC OXYGEN THERAPY

Wound repair can often be facilitated by increasing the partial pressure at which oxygen is supplied to wounds.[2] Clinical experience with adjunctive hyperbaric oxygen therapy in the treatment of chronic wounds[8] has shown that wound hyperoxia increases wound granulation tissue formation and accelerates wound contraction and secondary closure.[9,10] Nevertheless, the physiological basis for this modality remains largely unknown. Such ignorance adversely affects our ability to establish definitive criteria for the selection of patients and also to predict success in treatment. To date, there are few clinical studies that attempt to define the fundamentals underlying hyperbaric oxygen therapy. Hyperbaric studies have been criticized for the lack of well-defined wound care protocols, the absence of precise wound-healing measures, and poorly defined wound healing endpoints.[11] Evidence supporting the mode of action of hyperbaric oxygen in promoting wound healing is sketchy at best. For example, hyperbaric oxygenation above 2 atmospheres inhibits proliferation of fibroblasts and keratinocytes in cell monolayer cultures (e.g., a 10-day treatment at 3 atmospheres appeared cytostatic to keratinocytes). In contrast, hyperbaric treatment up to 3 atmospheres dramatically enhances keratinocyte differentiation, and epidermopoiesis in complete human skin equivalents.[12]

Hyperbaric oxygen therapy includes two key components: high (2–3 atm) pressure and close to 100% oxygen. What is the relative contribution of the pressure and oxygen factors? Do we need a combination of both for successful wound therapy or is normobaric oxygen treatment good enough? In the case of an exposed dermal wound, is it important to administer oxygen systematically or is topical oxygen applied locally to the wound site effective? While there are many opinions about these important questions, at present we do not have any firm evidence-based scientific conclusions. Systemic oxygen therapy is contraindicated in numerous situations and poses significant risk to organs such as the eye, brain, and lung. Under certain circumstances, negative pressure oxygen therapy has been claimed to be more effective than hyperbaric oxygen therapy.[13] A reasonable evaluation of the risk:benefit ratio of systemic oxygen therapy in the treatment of wound healing would require mechanism-oriented translational research.

TOPICAL OXYGEN THERAPY

Topical oxygen therapy represents a less explored modality in wound care.[14] Pure oxygen is locally administered to an affected region of the body at 1.03 atmospheres of pressure and can be done in the patient's own home (see FIGURE 1). It is indicated for the treatment of open wounds. The advantages of topical oxygen therapy include low cost, the lack of systemic oxygen toxicity, possibility of home treatment, and effectiveness, allowing this treatment to be prescribed for many patients early in the course of their disease rather than as a last resort.[15] Systemic hyperbaric therapy requires that patients be placed in special chambers in the presence of trained physician specialists with the delivery of oxygen in the chamber at 2–3 atmospheres of pressure. Whether topical oxygen therapy has similar efficacy as systemic hyperbaric oxygen therapy remains to be established. A few brief studies have reported the effects of topical oxygen therapy on wound healing. These studies are mostly observational and do not address underlying mechanisms.[16–18] It is claimed that topical oxygen alone or in combination with a low power laser may be useful to treat diabetic foot ulcers.[19] On the basis of prospective randomized clinical studies it has been inferred that topical oxygen therapy represents a cost-effective approach[20] to promote wound angiogenesis.[21] If indeed topical oxygen therapy emerges as a successful therapeutic modality in the treatment of wounds, it could significantly decrease the cost of caring for chronic wounds and substantially broaden the scope of patients eligible for treatment.

A RADICAL HYPOTHESIS IN SUPPORT OF OXYGEN THERAPY

The search for the mechanisms by which oxygen exerts its vital functions in wound healing has evolved another step. Reactive oxygen species (ROS, includes oxygen-derived radical as well as non-radical oxidants), often loosely termed "oxidants," are a vital part of healing.[22,23] Oxygen is the rate-limiting factor for activation of NADPH oxidase that triggers respiratory burst. Respiratory burst is a mechanism by which phagocytic cells generate oxidants from oxygen. Hyperbaric oxygen has been shown to stimulate respiratory burst activity.[24,25] Micromolar concentrations of hydrogen peroxide promote vascular endothelial growth factor (VEGF) expression in keratinocytes.[23] VEGF is an endothelial-cell–specific mitogen. The finding that VEGF was potent and specific for vascular endothelial cells and, unlike basic fetal growth factor freely diffusible, led to the hypothesis that this molecule plays a unique role in the regulation of physiological angiogenesis.[26]

Wound healing occurs in "phases." The main phases of wound healing include coagulation, which begins immediately after injury; inflammation, which initiates shortly thereafter; a migratory and proliferative process, which begins within days and includes the major processes of healing; and a remodeling process, which may last for up to a year and is responsible for scar tissue formation and development of new skin.[27] In the inflammation phase, one of the first lines of defense are migrating polymorphonuclear cells (PMNs) which locate, identify, phagocytize, kill, and digest microorganisms and eliminate wound debris. These cells, through their characteristic "respiratory burst" activity, produce $O_2^{-\cdot}$ (superoxide anion radical), which

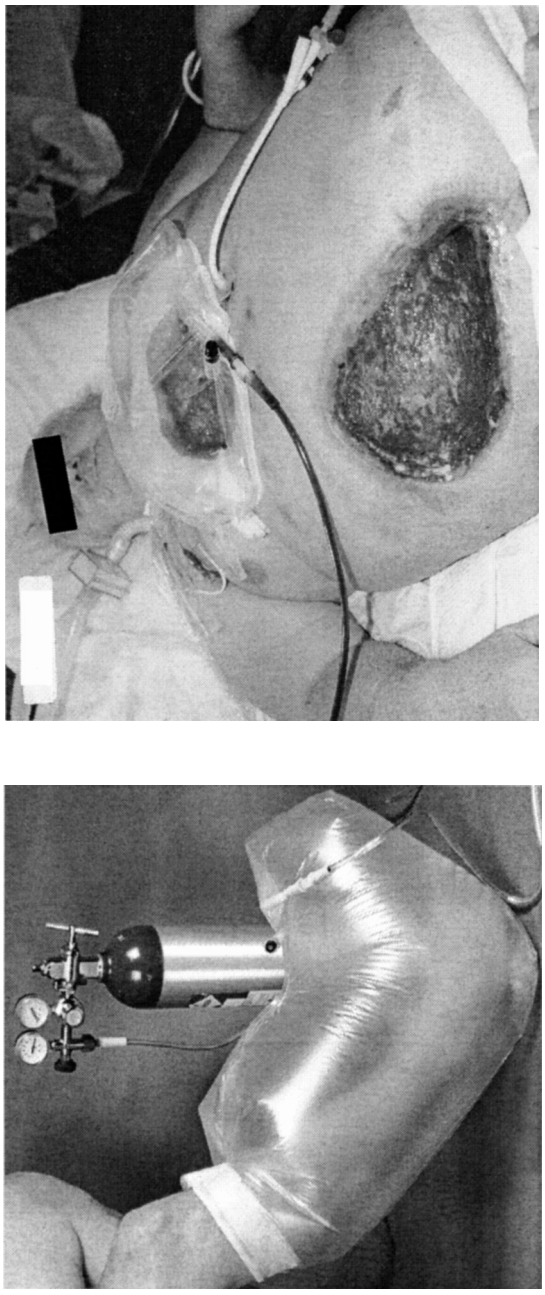

FIGURE 1. Devices for topical oxygen therapy.

is well known to be critical for defense against bacteria and other pathogens.[28] Superoxide is rapidly converted to membrane permeable form, H_2O_2, by superoxide dismutase activity or even spontaneously. Release of H_2O_2 may promote formation of other oxidants that are more stable (longer half-life) including, hypochlorous acid, chloramines, and aldehydes. The production of oxidants at the wound site is not restricted to neutrophils alone but may be also produced by macrophages, which appear and orchestrate a "long term" response to injured cells subsequent to the acute response. Taken together, this suggests that the wound site is rich in oxidants along with their derivatives such as chloramine, mostly contributed by neutrophils and macrophages. A clinically relevant model documented treatment of ischemia-induced ulcers with hydrogen peroxide cream and reported enhanced cutaneous blood recruitment not only to ulcers and adjacent sites, but also to distant sites.[29] Oxidants serve as cellular messengers that drive numerous aspects of molecular and cell biology.[30,31] While it is plausible that this information is of outstanding significance to the practice of oxygen therapy, at present it remains largely unexplored.

Consistent with the hypothesis that wound-related oxidants support the healing process, clearing oxidants from the wound environment of old rats during the early inflammation phase of the healing process decreased blood flow.[32] Exposure to mild concentrations of oxidants triggers expression of antioxidant defense proteins such as heme oxygenase 1[33] and keratinocyte growth factor[34] that are likely to protect the regenerating tissue against oxidant damage. Some would argue against the role of oxidants in wound healing. For example, Senel *et al.* have claimed that oxygen free radicals may be detrimental to ischemic skin wound healing.[35] Interpretation of results reported in this study requires careful consideration. It has been shown that treatment of the wound by allopurinol or superoxide dismutase increased tensile strength of the healing tissue. Allopurinol inhibits xanthine oxidase, a source of superoxide in endothelial cells, but does not have any effect on phagocytic or non-phagocytic oxidases that are known to be responsible for the respiratory burst phenomenon. Furthermore, superoxide dismutase accelerates the formation of hydrogen peroxide from superoxide. Hydrogen peroxide is a potent oxidant. Therefore, it is indeed plausible that the reported effects of superoxide dismutase were mediated by hydrogen peroxide. The concentration of oxidants in question is critically important. Although at micromolar concentrations oxidants such as hydrogen peroxide may favorably influence signal transduction processes that support healing, at millimolar concentrations hydrogen peroxide is likely to overwhelm the antioxidant defense system of the healing tissue[36] and trigger indiscriminate tissue damage thereby delaying healing.[37]

The effects of many growth factors and cytokines, recognized as key elements of the wound healing process, are mediated by oxidants. TGF-β1 is a pleiotropic cytokine that plays a key role in wound healing. Some fibrogenic actions of TGF-β1, necessary for extracellular matrix production, are mediated via formation of hydrogen peroxide.[38] Oxidants also promote fibroblast migration and proliferation.[39,40] Hydrogen peroxide generated by phagocytic cells in the wound site has also been shown to up-regulate endothelial-cell heparin-binding EGF mRNA, another key player in promoting wound healing.[41] Oxidants generated in response to Rac1 activation have been shown to be essential for nuclear factor κB–dependent transcriptional regulation of interleukin-1α, which, in an autocrine manner, induced

collagenase-1 gene expression. Remodeling of the extracellular matrix and consequent alterations of integrin-mediated adhesion and cytoarchitecture are central to wound healing. It has been proposed that activation of Rac1 may lead to altered gene regulation and alterations in cellular morphogenesis, migration, and invasion.[42] Recent studies in our laboratory provide the first evidence that Rac1 gene transfer accelerates contraction and healing of murine excisional dermal wounds (not shown).

Platelet-derived growth factor (PDGF), commonly used in clinical wound therapy, is found as PDGF-A, AB, and BB. It exerts its effects on cells by binding to one of two membrane-bound receptors, the α-receptor or the β-receptor. Both PDGF-BB and TGF-β1 alone are more effective than hyperbaric oxygen treatment by itself in accelerating the impaired wound healing produced by ischemia. In a recent study, acutely ischemic wounds in rabbit ears were treated with saline or PDGF-BB and then animals were treated with hyperbaric air or oxygen at 2 atm abs (202.6 kPa). Hyperbaric air was without significant effect compared with control rabbits breathing air at ambient pressure. Combined treatment with hyperbaric oxygen plus PDGF-BB was synergistic in up-regulating mRNA for PDGF-β receptor. Exposure to 85% oxygen has been shown to potently increase the expression of both the PDGF-B gene and the PDGF B-type receptor.[43] These findings lay a firm rationale to test the therapeutic significance of PDGF-BB and oxygen in synergism. The results of a preliminary clinical study support the use of combined therapy using topical becaplermin (trade name for PDGF) and hyperbaric oxygen therapy as a means of successfully treating the chronic diabetic ulcer patient with deficient nitric oxide production and local wound hypoxia.[9]

The hypothesis that cytokines such as PDGF and oxygen may function synergistically to promote wound healing is in line with predictions that could be made from cell biology studies. Cytokines such as PDGF, epidermal growth factor (EGF), tumor necrosis factor (TNF)-α or interleukin (IL)-1β generate oxidants upon binding to their receptors.[44] It has been specifically demonstrated that such oxidants play a key role in driving cellular signal transduction pathways of PDGF-treated cells. Inhibitors of oxidant production inhibit PDGF-induced activation of cell signaling.[45] Consistently, in a separate study over-expression of the antioxidant-enzyme superoxide dismutase blocked the PDGF-induced expression of genes and gene products. It was shown that nitric oxide synthase induced by PDGF is mediated in part by production of superoxide.[46] Pretreatment with catalase (decomposes hydrogen peroxide) completely abrogated hydrogen peroxide-induced PDGF receptor and c-Src tyrosine phosphorylation, suggesting that PDGF receptors send mitogenic signals utilizing oxidants as messengers.[47] Endothelial cells are not only capable of sensing oxygen tension, but are also able to discriminate and respond to even small differences in oxygen tension resulting in dramatic up-regulation of the PDGF-B chain gene.[48]

Nitric Oxide

A supporting role for reactive species in wound healing has been evident from numerous studies focusing on nitric oxide. While some questions have been raised,[49] it would be fair to summarize that nitric oxide produced during the healing process clearly promotes wound repair.[50] The earliest evidence demonstrating that nitric oxide may promote wound healing was presented only five years ago when it was demonstrated that nitric oxide synthesis is critical to wound collagen accumulation

and acquisition of mechanical strength.[51] Nitric oxide is expected to promote wound angiogenesis by inducing the expression of vascular endothelial growth factor.[52] Using knock-out mice and gene transfer approaches it has been established that both endothelial nitric oxide synthase[53] as well as inducible nitric oxide synthase play a key role in wound repair.[54]

HERBAL ANTIOXIDANTS IN WOUND HEALING

The search for "natural remedies" for a commonly occurring disorder such as wounds has drawn attention to herbals. From ancient times, herbals have been routinely used to treat wounds, and in many cultures their use in traditional medicine has persisted to the present. While it is possible that some time-tested herbal remedies are indeed effective, it seems to be often the case that the patient knows more about this form of medicine than the physician! In other words, lack of detailed mechanism-oriented and hypothesis-driven research poses a major drawback to the use of herbal medicine to treat wounds. For example, *Aloe vera* is commonly used for a wide range of dermatological applications including wound healing. The efficacy of *Aloe vera* in treating wound healing remains to be categorically established.[55] With the renewed interest in herbal cures, it is time to revisit the field.

There are numerous herbal derivatives that have been tried for their ability to promote wound healing. A complete discussion of these derivatives is beyond the scope of this work. While most studies are purely observational in nature, a few others have attempted to address the underlying mechanisms. For example, the polysaccharide-rich *Angelica sinensis* has a direct mucosal healing effect on gastric epithelial cells by increasing ornithine decarboxylase and c-Myc expression.[56] A *Eucommia ulmoides* Oliver leaf extract has been shown to favorably influence collagen metabolism and support wound healing. Oral administration of this herbal derivative accelerated granuloma maturation and the energy was supplied from fatty acid metabolism.[57] Eupolin extract increases fibroblast and endothelial cell growth.[58] The extract increases expression of several components of the adhesion complex and fibronectin by human keratinocytes. Eupolin reportedly stimulates the expression of many proteins of the adhesion complex and fibronectin by human keratinocytes. The adhesion complex proteins are thought to be essential to stabilize epithelium and this effect could contribute to the clinical efficacy of Eupolin in healing.[59]

Proanthocyanidins or condensed tannins are a group of biologically active polyphenolic bioflavonoids that are synthesized by many plants. Proanthocyanidins and other tannins are known to facilitate wound healing.[60,61] The mode of action, however, remains unclear. Grape seed proanthocyanidin extract, has been reported to have various clinically relevant redox-active properties.[62–66] It was recently observed that natural extracts derived from grape seeds facilitate oxidant-induced VEGF expression in keratinocytes. These results suggested that grape-seed–derived natural extracts may have beneficial effects in promoting dermal wound healing and other related skin pathologies.[23] Using a ribonuclease protection assay (RPA), the ability of GSPE to regulate oxidant-induced changes in several angiogenesis-related genes has been studied. While mRNA responses were studied using RPA, VEGF protein release from cells to the culture medium was studied using ELISA. Pretreatment

of HaCaT keratinocytes with GSPE up-regulated both hydrogen peroxide as well as TNFα-induced VEGF expression and release.[23] Studies with VEGF promoter linked to a luciferase reporter showed that the herbal extract influenced the transcriptional control of inducible VEGF expression. In a murine model of dermal excisional wound, a combination of grape seed extract and 5,000 ppm resveratrol markedly accelerated wound contraction and healing (not shown). In a previous section of this article, we have discussed how oxidants could support the wound healing process. Herbal extracts such as the grape seed extract are highly rich in antioxidants. This leads to an apparent paradox. How can both oxidants as well as antioxidants promote healing? While a definitive answer requires further experimentation, it should be noted that antioxidants do tend to possess signal transduction regulatory properties that may or may not be linked to their ability to detoxify oxidants.[30,31,67–69] In addition, under certain conditions such as a strong oxidizing environment lacking the support to regenerate (reduce) oxidized antioxidants, some antioxidants may assume the characteristics of a pro-oxidant.[70–73]

CONCLUSION

Recent advances in the molecular and cellular aspects of redox biology positions us well to revisit the apparently outstanding benefit of oxygen therapy in wound healing. It is likely that reactive derivatives of molecular oxygen, oxidants, for example, serve as cellular messengers to support the healing process. Strategies to manipulate the oxygen/oxidant environment in the wound are likely to be of outstanding significance.

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