OXYGEN-DERIVED HYDROGEN PEROXIDE AS A KEY SIGNAL FOR WOUND HEALING

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Abbreviated title: Hydrogen peroxide in wound healing

Word Count: 1915 (excluding references)

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ABSTRACT (215 words)

Background: Observations made just over a decade ago demonstrating that almost every cell of the human body has dedicated enzyme systems that reduce oxygen to superoxide anion radical led to question the long-held mindset that oxygen free radicals are indiscriminately bad for human health. Wound-related cells, phagocytic as well as non-phagocytic, contain NADPH oxidases which expend cellular reducing “currency” NADPH to generate superoxide radicals and hydrogen peroxide from molecular oxygen.

The Problem: What are the roles of oxygen derived free radicals and their derivatives, collectively known as reactive oxygen species, in the biology of wound healing? Are free radicals just the bad guys or do they have a bright side to them?

Basic/Clinical Science Advances: Reactive oxygen species, specifically hydrogen peroxide generated by the wound tissue, is now recognized as a key signaling mediator that orchestrates wound healing.

Clinical Care Relevance: In wounds that are limited in their ability to generate the required hydrogen peroxide for healing, either because of limitations in oxygen supply or in the necessary enzymatic system, delivery of low levels of hydrogen peroxide over time to mimic the environment of the healing wound should help improve wound outcomes.

Conclusion: Generation of hydrogen peroxide at the wound site is one key mechanism by which wound tissue oxygen supports healing responses to injury.

a. **Background** (194)

Leukocyte oxidase is an enzyme that was reported in 1959. The enzyme consumed oxygen to generate free radicals. In the late 1970s Bernard Babior linked the explosive production of superoxide radical anions by leukocyte oxidase to bacterial killing [1-2]. In the 1980s, limitations in methodological approaches to sensitively detect and monitor the extremely short-living reactive species clouded a true appreciation of the significance of oxygen-derived free radicals in health and disease. The primary identity of free radicals was that they were destructive to biological tissues, and that approaches to antagonize free radicals i.e. antioxidants should be helpful. Based on this crude preliminary concept, numerous clinical trials testing the efficacy of antioxidants were hastily started and the results were understandably disappointing. In 1999, the cloning of mox1 marked a major progress in categorically establishing the presence of distinct NADPH oxidases in non-phagocytic cells [3]. Later, Mox1 was renamed as Nox1 referring to NADPH oxidase. As we know today, Nox enzymes are present in nearly all cells of the human body, especially in wound-related cells [4]. Individuals suffering from genetic deficiency of Nox suffer from chronic granulomatous disease and impaired wound healing response (reviewed in [5]).

b. **Target Articles**

1. Niethammer P, Grabher C, Look AT, Mitchison TJ. A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish. ***Nature***. 2009;459:996-9.
2. Roy S, Khanna S, Sen CK. Redox regulation of the VEGF signaling path and tissue vascularization: Hydrogen peroxide, the common link between physical exercise and cutaneous wound healing. ***Free Radic Biol Med***. 2008;44:180-92.
3. Sen CK. Wound healing essentials: let there be oxygen. ***Wound Repair Regen***. 2009;17:1-18.

c. **Clinical Problem Addressed** (99)

Ischemic wounds have limited supply of oxygen and are therefore limited in their ability to support oxygen consuming processes, including hydrogen peroxide generation. Three major factors may contribute to wound tissue hypoxia: (i) peripheral vascular diseases garroting oxygen supply, (ii) increased oxygen demand of the healing tissue, and (iii) generation of reactive oxygen species by way of respiratory burst and for redox signaling. Other related factors such as arterial hypoxia (e.g. pulmonary fibrosis or pneumonia, sympathetic response to pain, hypothermia, anemia caused by major blood loss, cyanotic heart disease, high altitude) may contribute to wound hypoxia as well [5-6].

d. **Relevant Basic Science Context** (251)

Recent work have identified that oxygen is not only required to disinfect wounds and fuel healing but that oxygen-dependent redox-sensitive signaling processes represent an integral component of the healing cascade. Over a decade ago, it was proposed that in biological systems oxidants are not necessarily always the triggers for oxidative damage and that oxidants such as hydrogen peroxide could actually serve as signaling messengers and drive several aspects of cellular signaling. Today, that concept is much more developed and mature. The Nox family comprises seven members, Nox1-Nox7. Nox1, Nox2 (gp91phox-containing NADPH oxidase), Nox4 and Nox5 have been identified in the cardiovascular-renal systems and have been implicated in the pathophysiology of cardiovascular and renal disease. These enzymes are present in virtually all cell of the human body where they utilize oxygen and expend precious intracellular NADPH to generate superoxide anion radicals which in turn rapidly dismutate to form hydrogen peroxide. Evidence supporting the role of oxidants such as hydrogen peroxide as signaling messenger is compelling [7-9]. A complete understanding of the continuum between the classical and emergent roles of oxygen requires a thorough consideration of current concepts in redox biology.

Hydrogen peroxide is now known to serve as a cell signaling messenger in numerous contexts including:

i. cell survival [10]

ii. transcriptional regulator [11]

iii. wound healing [6]

iv. vascular biology [12-13]

v. cancer [14]

vi. lymphocyte activation [15]

The signaling role of hydrogen peroxide is effective at very low levels. Excessive hydrogen peroxide poses the clear risk of oxidative stress [16].

e. **Experimental Model or Material - Advantages and Limitations** (125)

In the primary work reviewed in this chapter [17], the zebrafish larval tail fin wound model was investigated. This experimental system is valuable in studying the inflammatory and regenerative responses to wounds [18]. Specifically, rapid recruitment of leukocyte to the wound site can be easily imaged [19]. The model is powerful in elucidating fundamental mechanisms implicated in wound healing. Results obtained should be interpreted with caution because the translational value of the observations made, as it relates to application to human wounds, are limited. If the primary interest is to understand human wound biology, results obtained from a zebrafish wound setting represent a valuable starting point to test the hypothesis in actual human wounds or in a pre-clinical setting [20] depending on the study design.

f. **Discussion of Findings and Relevant Literature** (681)

In the primary work reviewed in this chapter, Niethammer et al.[17] employed a powerful approach to image the spatiotemporal dynamics of hydrogen peroxide at the wound site. In addition, leukocyte motility was imaged in this setting where an intact vertebrate tissue was subjected to mechanical wounding. Hydrogen peroxide was measured using a genetically encoded ratiometric sensor called HyPer that is highly selective for hydrogen peroxide over other forms of reactive oxygen species. HyPer is made up of the bacterial hydrogen peroxide-sensitive transcription factor OxyR fused to a circularly permuted yellow fluorescent protein (YFP). Cysteine oxidation of the OxyR part induces a conformational change that increases emission excited at 500 nm (YFP500) and decreases emission excited at 420 nm (YFP420). This change is rapidly reversible within the reducing cytoplasmic environment, allowing dynamic monitoring of intracellular hydrogen peroxide concentration. HyPer was introduced by injection of messenger RNA into zebrafish embryos to induce global cytoplasmic expression. This is a powerful approach to image hydrogen peroxide in the zebrafish system.

In murine cutaneous wounds, the wound-edge tissue is known to be a major source of reactive oxygen species which are generated via Nox [4]. In the murine work reported in 2006 it was shown that the wound fluid is very rich in reactive oxygen species containing over 0.2mM hydrogen peroxide during the inflammatory phase [4]. The current 2009 work by Niethammer et al. showed consistent results from the zebrafish system demonstrating rapid rise in hydrogen peroxide concentration at the wound margin tissue. The hydrogen peroxide signal peaked 20 min (inflammatory phase) after

wounding. At this time, the recorded hydrogen peroxide gradient extended 0.1-0.2 mM inward from the wound margin. These concentrations of hydrogen peroxide are very similar in total to the concentration of hydrogen peroxide recorded in murine wound fluid at the inflammatory phase [4]. The Nox enzyme system was the primary source of wound-site hydrogen peroxide in both mice as well as zebrafish [4,17]. In the zebrafish system, hydrogen peroxide generated at the wound margin tissue

Niethammer e al. reported that hydrogen peroxide generated in the wound margin tissue was required to recruit leukocytes to the wound [17]. This observation is consistent with a 1996 report demonstrating that in mice micromolar hydrogen peroxide can induce neutrophil chemotaxis [21]. Directed locomotion of murine peritoneal neutrophils under agarose was studied, and activity of hydrogen peroxide as a chemoattractant was examined in the 1996 study.

Observations of the Niethammer study fits well with the current literature on the significance of hydrogen peroxide in inflammation. Hydrogen peroxide has a fine-tuning regulatory role, comprising both a proinflammatory control loop that increases pathogen removal and an anti-inflammatory control loop, which avoids an exacerbated harmful inflammatory response [22]. Monocytes are recruited to the wound-site by specific chemoattractants such as fragmented extracellular-matrix protein, transforming growth factor β (TGFβ), MCP-1, and macrophage inflammatory protein (MIP). Reactive oxygen species induce MIP1a, MIP2 as well as MCP1. ROS induces TGFβ expression as well

as its activation by oxidatively displacing the latency conferring peptide [23]. In certain cell types, hydrogen peroxide is required for TGFβ-induced cell signaling [24].

Hydrogen peroxide directly regulates monocyte function [25]. High mobility group box 1 (HMGB1) can be actively secreted by macrophages/monocytes in response to exogenous and endogenous inflammatory stimuli (such as bacterial endotoxin, TNF-α, IL-1, and IFN-γ) or passively released by necrotic cells and mediates innate and adaptive inflammatory responses to infection and injury. At doses found in the wound fluid, hydrogen peroxide induces HMGB1 cytoplasmic translocation and active release within 3-24 h. Inhibitors specific for the JNK (SP600125) and MEK (PD98059), but not p38 MAPK (SB203580), abrogate hydrogen peroxide-induced, active HMGB1 release suggesting a key role of hydrogen peroxide in inducing active HMGB1 release, potentially through a MAPK- and CRM1-dependent mechanism [26].

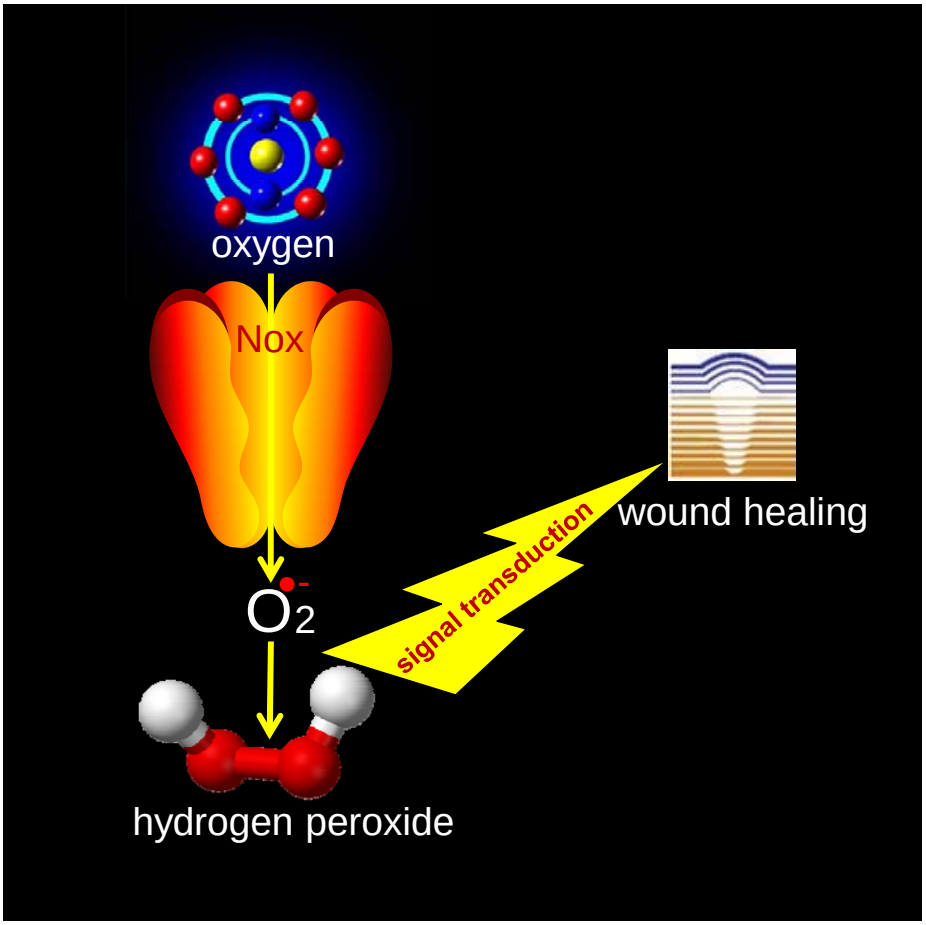
Monocytes adhere to specific proteins of the extracellular matrix by their integrin receptors. Such adhesion triggers the differentiation of monocytes to reparative macrophages and stimulates phagocytosis of micro-organisms and fragments of extracellular matrix. Hydrogen peroxide induces LFA-1-dependent neutrophil adherence and Mac-1 dependent macrophage adherence [27]. The significance of hydrogen peroxide in inflammation has been recently reviewed [6].

g. **Innovation** (42)

The most innovative aspect of the study by Niethammer et al.[17] is the elegant approach to image the spatiotemporal dynamics of hydrogen peroxide at the wound site. The observations made are interesting and consistent with the current literature on murine wound biology.

h. **Summary Illustration**

Fig. 1: **Oxygen is utilized by the Nox enzyme system to generate low levels of hydrogen peroxide which in turn serves as a signaling messenger to enable leukocyte recruitment and wound healing**. For detailed review see Sen and Roy [6].



i. **Take home messages**

Basic Science Advances (73)

- An elegant approach to image hydrogen peroxide in zebrafish wounds is available
- Wound-site cells contain enzymes (Nox family) that consume oxygen and expend NADPH to make hydrogen peroxide
- Wound-induced hydrogen peroxide generation is rapid
- Wound-induced production of hydrogen peroxide is required to recruit leukocytes to the wound site
- Hydrogen peroxide is a signaling messenger. The field of hydrogen peroxide induced cell signaling is widely known as redox signaling

Clinical Science Advances (54)

- Appropriate tissue oxygenation will allow hydrogen peroxide signaling enabling leukocyte cell recruitment and wound healing
- Limitation in wound tissue oxygenation will limit hydrogen peroxide production and redox signaling
- Genetic deficiencies, such as noted in patients with chronic granulomatous disease, may limit Nox function at the wound site and limit healing responses

Relevance to Clinical Care (43)

- Appropriate wound tissue oxygenation is needed to enable the healing response
- In ischemic wounds suffering from hypoxia or in patients with Nox deficiency, therapeutic strategies aimed at delivery of low-levels of hydrogen peroxide over a period of time warrant clinical testing

j. **Caution, Critical Remarks and Recommendations** (139)

Although low levels of hydrogen peroxide may drive cell signaling relevant to wound healing, one must be cautious about the fact that excessive hydrogen peroxide may be toxic and hurt wound healing. Delivery of small doses over an extended period of time is therefore likely to be helpful. Initial rinsing of the wound with higher concentrations of hydrogen peroxide may be of some value with respect to cleansing the wound but such strong hydrogen peroxide solution may not support the healing response on a long-term basis. Decomposition of wound-site hydrogen peroxide by overexpression of catalase is known to impair wound closure in mice[4]. While topical hydrogen peroxide treatment has been reported to be effective in improving wound closure in mice [4], results from humans are missing. Clinical studies testing the efficacy of hydrogen peroxide in treating wounds are warranted.

k. **Future Development of Interest** (35)

Additional studies testing the significance of endogenous hydrogen peroxide on other aspects of wound healing are warranted. Such studies will help develop a comprehensive picture of the overall significance of hydrogen peroxide in wound healing.

I. Abbreviation list

CRM1, an evolutionarily conserved protein, was shown to be a receptor for leucine-rich nuclear export signal (NES)-dependent protein transport

HMGB1, High-mobility group box 1

HyPer, genetically encoded ratiometric hydrogen peroxide sensor

IFN, interferon

IL, interleukin

JNK, Jun N-terminal kinase

LFA, lymphocyte function-associated antigen 1

Mox, the older name for Nox

MAPK, mitogen-activated protein kinase

MEK, MAPK kinase

MCP, monocyte chemoattractant protein

NADPH, nicotinamide adenine dinucleotide phosphate

Nox, NADPH oxidase

OxyR, bacterial hydrogen peroxide-sensitive transcription factor

ROS, reactive oxygen species

TGF, transforming growth factor

YFP, yellow fluorescent protein

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n. Acknowledgments and Funding Sources

Wound healing research in the author's laboratory is funded by NIH awards GM 077185 and GM 069589.

o. Author Disclosure and Ghostwriting

The author has no relevant conflicts to disclose. The work was not written by any writer other than the author.