Therapeutics

FORUM REVIEW ARTICLE

Hyperbaric Oxygen, Vasculogenic Stem Cells, and Wound Healing

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Abstract

Significance: Oxidative stress is recognized as playing a role in stem cell mobilization from peripheral sites and also cell function. ***Recent Advances:*** This review focuses on the impact of hyperoxia on vasculogenic stem cells and elements of wound healing. ***Critical Issues:*** Components of the wound-healing process in which oxidative stress has a positive impact on the various cells involved in wound healing are highlighted. A slightly different view of wound-healing physiology is adopted by departing from the often used notion of sequential stages: hemostatic, inflammatory, proliferative, and remodeling and instead organizes the cascade of wound healing as overlapping events or waves pertaining to reactive oxygen species, lactate, and nitric oxide. This was done because hyperoxia has effects of a number of cell signaling events that converge to influence cell recruitment/chemotaxis and gene regulation/protein synthesis responses which mediate wound healing. ***Future Directions:*** Our alternative perspective of the stages of wound healing eases recognition of the multiple sites where oxidative stress has an impact on wound healing. This aids the focus on mechanistic events and the interplay among various cell types and biochemical processes. It also highlights the areas where additional research is needed. *Antioxid. Redox Signal.* 21, 1634–1647.

Introduction

WOUND HEALING AFTER an insult or injury is a complex process that involves the coordination of multiple mediators and components. Research continues to expand our understanding of the wound-healing process, which is central to normal physiology and also a growing concern in clinical medicine. For example, more than 6 million individuals in the United States per year are affected by chronic wounds (150). Often, these individuals have other medical disorders, such as diabetes mellitus. As estimated by the Kaiser Foundation in 2012, those with diabetes and lower-extremity wounds in the US Medicare program accounted for $41 billion in cost, which is ~1.6% of all Medicare health care spending. Apart from the costs, wounds that fail to heal may result in limb amputations and death. Lower-extremity amputation in those with diabetes is associated with a risk of mortality of about 20% per year (107, 108, 132).

Attempts to use so-called adjunctive measures to improve the rate of wound healing have added to our understanding of wound physiology. One rather novel approach that has provided an interesting perspective on the role of oxidative stress in wound healing is hyperbaric oxygen (HBO_2). HBO_2 therapy is a treatment modality in which a person breathes 100% O_2 while exposed to increased atmospheric pressure. Treatments are carried out in either a monoplace (single person) or multiplace (typically 2–14 patients) chamber. Pressures applied while in the chamber are usually 2 to 3 atmospheres absolute (ATA), the sum of the atmospheric pressure (1 ATA) plus additional pressure equivalent to 1 or 2 atmospheres (1 atmosphere = a pressure of 14.7 pounds per square inch or 101 kPa). Treatments are usually about 1.5–2 h long and may be performed once or twice daily.

Results

Physiology overview

During HBO_2 treatment, the arterial O_2 tension typically exceeds 2000 mmHg, and levels of 200–400 mmHg occur in tissues (167). It is well accepted that an increase in the

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production of reactive oxygen species (ROS) occurs during hyperoxia. While O_2 toxicity is a risk, clinical protocols have maintained the incidence of adverse effects very low (27). The beneficial aspect to ROS and also reactive nitrogen species (RNS) is that they serve as signaling molecules in transduction cascades, or pathways, for a variety of growth factors, cytokines, and hormones (5, 23, 113, 176). As such, reactive species can generate either ''positive'' or ''negative'' effects depending on their concentration and intracellular localization. Since exposure to hyperoxia in clinical HBO_2 protocols is rather brief, studies show that antioxidant defenses are adequate so that biochemical stresses related to increases in reactive species are reversible (31, 32, 120, 141).

Oxygen and wound healing

Soon after tissue injury, as a part of the repair process, devitalized tissue is removed, keratinocytes migrate and proliferate to the wound edge, and granulation tissue, which is primarily composed of fibroblasts and endothelial cells, begins to form. Granulation tissue contains excessive neovascular proliferation. This process includes the repair, restoration, and regeneration of blood vessels.

Postnatal neovascularization involves two complementary processes. One is the sprouting of the endothelium from preexisting blood vessels (angiogenesis); the second involves endothelial stem/progenitor cells (SPCs) released from the bone marrow as well as peripheral tissue sites that home to foci of ischemia in a process termed vasculogenesis (59, 138, 180). SPCs likely orchestrate vascular repair by differentiating into endothelial cells as well as supporting structures that give rise to repaired and/or regenerated blood vessels. A variety of cell surface markers have been used to identify these cells and since they change as the cells mature and/or assume different functions, we have chosen the moniker SPCs *versus* the term endothelial progenitor cell as used by some investigators because these cells are defined by a narrow range of surface markers and their nature is debated (10, 52, 195).

The notion of redox regulation and varied roles for O_2 in wound healing is commonly discussed and has been outlined by many in recent years (126, 146, 152). There is also a burgeoning literature on the role of oxidants in embryonic and hematopoietic stem cells, which is beyond the scope of this review (86, 92, 93, 118, 158, 166, 183). HBO_2 has effects on a number of cell types and will influence both angiogenesis and vasculogenesis. In this review, we will frame our discussion around recognized mediators of wound healing to emphasize that HBO_2 merely acts by modifying established regulatory pathways. The classical view of wound healing envisages a number of sequential phases (*e.g.*, hemostatic, inflammatory, proliferative, and remodeling), and this perspective has been effective for focusing areas of investigation for $\sim$75 years. We believe that a useful alternative wound-healing paradigm which eases discussion of HBO_2—or more generally the availability of O_2, ROS, and RNS—involves three overlapping events or ''waves.'' Biochemical energy is generated from O_2 for the increased energy demands of repair processes such as cell proliferation, bacterial defense, and collagen synthesis. The second role of O_2 is cell signaling that is mediated by the generation of reactive species. A convenient division for cell signaling involves roles for ROS, elevations in wound lactate, and also nitric oxide ($^{\bullet}$NO), as all of them converge to influence cell recruitment/chemotaxis and gene regulation/protein synthesis responses that mediate wound healing.

The ROS wave

O_2-derived free radicals as well as O_2-derived nonradical species such as H_2O_2 and hypochlorous acid are generated as a part of normal metabolism by mitochondria, endoplasmic reticulum (ER), peroxisomes, various oxidase enzymes, and phospholipid metabolism. ROS act in conjunction with several redox systems involving glutathione, thioredoxin, and pyridine nucleotides, and they play central roles in coordinating cell signaling and also antioxidant, protective pathways (26, 75, 190). The main physiological source of extracellular H_2O_2 in wounds is considered a family of NADPH oxidases, which transport electrons from cytoplasmic NADPH to generate superoxide radicals ($O_2^{-\bullet}$) or H_2O_2 (15). The so-called Nox (NADPH oxidase) group of five genetically distinct enzymes generates superoxide, which can be converted to H_2O_2 by superoxide dismutase (SOD); whereas two Duox (dual oxidase) enzymes generate H_2O_2 without requiring SOD (7). An overview of components in the ROS wave is shown in Figure 1.

Cell migration/chemotaxis.   Acting in a paracrine manner, H_2O_2 serves as a chemotactic signal in the first minutes after wounding. Mechanical or chemical stress triggers a burst of H_2O_2 from epithelial cell Duox enzyme activity (124). Postwound extracellular H_2O_2 can reach concentrations of $\sim$0.5–50 μM near the wound margin, with a gradient extending $\sim$200 μm. H_2O_2 diffusion across many cell widths appears to occur *via* aquaporin-like channels (17). It would seem reasonable that there may also be roles for antioxidants such as catalase and peroxidase in this process, but this has not been clearly established. SOD activity decreases in some vascular injury models, and supplementation of SOD either *via* adenoviral vector gene transfer or from SPCs recruitment can improve healing in animal models of diabetes mellitus (91, 101, 109). Neutrophils exhibit a chemotactic response to exogenous H_2O_2 (although the molecular details for this response are unknown), and they appear at the wound edge within 10 min after wounding (82, 124). HBO_2 will increase production of reactive species within neutrophils (primarily from Nox2, although multiple sources may contribute) and can improve bacteriocidal efficacy (103, 104, 170). It is unclear whether this oxidant source also contributes to cell recruitment.

Platelet aggregation during the early stages postinjury generate ROS, which are derived from Nox as well as from xanthine oxidase (142, 143, 177). Vascular smooth muscle cells synthesize thrombogenic tissue factor in a Nox-dependent fashion, which may perpetuate the thrombogenic process within injured vessels that is initiated by platelets (62). Skin keratinocytes and fibroblasts also use Nox to generate H_2O_2, as do recruited leukocytes. In addition to its well-recognized antibacterial function, H_2O_2 increases epithelial cell, smooth muscle cell, endothelial cell, and monocyte/macrophage migration (94, 125, 127, 139, 164), and it may increase leukocyte integrin adhesion (100). HBO_2 does not alter platelet function and inhibits neutrophil β_2 integrin adhesion at
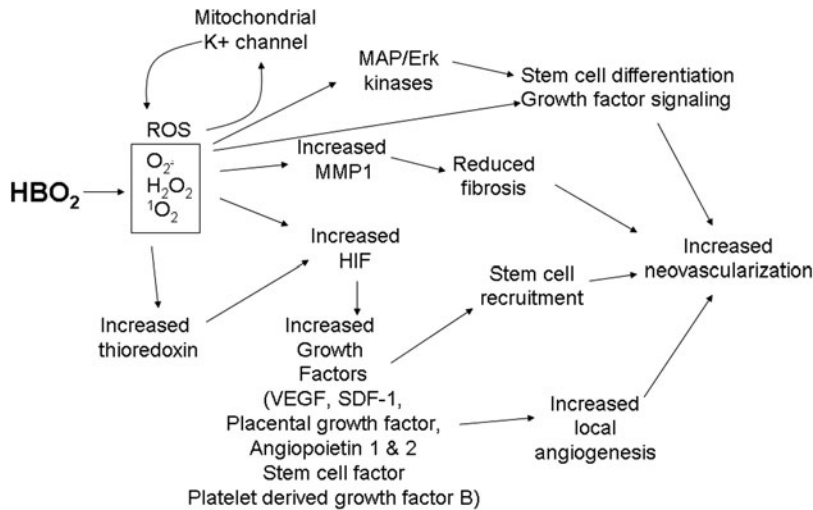
FIG. 1. ROS wave: Summary of wound-healing events related to reactive oxygen species (ROS). HIF, hypoxia-inducible factors; MAP, mitogen-activated protein kinase; MMP1, matrix metalloproteinase-1; SDF-1, stromal-derived factor-1; VEGF, vascular endothelial growth factor.

pressures of 2.8 ATA or more, beyond those used in wound-healing protocols (168, 170, 173).

In an *in vivo* Matrigel wound model, HBO$_2$ increases Nox-derived H$_2$O$_2$ synthesis, which contributes to SPCs recruitment as well as to growth factor synthesis (116). Effects on SPCs and cells that have undergone greater differentiation will be outlined in greater detail in subsequent sections. In an *in vitro* model, transient DNA oxidative stress from short-term HBO$_2$ was also shown to improve endothelial cell tolerance to subsequent oxidant exposure (187). The sources for H$_2$O$_2$ in wound healing are still not entirely clear, and overlapping roles may exist. Exogenous addition of H$_2$O$_2$ can activate Nox (140). Although not shown for the endothelium, mitochondrial H$_2$O$_2$ can regulate Nox activity in smooth muscle cells (*via* protein kinase C) and human 293T cells (*via* phosphoinositide 3 kinase and Rac1), and activated Nox can mediate mitochondrial ATP-sensitive potassium channels and thus mitochondrial H$_2$O$_2$ production (29, 90, 140).

Gene regulation/protein synthesis. The interruption of blood flow associated with acute injuries rapidly causes wound hypoxia, which contributes to stabilization of hypoxia-inducible factors (HIF) and these transcription factors activate many genes, resulting in the synthesis of a variety of proteins required for wound healing (*e.g.*, vascular endothelial growth factor [VEGF], stromal-derived factor 1 [SDF-1], placental growth factor, angiopoietin 1, angiopoietin 2, platelet-derived growth factor B, and stem cell factor [SCF]) (148, 149). Early elevations in H$_2$O$_2$ will also stabilize HIF *via* decreased ascorbate availability and secondarily by decreasing prolyl-hydroxylase activity (130).

Among the proteins synthesized in response to ROS is thioredoxin, which not only acts in antioxidant pathways, but also functions as a transcription factor to increase HIF synthesis (193). This pathway is triggered by HBO$_2$ in localized vasculogenic stem cells, which augments VEGF and SDF-1 synthesis, enhancing neovascularization (115, 116). Subsequent signaling between SDF-1 and its cognate cellular receptor, CXCR4, also involves ROS (89). Likely a synergistic process, lactate can also mediate HIF stabilization in endothelial cells by metabolic conversion to pyruvate that inhibits prolyl hydroxylases (160). In ischemic peripheral wounds,

placement of SDF-1 into the margins will markedly augment SPCs recruitment associated with HBO$_2$ treatments (48).

ROS (particularly O$_2^{-\bullet}$) generated by platelets and other cells early in the wound process modulate activation of cell surface latent tissue factor (133). Activated tissue factor activates thrombin, which contributes to hemostasis and also activates vascular cell Nox oxidases (thus adding to H$_2$O$_2$ production in the early wound). ROS production modulates responses of endothelium, lymphoid, and monocytic cells and also smooth muscle cells by influencing NFkB activation (111, 114) and H$_2$O$_2$ augments macrophage, keratinocyte, and fibroblast-mediated VEGF and VEGF-receptor 2 synthesis (11, 25, 55, 113, 144, 151). Ambient pressure hyperoxia as well as HBO$_2$ increases VEGF synthesis in soft tissue wounds as well as in healing bone (43, 155). As mentioned earlier, enhanced H$_2$O$_2$ production by HBO$_2$ will result in increases in VEGF and SDF-1 synthesis by SPCs and, as one might expect, supplementing the local environment with catalase will abrogate these responses (115, 116). HBO$_2$ enhances placental growth factor production by bone marrow-derived mesenchymal stem cells through elevations of ROS (156). H$_2$O$_2$ also increases cellular synthesis of pro-inflammatory tissue necrosis factor in wounds (57, 58).

Singlet oxygen activates plasminogen, which will then activate matrix metalloproteinase 1 (MMP1) to reduce fibrosis during wound remodeling (56). ROS are also involved in cell responses to growth factors. For example, a variety of growth factors influence endothelial cell function by activating protein kinases such as Erk. The pathways by which growth factors activate Erk involve elevations in intracellular ROS, which, in turn, inhibit phosphatase enzymes that impede protein kinase phosphorylation (70). Erk plays a complex role in HBO$_2$-mediated stimulation of SPC-mediated neovascularization (115, 116). H$_2$O$_2$ as well as other ROS increases signaling by platelet-derived growth factor (PDGF) and transforming growth factor (TGF) beta in several cell types (71). HBO$_2$ was shown to up-regulate PDGF receptors in experimental wounds (19).

A separate and also critically important role for O$_2$ in newly synthesized tissue is collagen cross-linking. O$_2$ is a required co-factor for this process (67), and collagen synthesis by fibroblasts is proportional to local O$_2$ concentration in the range of 0–200 mmHg (80, 155).

ROS play an important role in whether stem cells enter the cell cycle. In embryonic stem cells, p38 inhibition sustains self-renewal; whereas ROS-mediated p38 activation enhances cell turnover (68, 136). Activity of p38 will also increase the transcriptional coactivator peroxisome proliferator-activated receptor γ coactivator 1α, which plays a central role in mitochondrial biogenesis that influences stem cell differentiation (135). ROS also play a role in the stem cell pluripotency, the ability to differentiate into different cell lineage types (73, 157). For example, at progressively higher concentrations of H_2O_2, cells exhibited greater differentiation into cardiac myocytes (21). Mesenchymal stem cells transplanted into infarct zones using a rat myocardial infarction model exhibit greater engraftment and improved cardiac function with HBO_2 treatment (77, 78). If alternative protein kinase pathways are activated by ROS, stem cell lineage patterns can be modified (69). Recent studies have shown HBO_2-mediated enhancement of chondrocyte-specific gene expression and also osteogenesis in differentiating between human and animal adipose-derived stem cells (a peripheral, mesenchymal stem cell type) (24, 33, 46, 153). Neuronal differentiation of mesenchymal stem cells involves up-regulation of Nox and increased ROS synthesis, although whether these changes are cause or effect is unclear (179). Perhaps further insights into the phenomenon can be gleaned by observations that HBO_2 also promotes neural progenitor cell neurogenesis *ex vivo,* possibly by modification of the Wnt pathway, and they may promote proliferation of endogenous central nervous system stem cells to form neurons and vascular channels after hypoxic or ischemic insults (185, 188, 191, 192).

The ˙NO wave

Nitric oxide is synthesized by one of three nitric oxide synthase (NOS) isoforms present in a large variety of cells. All enzyme isoforms use O_2, the amino acid arginine, and a variety of cofactors to synthesize ˙NO. NOS activity increases early after wounding and persists at an elevated level for many days. The primary source of NOS in early wound healing is macrophages, although many other cells (*e.g.*, fibroblasts) contribute to local production (88). Oxygen availability influences activity of NOS enzymes differently, in part because they have different Km values for binding O_2 and as the active sites and rates of turnover are different, making them more or less sensitive to oxidation. Reported Km values for type 1 (nNOS) is 350 μM, type 2 (iNOS) is 135 μM, and type 3 (eNOS) is 23 μM (165). Hence, the rate of ˙NO formation by nNOS will be much more affected by O_2 fluctuations over a greater physiological range than eNOS. Figure 2 summarizes roles for ˙NO in neovascularization.

HBO_2 can augment activity all three NOS isoforms. Activation of nNOS and also eNOS appears to be mediated through enhanced binding of heat shock protein 90 (22, 169). Activation of iNOS, at least in neutrophils, occurs due to an increase in short filamentous actin synthesis and secondary iNOS linkage (171). Consequences linked to NOS activation by HBO_2 will be discussed next and in this regard, it is important to remain cognizant that since exposures are transient, enzyme activation is likely relatively brief. In neutrophils, iNOS activity is transient because enzyme association to filamentous actin ceases due to breakdown of a poly-protein complex (171). The notion that transient ˙NO synthesis is important, because while ˙NO is required for wound healing to occur, too much hinders healing (14, 81).

Cell migration/chemotaxis. MMPs play critical roles in matrix remodeling and cell migration. ROS and also ˙NO regulate MMPs at the transcriptional and post-translational levels (121, 128, 159). For example, ˙NO enhances endothelial cell migration by increasing the local extracellular concentration of MMP-13. MMP-13 activity is usually constrained, because it is bound to membrane caveolae. This linkage is broken by locally synthesized ˙NO, which increases collagen breakdown (97). Elevated local concentrations of ˙NO synthesized by iNOS will stimulate keratinocyte migration during re-epithelialization (161). Activating eNOS in bone marrow stroma secondarily nitrosylates MMP-9, which releases the stem cell active cytokine, soluble Kit ligand (SCF) (60). This agent shifts SPCs from a quiescent to the proliferative niche, and stimulates their mobilization to the peripheral blood (2, 3, 60, 61, 119, 137). By directly activating eNOS, HBO_2 mobilized bone marrow SPCs in both animal models and humans (48, 54, 172).

Gene regulation/protein synthesis. Nitric oxide plays a central role in synthesizing VEGF (45), cytokines, and growth factors (6, 145, 182). Synthesis of ˙NO by eNOS (*versus* other isoforms) plays a predominant role in VEGF-mediated angiogenesis (47), possibly by stabilizing HIF-1 (35, 149). Many down-stream effects of VEGF are also stimulated *via* ˙NO (9, 131). In addition to its local effects within the wound, VEGF gets into the circulation and eventually the bone marrow, where it activates stem cell mobilization *via* NOS activation, which as described earlier, causes S-nitrosylation and activation of MMP-9, release of soluble Kit ligand, and SPCs mobilization (2, 3).

The lactate wave

Wounding impairs blood flow due to damaged vessels along with local consumption of O_2 by the varied Nox isoforms, which rapidly establishes hypoxia. An immediate consequence is anaerobic metabolism by local wound cells, which generates lactate. This is only likely transient, and oxygenation improves with any restoration of blood flow. Wound margin lactate concentrations remain elevated, however, because of endothelial cells and recruited leukocytes that preferentially rely on glycolysis, even in an aerobic environment (18, 51, 122). Thus, hyperoxia does not reduce wound lactate concentration (66, 123). In fact, a study of HBO_2 metabolic effects in an *ex vivo* blood vessel model suggests that in the hours after hyperoxia (but not during oxygen exposure), lactate levels increase (186). When examining this report, as with all studies of HBO_2, readers need to be sensitive to the differences between *ex vivo* and *in vivo* studies. Tissue/cell oxygenation with just normobaric hyperoxia in an *ex vivo* setting equals or exceeds that achieved *in vivo* during hyperoxia. Thus, both normobaric and hyperbaric hyperoxia increased lactate levels in this *ex vivo* tissue model (186). The mechanism for the response was not identified and there are several possibilities. For example, elevated ROS as well as ˙NO can impede tricarboxylic acid (TCA) cycle metabolism and mitochondrial oxidative

phosphorylation (12, 181, 184). A more complex process could be *via* augmentation of HIF levels, because HIF can suppress metabolism through the TCA cycle and also up-regulate expression of lactate dehydrogenase (LDH) (in fact, this model also reported elevated LDH in the tissue medium, while suggesting there was no oxidative stress-mediated enzyme –''leakage'') (63, 79). In a situation where actively metabolizing tissues are no longer under the influence of hyperoxia, the higher than normal NADH could lead to a reverse LDH effect, catalyzing conversion of pyruvate and NADH to lactate and NAD+ synthesis. Whatever the mechanism(s), there are numerous consequences to lactate elevations and some are synergistic with HBO_2. Figure 3 shows an overview of effects.

Cell migration/chemotaxis. Lactate is not itself a chemotactic stimulus but it can influence cell migration secondarily. Lactate stimulates hyaluronic acid synthesis (44, 162). Hyaluronic acid accumulation in the peri-wound extracellular matrix causes expansion of tissue, enabling easy cell movement into damaged tissues and recruitment of new fibroblasts that adhere to matrix *via* the CD44 receptor.

Gene regulation/protein synthesis. Lactate combined with normobaric O_2 stimulates angiogenesis (65). Lactate chelates iron in the ER and radicals generated by the concurrent presence of O_2^- and H_2O_2 *via* Fenton reaction (while confined to the ER) generates hydroxyl radical ($^\bullet OH$) that reduces HIF prolyl hydroxylase activity (4, 98, 99). Similar impairment of prolyl hydroxylase and increased HIF binding to DNA occurs with the glycolytic intermediates pyruvate and oxaloacetate, in addition to lactate (98, 160). Lactate modifies the gene expression pattern of mesenchymal stem cells to one more conducive to wound healing *versus* apoptosis (194). The pro-oxidant action of lactate improves the function of vasculogenic stem cells recruited from bone marrow to peripheral sites as a consequence of HBO_2 (117). Lactate *via* elevated ROS production will increase cell content of HIF factors (HIF-1 and HIF-2), resulting in elevated synthesis of VEGF and SDF-1, which then augment local neovascularization as well as recruit additional cells to the healing complex.

The metabolism of lactate by LDH increases intracellular concentration of NADH at the expense of the NAD pool. In addition to feeding reducing equivalents for ROS as mentioned earlier, the altered NAD/NADH ratio reduces the cell

content of polyADP-ribose (178). Although unclear whether entirely mediated by an altered oxidation/reduction set point, lactate stimulates collagen mRNA abundance and also collagen promoter activity by fibroblasts (49, 50). In endothelium, lactate-mediated reduction of poly-adenyl ribosylation of VEGF improves VEGF angiogenic potency (85).

Discussion: Efficacy of HBO_2

The outline described earlier highlights the multiple sites where ROS, lactate, and $^\bullet NO$ can influence wound healing, with special emphasis on SPCs. We believe this categorization has merit to examine concurrent processes in wound healing and lends itself to highlighting sites where HBO_2 has effects. In one regard, this approach may be too simplistic however, because tissues contain a variety of cell types and HBO_2 may influence each in different ways. Figure 4 is an attempt to highlight many of these events. HBO_2 increases synthesis of many growth factors, although the biochemical mechanisms have not been elucidated in detail. Synthesis of VEGF has been shown to be increased in experimental wounds by HBO_2 (41, 154). HBO_2 also stimulates synthesis of basic fibroblast growth factor and TGF (1 by human dermal fibroblasts (74), angiopoietin-2 by human umbilical vein endothelial cells (95), and as previously mentioned, it up-regulates PDGF receptor in experimental wounds (19).

HBO_2 appears to be a reliable way to mobilize SPCs in humans (102, 172, 174). Animal data indicate that the specific target which initiates this process is NOS-3 in the stromal cell compartment of the bone marrow with subsequent liberation of SCF (54, 172). With regard to this process, in is important to stress that contrary to many of the traditional agents which increase SPCs, HBO_2 does not concomitantly elevate the circulating leukocyte count, which may be thrombogenic (102, 134). Newly mobilized SPCs appear to have greater content of HIF-1, HIF-2, and thioredoxin, which in the murine model exhibit improved neovascularization (115, 116, 174). Subsequent to HBO_2 treatments of diabetic patients, most wound margin HIFs and thioredoxin appear to be derived from localized SPCs (174). This suggests that SPCs may play an important role in supplying critical factors during wound healing in diabetic patients.

The influence that HBO_2 has on HIF isoform expression appears to vary based on chronology (*e.g.*, looking early or late after wounding or an ischemic insult). One recent model showing accelerated wound healing by HBO_2 reported lower
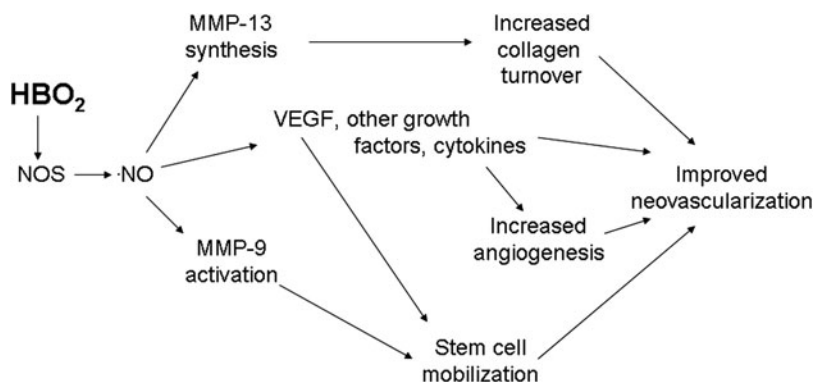

FIG. 2. **$^\bullet$NO wave: Summary of wound-healing events related to elevated synthesis of nitric oxide ($^\bullet$NO).** NOS, nitric oxide synthase.

FIG. 3. Lactate wave: Summary of wound-healing events related to elevated lactate concentrations in the region of a wound. LDH, lactate dehydrogenase; NAD, nicotine adenine nucleotide; NADH, nicotine adenine nucleotide, reduced; NOX, NAD(P)H oxidase; TCA, tricarboxylic acid.
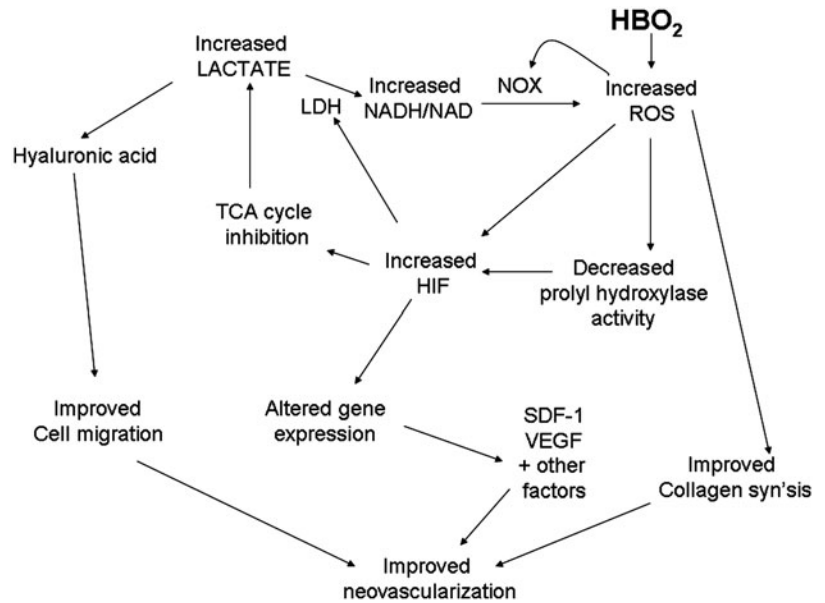
HIF-1 levels at wound margins with reduced inflammation and fewer apoptotic cells (189). In contrast, higher levels of HIF-1 have been linked to elevated VEGF in wounds in response to hyperoxia (64, 154).

Chronic wounds are said to have stalled in the inflammatory-healing phase, but this characterization does little to address specific flaws that vary depending on the underlying pathophysiology (87). We think this adds to the merit of viewing wound healing as ''waves'' of ROS, lactate, and ˙NO production. HBO$_2$ in current practice is used to treat refractory diabetic wounds and delayed radiation injuries. The pathophysiology of radiation injury is obviously different than diabetic wounds but the varied tissue abnormalities have been likened to a chronic wound (30). Common elements shared by both disorders include depletion of epithelial and stromal cells, chronic inflammation, fibrosis, an imbalance or abnormalities in extracellular matrix components and remodeling processes, and impaired keratinocyte functions (20, 30, 40, 110, 163, 175). Diabetic wound healing also is impaired by deceased growth factor production, lower ˙NO production due to low insulin levels, eNOS phosphorylation, and higher levels of asymmetric dimethylarginine and impaired SPCs mobilization; whereas in postradiation tissues, there appears to be an imbalance between factors mediating

FIG. 4. Summary of stem cell and peripheral wound site events impacted by HBO$_2$. Images in *lower left* are confocal microscope images similar to those reported in reference (116). They demonstrate vasculogenesis in a Matrigel implant placed in a mouse that was exposed to HBO$_2$. They show CD34+ SPCs (*green*) and Nile red beads (*red*) injected *via* the heart to demonstrate functional blood vessels. The overlay between CD34+ cells and beads is shown in *yellow*. Trx-S$_2$, oxidized thioredoxin; Trx-SH$_2$, reduced thioredoxin. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars
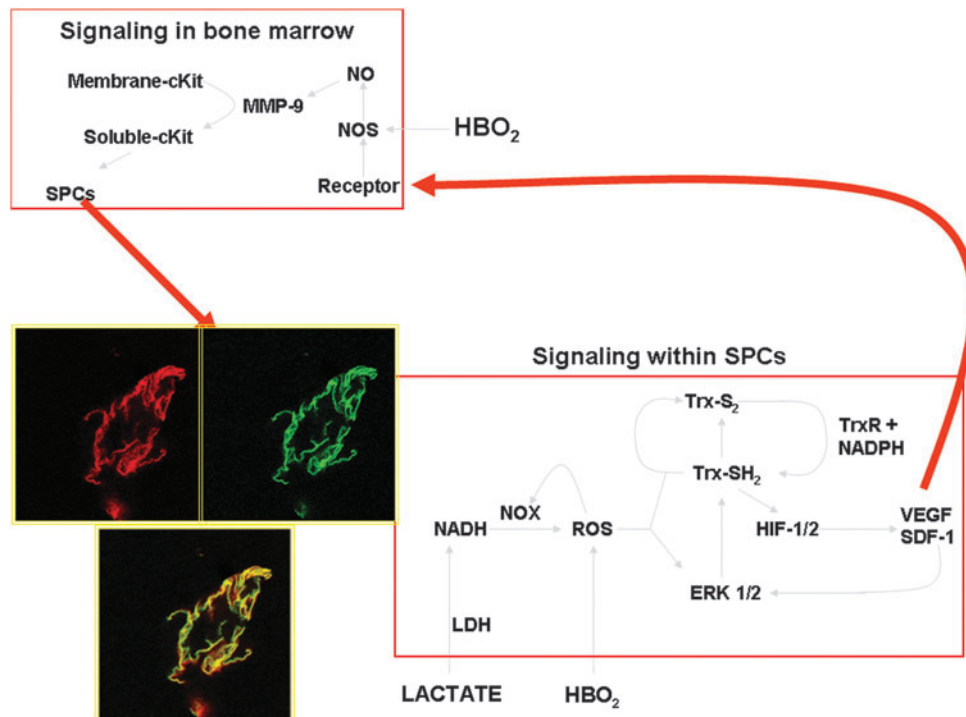
fibrosis and those promoting normal tissue healing (20, 30, 37, 147, 175).

The benefit of HBO_2 for radiation injury has been shown in randomized trials and is supported by independent evidence-based reviews (16, 28, 112). With regard to use of HBO_2 as a component to refractory diabetic wound management, the most recent meta-analysis involved eight trials and pooled data from three showed an increase in the rate of ulcer healing (odds ratio 5.20, 95% confidence interval [CI] 1.25–21.66; $p = 0.02$) with HBOT at 6 weeks, although benefit did not persist at 1 year follow up (84). Another analysis concluded that adjunctive use of HBO_2 as a component to diabetic wound care improves healing with an odds ratio of 11.64 (95% CI 3.457–39.196) (53). This analysis was based on clinical trials conducted through 2007 (1, 13, 34, 38, 39, 42, 72, 76, 129). Another meta-analysis concluded that only four patients needed to be treated with HBO_2 to prevent one amputation (83). Since this publication, two additional groups have reported benefits to use of HBO_2; one was a double-blinded randomized trial (36, 96). Controlled trials continue to demonstrate that HBO_2 improves outcome but there is room for further investigation, as will be emphasized later. The double-blinded trial was a single-center study that enrolled individuals with diabetic foot ulcers. Individuals were randomized to receive either HBO_2 (100% oxygen, 2.5 ATA for 85 min 5 days per week for 8 weeks) or control (room air, 2.5 ATA for 85 min 5 days per week for 8 weeks) and standardized wound care. The outcome was a healed wound by 12 months after the commencement of therapy. A total of 94 individuals with wounds present for more than 3 months were evaluated. In the intention-to-treat analysis, complete healing of the index ulcer was achieved in 37 patients at 1 year of follow up: 25 out of 48 (52%) in the HBO_2 group and 12 out of 42 (29%) in the placebo group ($p = 0.03$). In a sub-analysis of those patients completing > 35 HBO_2 sessions, healing of the index ulcer occurred in 23 out of 38 (61%) in the HBO_2 group and 10 out of 37 (27%) in the placebo group ($p = 0.009$).

It is important to state that for both diabetic wounds and radiation injuries, HBO_2 is used in conjunction with standard surgical management. Randomized trials show clinical benefit with HBO_2 when attention is paid to potential confounding issues and quality of baseline care. When used by itself or if used only in the postoperative period, however, HBO_2 is likely to have no benefit (8, 105). In randomized trials, clinicians are constrained to follow a rigorous wound care plan that may be as important as is HBO_2 for improved outcomes. The optimal timing for intervention with HBO_2 in relation to more standard forms of therapy, as well as the most appropriate endpoints to be used for evaluating outcomes remains elusive. This can be seen in a "real world" comparative effectiveness study involving records review of 6, 259 individuals with foot wounds related to diabetes with adequate lower limb arterial perfusion (106). Individuals receiving HBO_2 were less likely to heal their foot ulcer (propensity score odds ratio 0.68 [95% CI: 0.63–0.73]) and more likely to have an amputation (odds ratio 2.37 [95% CI: 1.84–3.04]). However, utilization and how HBO_2 was coordinated with other interventions was uncertain. The mean number of treatments was 29 but with a broad range (25th%–75th%: 15–48). If the minimum number of treatments was taken as eight and only this population was studied, the impact of HBO_2

was less clear with regard to amputation (odds ratio 2.03 [95% CI: 1.49–2.77]) and with regard to a healed wound (odds ratio 0.73 [95% CI: 0.66–0.81]). These data indicate that basic science and insight into HBO_2 is improving, whereas there is still more to learn regarding the coordination of HBO_2 with other treatments and there remains a need for further clinical research.

Innovation

This review highlights the components of wound healing where oxidative stress has a positive impact on the various cells involved in wound healing. It departs from the notion of sequential wound-healing stages by organizing the cascade of wound healing as overlapping events or waves pertaining to reactive oxygen species, lactate, and nitric oxide. This was done because hyperoxia has effects of a number of cell signaling events that converge to influence cell recruitment/chemotaxis and gene regulation/protein synthesis responses which mediate wound healing. This aids the focus on mechanistic events and the interplay among various cell types and biochemical processes.

Acknowledgments

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Abbreviations Used	
ATA = atmospheres absolute	RNS = reactive nitrogen species
Duox = dual oxidase	ROS = reactive oxygen species
ER = endoplasmic reticulum	SCF = stem cell factor
HBO₂ = hyperbaric oxygen	SDF-1 = stromal-derived factor-1
HIF = hypoxia-inducible factor	SOD = superoxide dismutase
LDH = lactate dehydrogenase	SPCs = stem/progenitor cells
MMP = matrix metalloproteinase	TCA cycle = tricarboxylic acid cycle
NAD = nicotine adenine nucleotide	TGF = transforming growth factor
NADH = nicotine adenine nucleotide = reduced	TGF-β = transforming growth factor-beta
NOS = nitric oxide synthase	Trx = thioredoxin
Nox = NADPH oxidase	TrxR = thioredoxin reductase
PDGF = platelet-derived growth factor	VEGF = vascular endothelial growth factor

HBO_2 = hyperbaric oxygen