THE ROLE OF HYDROGEN PEROXIDE AND OTHER REACTIVE OXYGEN SPECIES IN WOUND HEALING

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Summary: Wound healing is a complex physiological process important for tissue homeostasis. An acute injury initiates massive cell migration, proliferation and differentiation, synthesis of extracellular matrix components, scar formation and remodelling. Blood flow and tissue oxygenation are parts of the complex regulation of healing. Higher organisms utilize molecular oxygen as a terminal oxidant. This way of gaining energy for vital processes such as healing leads to the production of a number of oxygen compounds that may have a defensive or informatory role. They may be harmful when present in high concentrations. Both the lack and the excess of reactive oxygen species may influence healing negatively.

Key words: Wound healing; Oxygen; ROS

REVIEW ARTICLE

Molecular oxygen is a terminal electron acceptor in organisms that gain energy by controlled oxidation of carbon compounds. The oxygen molecule O\(_2\) in its ground state (triplet oxygen) contains two unpaired electrons with parallel spin states. Four electrons are sequentially accepted by the oxygen molecule when it is reduced to water. This process gives birth to a number of intermediates and side products. Most of them are radicals containing an unpaired electron. Other substances (hydrogen peroxide, hypochlorous acid) are reactive but do not have a character of a radical. They are collectively called reactive oxygen species (ROS).

Superoxide anion radical is formed when the oxygen molecule accepts one electron. It can function both as a reductant and as an oxidant. Its reactivity is relatively low but it can accept a hydrogen proton and form a hydroperoxyl radical that is a stronger oxidant than O\(_2\)\(^{-}\). Hydrogen peroxide is formed in the reaction of two superoxide radicals called disproportionation. This spontaneous reaction is greatly accelerated by superoxide dismutase (SOD). It is generally accepted that the reaction of superoxide radical with hydrogen peroxide in the presence of ferrous ions (the Haber-Weiss reaction) produces an extremely reactive hydroxyl radical. Peroxynitrate is produced in the reaction of superoxide radical with nitric oxide, which is also a radical.

When two electrons attach to the oxygen molecule, peroxide anion is produced that yields H\(_2\)O\(_2\) by accepting two hydrogen protons. Hydrogen peroxide is also formed in the reaction of superoxide radical with hydroperoxyl radical. Ferrous ions (Fe\(^{2+}\)) reduce hydrogen peroxide; ferric ions (Fe\(^{3+}\)), a hydroxyl radical and a hydroxyl anion are formed in the so-called Fenton reaction. Fe\(^{3+}\) ions are reduced back to Fe\(^{2+}\) by another molecule of hydrogen peroxide.

Peroxynitrate can be reduced by accepting a proton and then it can split to other reactive radicals. Peroxynitrate is produced in the reaction of superoxide radical with nitric oxide, which is also a radical.

Spectroscopic measurements indicate that the Haber-Weiss reaction produces the singlet form of molecular oxygen and not the hydroxyl radical. Singlet oxygen contains two electrons with antiparallel spins in contrast to the ground triplet form containing two electrons with parallel spins. It is highly reactive as it forms covalent bonds much more easily than the common triplet oxygen. Peroxynitrate is produced in the reaction of superoxide radical with nitric oxide, which is also a radical.

The reactions describing the production of various ROS are summarized in Table 1.

Production and detoxification of ROS in healing wounds

Wound healing is characterized by four distinct but overlapping phases. As blood spills into the site of injury, platelets come into contact with collagen and other extracellular matrix (ECM) components. Blood clotting factors are released and within minutes the wound is covered by a fibrin clot. Hemostasis is followed by the inflammatory phase. Neutrophils enter the wound site and remove damaged tissue and bacteria. Macrophages appear later and continue the process of phagocytosis. Both platelets or macrophages release growth factors and profibrotic cytokines such as the platelet-derived growth factor (PDGF) and the transforming growth factor beta (TGF-β). Mast cells release granules containing histamine and other mediators that cause surrounding vessels to become leaky and allow the passage
of cells into the wound. In the proliferative phase endothelial cells form new capillaries. Fibroblasts migrate into the cleaned wound from the neighbouring tissue, proliferate and deposit fibronectin, collagen and other components of new ECM. Keratinocytes proliferate, migrate and restore surface integrity. Finally, during the remodeling phase, fibroblast-rich granulation tissue is gradually replaced with a relatively acellular scar. Crosslinked collagen molecules give the scar tensile strength that approximates that of the intact skin (37, 49).

A characteristic feature of the inflammatory phase is the oxidative burst. Polymorphonuclear cells and macrophages migrating into the wound release large amounts of superoxide radical that is converted to hydrogen peroxide by SOD. Superoxide is produced by NADPH oxidase. This enzymatic complex transfers electrons from NADPH to molecular oxygen and produces the superoxide radical. It is composed of proteins localized in phagosomal membranes and in the plasma membranes of phagocytic cells. Some components of the complex are found in the cytosol; they move to the membrane as a consequence of phagocytosis or under the influence of soluble stimulators. The activity of NADPH oxidase in phagocytes is regulated by a small GTPase Rac2 (42).

The increase in the concentration of superoxide and hydrogen peroxide is transient as the neutrophil and macrophage migration diminishes and as a result of the action of antioxidative defense systems (27). The ability of macrophages to produce ROS is much smaller than that of neutrophils but ROS and particularly H$_2$O$_2$ are important second messengers regulating physiological functions of macrophages (12). Myeloperoxidase contained in granulocytes and monocytes catalyzes the reaction of hydrogen peroxide with chloride anions; powerful oxidant hypochlorous acid is produced (42). Myeloperoxidase reaction is also a source of singlet oxygen (51).

Also nonphagocytic cells like fibroblasts contain the NADPH oxidase complex (34) or another enzymatic system generating superoxide (38). Fibroblasts secrete ROS when they are stimulated with inflammatory cytokines interleukin-1 or TNF-alpha or with growth factors EGF and PDGF (34). The level of ROS produced by nonphagocytic cells is about hundredfold less than that generated by activated phagocytes but it still may be important in signal transduction. NADPH oxidase of nonphagocytes is regulated by the protein Rac1, a member of GTPase family (16).

The lifetime and reactivity of various ROS are very different, which influences the range of their activity in the cells and the type of organelles they can affect. There are two types of defense systems that cells developed to detoxify ROS. The non-enzymatic systems comprise small antioxidant molecules, such as vitamin C, vitamin E, β-carotene, glutathione, coenzyme Q, bilirubin and urate. The reduced form of glutathione and ascorbate function in water solutions, the other compounds are effective in membranes and other hydrophobic media. These systems have different standard redox potentials and thus they can collaborate (40).

The enzymatic systems include three types of SOD (cytosolic, mitochondrial and extracellular), glutathione peroxidase and catalase. High levels of SOD gene expression are found at early stage of wound repair (27). Hydrogen peroxide produced by SOD can be either decomposed by catalase or used to oxidize a suitable substrate, e.g. glutathione. The expression of cytosolic and mitochondrial SOD is coordinated with the expression of catalase in the cutaneous wound (50). The oxidation of glutathione is catalyzed by glutathione peroxidase. Reduced glutathione is regenerated by the action of glutathione reductase (42).

### Function of ROS in healing wounds

Majority of ROS is released by neutrophils and macrophages during the inflammatory phase of healing. Wound fluid contains micromolar concentrations of H$_2$O$_2$. ROS protect the host against bacterial and fungal infection. Partial bacteriostatic effect of H$_2$O$_2$ was observed at concentrations between 25–50 µM, cell killing at concentrations exceeding 500 µM, when the viability of E.coli was tested in vitro.

<table>
<thead>
<tr>
<th>Chemical Reaction</th>
<th>Description</th>
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<tbody>
<tr>
<td>O$_2$ + e$^-$ → O$_2^-$</td>
<td>Superoxide anion radical</td>
</tr>
<tr>
<td>O$_2^-$ + H$^+$ → OOH$^-$</td>
<td>Hydroperoxyl radical</td>
</tr>
<tr>
<td>O$_2^-$ + O$_2^-$ + 2H$^+$ → H$_2$O$_2$ + O$_2$</td>
<td>(disproportionation) hydrogen peroxide</td>
</tr>
<tr>
<td>O$_2^-$ + H$_2$O$_2$ → O$_2$ + OH$^-$ + OH$^-$</td>
<td>(Haber-Weiss reaction) hydroxyl radical</td>
</tr>
<tr>
<td>O$_2^-$ + NO → ONOO$^-$</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>ONOO$^-$ + H$^+$ → NOO$^-$ + NO + H$_2$O</td>
<td>Nitric oxide (NO)</td>
</tr>
<tr>
<td>O$_2^-$ + 2e$^-$ → O$_2$</td>
<td>Peroxide anion</td>
</tr>
<tr>
<td>O$_2^-$ + 2H$^+$ → H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>O$_2^-$ + OH$^-$ + H$^+$ → H$_2$O$_2$ + O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>H$_2$O$_2$ + Fe$^{2+}$ → Fe$^{3+}$ + OH$^-$ + OH$^-$</td>
<td>(Fenton reaction) hydroxyl radical</td>
</tr>
<tr>
<td>H$_2$O$_2$ + Fe$^{3+}$ → Fe$^{2+}$ + OOH$^-$ + H$^+$</td>
<td>Hydroperoxyl radical</td>
</tr>
<tr>
<td>H$_2$O$_2$ + Cl$^-$ + H$^+$ → HClO + H$_2$O</td>
<td>Hypochlorous acid</td>
</tr>
<tr>
<td>H$_2$O$_2$ + HClO → O$_2$ + H$_2$O + HCl</td>
<td>Singlet oxygen</td>
</tr>
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</table>
If the inflammatory phase does not resolve in time and the concentration of ROS exceeds the antioxidant capacity of the cell, the condition called oxidative stress results (32). Oxidative stress mediated by radical ROS (superoxide anion, hydroxyl radical) and nonradical ROS (hydrogen peroxide, singlet oxygen) may inhibit cell migration and proliferation and cause tissue damage and perpetuation of inflammation (50, 60). On the other hand, neutrophil defect leads to severe infections and poor wound healing. Decreased level of Rac2, the regulatory protein of phagocyte NADPH oxidase, may be a cause of this problem in patients (2).

While ROS at high concentrations have pronounced bacteriostatic effects, at low concentrations they function as second messengers. A prominent role is played by H$_2$O$_2$. It is easily synthesized, easily degraded and present within all types of cells. It has a longer half-life than radical ROS and its small uncharged molecules diffuse easily through tissues. It does not react indiscriminately with the neighbouring molecules like many radicals (11, 14).

Low concentrations of H$_2$O$_2$ (10 µM) act as a chemottractant on mouse peritoneal neutrophils. This activity is not dependent on the presence of blood serum (30). H$_2$O$_2$ stimulates the proliferation of human fibroblasts and vascular endothelial cells of mammalian origin in a similar range of concentrations (36, 52). Overexpression of catalase in the wound can be achieved by gene transfer. The degradation of H$_2$O$_2$ by this enzyme results in the impairment of wound angiogenesis and wound closure (43). The response of cultured smooth muscle cells to PDGF, which includes chemotaxis and DNA synthesis, is blocked by catalase and by the antioxidant N-acetylcystein and thus seems to be mediated by H$_2$O$_2$ (53).

Diminished peripheral blood flow and impaired angiogenesis and vasculogenesis are characteristic of poorly healing wounds in diabetic patients, particularly in lower extremities (4, 15). Low partial pressure of oxygen reduces the activity of enzymes producing ROS (19). As a consequence, the differentiation of myofibroblasts (MFB), the cells responsible for wound contraction, is impaired. The expression of α-smooth muscle actin, the main component of myofibroblast cytoskeleton, decreases with reduced supply of oxygen, as shown in the cells transferred from 2% to 2% oxygen atmosphere. MFB lose their ability to contract in vitro and possibly the wound in vivo (35). On the other hand, when human dermal fibroblasts are exposed to 2% oxygen, they produce more TGF-β1 that mediates the increase in mRNA levels of procollagen I (10). Low oxygen tension is also a stimulus for the proliferation of human adult and neonatal dermal fibroblasts (9).

Higher concentration of H$_2$O$_2$ (0.5 mM) stimulates the production of macrophage inflammatory protein-1α that is chemotactic for mononuclear phagocytes, neutrophils, eosinophils, basophils and lymphocytes (47). Macrophages incubated with 0.1 mM H$_2$O$_2$ produce vascular endothelial growth factor (VEGF) that stimulates angiogenesis. Wound angiogenesis is an important part of healing (6). However, high concentrations of H$_2$O$_2$ (50 mM) applied on the excisional dermal wound in mice retard wound closure (43). Sources of ROS, phenazine methosulfate applied intraperitoneally or zymosan applied topically, reduce the breaking strength of the incisions made on the back of rats (13). ROS produced by xanthine and xanthine oxidase system decrease collagen and increase glycosaminoglycan synthesis in cultured human dermal fibroblasts (55). H$_2$O$_2$ plays a role in the activation of latent neutrophil collagenase that may degrade collagen in the wound (58). The activity of metalloproteinases in non-healing chronic ulcers is upregulated (56). H$_2$O$_2$ in concentrations 0.05 to 0.5 mM causes apoptosis of fibroblasts (54).

Burn injury is followed by a decrease in blood flow and ischemia. Reperfusion occurs during the burn shock resuscitation, which is accompanied by an invasion of neutrophils in various tissues. Activated neutrophils produce a huge amount of ROS that cause peroxidation of membrane lipids and disruption of membranes. Damage to proteins by ROS includes oxidation of sulfhydryl groups, modification of amino groups and peptide fragmentation. Oxidative damage to DNA causes formation of adducts of base and sugar moieties as well as strand breaks (5, 41). Excessive release of proteases may contribute to the damage caused by neutrophils (24).

**Treatment of poorly healing wounds**

Hyperbaric oxygen therapy (HBOT) is a treatment in which patients breathe 100% oxygen in a pressurized chamber. At normal atmospheric pressure, the saturation of hemoglobin with oxygen is 97% and it can reach 100% in a hyperbaric chamber. The amount of oxygen dissolved in blood increases greatly when patients breathe pure oxygen at 3 ATA and large amounts of ROS are then generated (17). An improvement in wound healing and reduction in ulcer size in diabetic patients after HBOT was reported (28, 31, 39). HBOT is recommended as an adjunctive therapy (7). In an experiment with human dermis in vitro, hyperbaric oxygen treatment enhanced keratinocyte migration and maturation (25). HBOT improved healing of ischemic wounds made in rats. The expression of hypoxia-inducible factor-1α that plays a key role in oxygen homeostasis and subsequent downstream signalling including apoptosis was reduced (61).

Topically applied hyperbaric oxygen improved wound healing in both diabetic and non-diabetic patients. Its action was correlated with vascular endothelial growth factor (VEGF) expression (20). Topical treatment with oxygen solution in perfluorocarbon increased epithelialization of partial thickness wounds and second-degree burns made in the skin of pigs (8). Hydrogen peroxide cream containing 1.5% H$_2$O$_2$ improved blood flow that was measured by la-
ser Doppler technique in the wounds induced in the skin of guinea pigs (57).

In contrast to the favourable outcome of O2 or H2O2 treatment in patients, there are reports suggesting the need to suppress ROS formation in wounds. Antioxidant defense, reduced glutathione, ascorbic acid and vitamin E contents as well as superoxide dismutase, glutathione peroxidase and glutathione-S-transferase activities are decreased in cutaneous wounds in rats 2 to 7 days after tissue excision (44). Allopurinol or SOD solution applied on skin wounds in rats increased collagen content and breaking strength of the wounds (46). Antioxidant Trolox accelerated wound closure in diabetic rats (21). Honey has a favourable effect on wound healing because it possesses both antibacterial activity and antioxidant capacity (22).

Conclusions

Cells activated by extracellular stimuli produce ROS. Cellular signalling pathways contain redox-sensitive sites and are subjected to redox regulation. Oxidants also react with nuclear transcription factors and modulate gene transcription. ROS are messengers for certain growth factors. ROS are messengers for certain growth factors. They also facilitate healing of chronic foot ulcers in patients with diabetes. Possible role in the beneficial activity of phagocytes. Free Rad Biol Med 2000; 28:463–499.

References


Received: 12/01/2011
Accepted in revised form: 18/03/2011

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