



Review

Role of nitric oxide, nitroxidative and oxidative stress in wound healing

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Abstract:

Redox-regulated processes are relevant to wound healing. A balance between bioavailable nitric oxide (NO) concentration and a level of oxidative and nitroxidative stress in wounds may be crucial in wound repair. The highly beneficial effect of bioavailable NO is attributed to scavenging of superoxide, which is the main component of oxidative stress. Also, the high level of NO can influence angiogenesis and endothelial/skeletal muscle cell remodeling and proliferation. However, under conditions of excessive and prolonged production of O_2^- in wounds, the supplementation of NO can be evolved in significant increase in nitroxidative stress due to production of peroxynitrite ($ONOO^-$) and peroxynitrous acid ($ONOOH$). $ONOOH$ can trigger a cascade of events leading to the generation of highly reactive and damaging radicals and oxidative species. These species (mainly $CO_3^{\cdot-}$, NO_2^+ , NO_2 , N_2O_3 , OH^{\cdot}) can impose significant damage in biological milieu and impair the process of wound healing. Therefore, a general strategy for an acceleration of the wound healing process may include an intervention(s) leading to the decrease in oxidative stress (treatment with antioxidants and/or prevention of O_2^- generation by uncoupled constitutive nitric oxide synthase, cNOS) and delivery of NO (treatment with NO donors, cNOS gene therapy). Here we briefly review the role of NO, and focus on O_2^- and $ONOOH$ (major components of oxidative and nitroxidative stress respectively) in the normal and impaired process of wound healing.

Key words:

nitric oxide, wound healing, oxidative stress, nitroxidative stress, acute wounds, chronic wounds, antioxidants, oxidative damage

Abbreviations: bFGF – basic fibroblast growth factor, cGMP – 3',5'-cyclic guanosine monophosphate, CSF-1 – colony stimulating factor-1, ECM – extracellular matrix, FGF – fibroblast growth factor, IGF1 – insulin growth factor-1, NF- κ B – nuclear factor- κ B, NONOate – diazeniumdiolates, PAF – platelet activation factor, PDGF – platelet derived growth factor, PMN – polymorphonuclear, PVA – poly vinyl alcohol, RNS – reactive nitrogen species, SNAP – S-nitroso-N-acetylpenicillamine, TGF – tissue growth factor, TNF- α – tissue necrosis factor α , Trx – thioredoxin, VEGF – vascular endothelial growth factor

Introduction

Damage of a tissue triggers a cascade of repair events, which begin with the formation of a fibrin clot. The

clot, formed as a result of leakage of blood, provides protection to the underlying tissues, serves as a provisional matrix through which cells can move and also acts as a reservoir for growth factors and cytokines [11]. The growth factors initiate the inflammation, epithelization, wound contraction and angiogenesis process [32]. Platelet derived growth factor (PDGF) and tissue growth factor (TGF) released from platelets can act as chemoattractants for neutrophils and monocytes/macrophages. The major role of neutrophils is to kill the invading microorganisms by their characteristic respiratory burst activity and also to activate keratinocytes and fibroblasts [25]. The monocytes in the inflamed tissues can mature into macrophages and are responsible for phagocytosis of dying neutrophils,

damaged tissue and microorganisms. Macrophages also plays an important role in the long term repair response by releasing a battery of cytokines and growth factors (FGF, PDGF, TGF and IGF1) to amplify the inflammatory response and also to initiate the proliferative phase of wound healing [11]. A reepithelization of wounds is marked by the migration of keratinocytes across the fibrin clot and at the same time proliferation of keratinocytes at the wound edge. Angiogenesis leads to rebuilding of the damaged vessels, restoration of the blood flow and restoration of the oxygen supply to the tissue. During the process of angiogenesis, proteases are released from the activated endothelial cells. The proteases are involved in the degradation of endothelial cells' basement membrane. This process allows migration of endothelial cells and their differentiation into mature capillary blood vessels [76]. About 7 days after wounding the clot is completely replaced by fibroblasts that synthesize and remodel a new collagen rich matrix. At the same time some proportion of fibroblasts transform into myofibroblasts which resemble smooth muscle and cause wound contraction [11].

Redox homeostasis

Molecular oxygen can be reduced in biological milieu in one, two or four electron transfer to produce superoxide anion (O_2^-), peroxide anion (HO_2^-) and finally to hydroxyl ion (HO^-) respectively. Both superoxide and peroxide are strong oxidants and can contribute to oxidative damage in cells [9]. There is a delicate balance in biological systems between an amount of oxidant and antioxidants to prevent oxidative damage to the cells. The ability of cells to maintain homeostasis by preventing accumulation of excess oxidants is termed as redox homeostasis. Oxidants play an important role in wound healing [59] providing signaling and defence against microorganisms. However the oxidants have to be detoxified in order to prevent damage to host cells. An antioxidant defense system involves reduction (scavenging) and/or dismutation of O_2^- and/or HO_2^- and their protonated forms. When the antioxidant defense system fails to eliminate the oxidants, the alteration in homeostasis leads to oxidative stress.

Reactive oxygen species and oxidative stress

Daily, humans use about 250 g of oxygen out of which 2–5% is converted to reactive oxygen species (ROS). ROS means all oxygen associated species that have higher oxidative potential (higher reactivity) than molecular oxygen: singlet oxygen, superoxide anion, hydrogen peroxide and hydroxyl radical (OH^\bullet) [16]. In the ground state, molecular oxygen is in a relatively inert triplet state, ${}_3O_2$ (Fig. 1). The initial event that activates oxygen in biological systems is a change of electron spin pairing. This change results from one of at least three different chemical mechanisms.

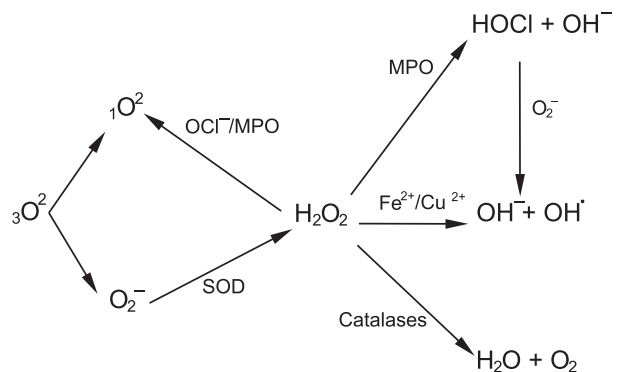


Fig. 1. Reduction of molecular oxygen (${}_3O_2$) and generation of oxidative stress. (MPO – myeloperoxidase)

The first mechanism involves an elevation of one electron to a higher energy level and production of unpaired electrons with antiparallel spins. By this mechanism, organic endoperoxides and UV or near-UV radiation in combination with photosensitizing chemicals produce singlet oxygen, (${}_1O_2$). The second mechanism involves one molecule of oxygen to be reduced by one electron. Transition metals and organic electron donors reduce ${}_3O_2$ by this mechanism and produce superoxide (O_2^-) and metal-oxygen complexes such as perferryl and related species. The third mechanism involves abstraction of one electron (or hydrogen) from an organic compound. In this manner, carbon radicals resulting from hydrogen abstraction by hydroxyl radicals react with ${}_3O_2$ and produce peroxyl radicals.

Tab. 1. Main components of oxidative and nitroxidative stress

oxidative stress		nitroxidative stress	
singlet oxygen	$^1O^2$	peroxynitrite anion	$ONOO^-$
superoxide	O_2^-	peroxynitrous acid	$ONOOH$
hydroperoxyl radical	O_2H^\bullet	nitronium ion	NO_2^+
hydrogen peroxide	H_2O_2	nitrogen dioxide radical	NO_2^\bullet
hydroxyl radical	OH^\bullet	nitryl chloride	NO_2Cl
hypochlorous acid	$HOCl$	nitrite ion	NO_2^-
trioxocarbonate radical	$CO_3^{\bullet-}$		

Superoxide is the main component of ROS. Superoxide can rapidly dismutate to H_2O_2 and O_2 by constitutive and inducible superoxide dismutases (SOD). H_2O_2 can also be detoxified by catalases to H_2O and O_2 . Alternatively, in the presence of transition metals (e.g. ferrous or cuprous ions), H_2O_2 is reduced to OH^- and to a highly reactive hydroxyl radical (OH^\bullet) [13]. O_2^- , H_2O_2 and OH^\bullet are components of the oxidative stress (Tab. 1). During the process of wound healing, various inflammatory cells like neutrophils, macrophages (phagocytes), endothelial cells and fibroblasts produce superoxide. Activated neutrophils and macrophages produce large amounts of superoxide and its derivatives *via* the phagocytic isoform of NADPH oxidases. Thrombin, PDGF and tissue necrosis factor α (TNF- α) stimulate release of superoxide from endothelial cells whereas interleukin (IL-1), TNF- α and platelet activation factor (PAF) stimulate superoxide release from fibroblasts [17]. Endothelial cells can also produce high concentrations of O_2^- , H_2O_2 and OH^\bullet under ischemic conditions in wounds. The main source of O_2^- in endothelial cells are NAD(P)H oxidase and endothelial nitric oxide synthase (eNOS) [36].

Reactive nitrogen species and nitroxidative stress

The main components of nitroxidative stress are listed in Table 1. NO and peroxynitrite ($ONOO^-$) are major reactive nitrogen species in biological systems [35]. NO can be produced by two constitutive nitric oxide

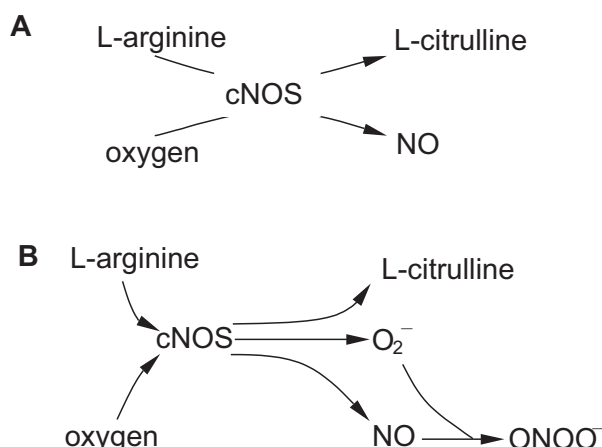


Fig. 2. Production of NO or NO and O_2^- by coupled (A) and uncoupled (B) constitutive nitric oxide synthase

synthase (cNOS) isoforms: endothelial NOS (eNOS), neuronal NOS (nNOS) and one inducible NOS isoform (iNOS). There are many different cells in a wound that have the capability to produce NO (Fig. 2). These include platelets (nNOS and iNOS), macrophages (iNOS), fibroblasts (eNOS and iNOS), endothelial cells (eNOS) and keratinocytes (eNOS and iNOS) [57]. Nitroxidative stress is generated by rapid oxidation of NO [9, 26, 27] (Fig. 3). NO can react with several oxidative molecules like molecular oxygen, ROS, transition metals and thiols to yield various reactive nitrogen species (RNS): nitrosyl-metal com-

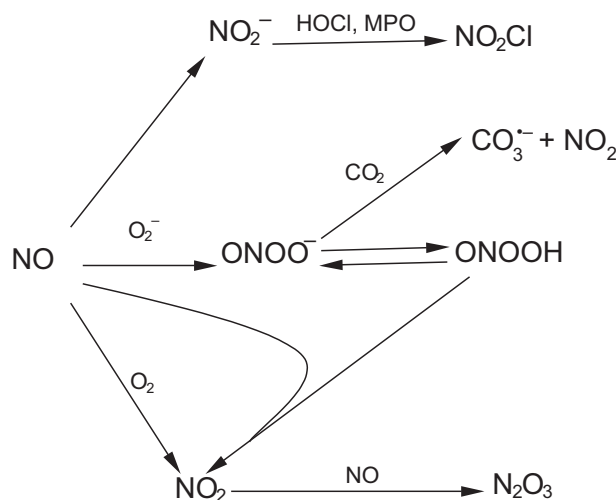


Fig. 3. General pathway of NO oxidation and generation of nitroxidative stress in biological milieu

plexes, S-nitrosothiols, N_2O_3 , NO_2^- and $ONOO^-$. Also highly reactive nitryl chloride results from oxidation of nitrate by myeloperoxidase-dependent pathways [39]. $ONOO^-$ can be produced in an extremely rapid, diffusion controlled ($k = 9.6 \times 10^{10} \text{ mol}^{-1} \text{ s}^{-1}$) reaction between NO and superoxide. The main source of both NO and O_2^- can be cNOS [9, 35, 40]. A coupled cNOS produces mainly NO from L-arginine and oxygen. However the uncoupled cNOS dimers due to shortage of L-arginine, tetrahydrobiopterin and/or oxygen can generate during short interval times both NO and O_2^- . Therefore uncoupled cNOS can be a powerful generator of peroxynitrite [40].

At low concentrations $ONOO^-$ can isomerize to the harmless nitrate ion (Fig. 4). However when generated at high concentration ($> 30\text{--}50 \text{ nmol/l}$) peroxynitrite can diffuse and during the diffusion process, can undergo symmetrical or non-symmetrical cleavage to form NO_2^\bullet and OH^\bullet or NO_2^+ and OH^- respectively.

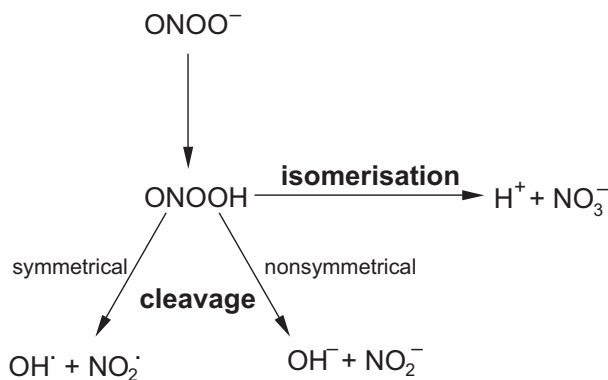


Fig. 4. An isomerisation or cleavage of protonated peroxynitrite (peroxynitrous acid) and generation of components of nitroxidative stress

Three of these species are powerful oxidants and contribute to increase in oxidative (OH^\bullet) and nitroxidative (NO_2^\bullet , NO_2^+) stress [26]. In addition, peroxynitrite can also react with carbonate ion to form nitroso-peroxocarbonates. The putative intermediate, 1-carboxylato-2-nitrosodioxidane, fragments into the free radicals trioxocarbonate ($CO_3^{\bullet-}$) and nitrogen dioxide (NO_2^\bullet). Both radicals ($CO_3^{\bullet-}$ and NO_2^\bullet) are considered to also convey the damaging potential of peroxynitrite *in vivo* due to oxygenation, nitrosation, and nitration of biomolecules [38].

Reduction of oxidative and nitroxidative stress

Antioxidants are important to maintain low levels of free radicals and reactive nonradical species derived from radicals. These include the enzymes SOD, glutathione peroxidase, and catalase and also nonenzymatic compounds such as vitamin E, β -carotene, ascorbate, and glutathione. Trace minerals such as zinc and selenium are also used as antioxidants. Besides, there are compounds that have lower antioxidative capacity but since they are present in high concentrations they can significantly contribute to the overall ROS scavenging capacity. Examples of low efficiency antioxidants include free amino acids, peptides and proteins [17]. These antioxidants may have a role in quenching the oxidants as there is high level of protease activity in early phases of wound healing [8]. The most effective antioxidant is NO, which is a scavenger of superoxide. However, the process of scavenging superoxide by NO decreases the oxidative stress while increasing the nitroxidative stress.

ROS and RNS generated during the inflammatory phase are also involved in the process of reestablishing the original redox state of the cells. Nitric oxide has a negative feedback on NO generation by inactivating the cytochrome P450 subunit of cNOS [2]. This process is important in preventing cNOS uncoupling and O_2^- generation. Also, high levels of NO released by iNOS show anti-inflammatory effects as NO switches off nuclear factor- κ B (NF- κ B), an important transcriptional activator of inflammatory proteins [12]. Elevated ROS can also induce expression of genes in cells whose products show antioxidative capacity. Lymphocytes, recruited into the inflammatory environment, also activate powerful protective mechanisms against oxidative stress. An increase in the expression of the oxidoreductase thioredoxin (Trx) was activated by lymphocytes and keratinocytes under conditions of oxidative stress [55]. Trx maintains reducing intracellular redox state. Macrophages when exposed to ROS express heme oxygenase (HO) and cystine transporter in order to protect themselves from oxidative damage [28]. The presence of HO in the skin protects against the heme-mediated prooxidative and proinflammatory microenvironment that is instantly created on injury, and might play a role in down-regulation of inflammatory cell recruitment and resolution of inflammation [69]. As the

levels of cystine are lower in plasma, cystine transporter plays an important role in cellular supply of cystine and in the biosynthesis of glutathione in macrophages and lymphocytes [17].

Oxidative and nitroxidative stress in wounds

In acute wounds a temporary increase in the level of oxidants has been observed. Antioxidant defence mechanisms are based on gradual detoxification of oxidants and on a gradual return of cells to the state of redox homeostasis. However, in chronic wounds the detoxification process is hindered due to persistent and uncontrolled production of ROS and RNS during the inflammatory phase. Antioxidative response though elevated in chronic wounds is not able to quench oxidants because of reduction in antioxidative activity caused by excess oxidants [17]. As a result homeostasis is shifted due to increase in oxidant concentrations to oxidant levels much higher than normal. Chronic wounds thus become stagnant in the inflammatory phase and show no spontaneous tendency to produce granulation tissue [16]. Clinically, chronic wounds show no signs of healing even after local basic treatment for 8 weeks. In the case of chronic wounds, upregulated expression of iNOS and oxidants will result in sustained production of components of oxidative and nitroxidative stress. In other words, the indirect effects of NO will prevail in chronic wounds due to reaction of NO with other oxi-

dants [71]. Increased NOS activity and nitrite levels have been shown to be responsible for diabetic foot and chronic venous ulcers [1, 33]. Processes and targets involving oxidative and nitroxidative stress in wound healing are listed in Table 2.

Positive effects of oxidative stress in wound healing

Coagulation

ROS generated by oxidative stress plays an important role in potentiating the clotting process as it is involved in the induction of tissue factor (TF)-mRNA. TF, released as a result of tissue injury, causes initiation of the extrinsic coagulation pathway and subsequent formation of thrombin. ROS released by thrombin induces TF-mRNA and also enhances TF dependent surface procoagulant activity to potentiate the thrombogenic (clotting) cycle in the damaged vessel [10, 11, 24]. ROS is also involved in increased platelet recruitment and collagen induced platelet activation [59]. Platelet activation and aggregation is essential for forming clots and also for the release of various growth factors and cytokines that kick start the healing process. Finally, activated platelets further potentiate clot formation by releasing ROS and RNS that upregulate TF expression [21].

Initiation and potentiation of inflammatory phase

Various growth factors released by platelets, fibroblasts and leucocytes, are responsible for recruitment and activation of neutrophils and monocytes to wounds, initiate wound reepithelization and angiogenesis. TGF released by fibroblasts and leucocytes induces these cells in an autocrine manner to generate additional cytokines like TNF- α , IL-1 β and PDGF that further potentiate the inflammatory response. PDGF activates the transcription factor NF- κ B and macrophage chemoattractant protein-1 to also stimulate the inflammatory response. H₂O₂ acts as a secondary messenger to growth factors like PDGF and TGF and thus H₂O₂ mediates the inflammatory response of these growth factors [34, 45]. Additionally, ROS and RNS can directly effect neutrophil chemotaxis as low-

Tab. 2. Processes and targets involving oxidative and nitroxidative stress in wound healing

Process	Target	Effect
Inhibition	Keratinocytes	Migration and proliferation
Induction	Fibroblasts	Senescence
Damage	DNA, lipids and proteins	DNA base modification, strand breakage, lipid peroxidation and protein oxidation
Inactivation	Protease inhibitors	Oxidation and inactivation of protease inhibitors
Decreased activity/depletion	Antioxidants	Decrease activity of enzymatic antioxidants, depletion of nonenzymatic antioxidants

ering of ROS/RNS by overexpression of antioxidants like thioredoxin suppressed LPS mediated leucocyte recruitment [50]. Macrophage inflammatory protein-1 α (MIP-1 α), a member of the CC subfamily of chemokines, has been shown to contribute to monocyte/macrophage and neutrophil chemotaxis and activation. Oxidative stress was responsible for upregulating the transcriptional and posttranslational stabilization of MIP-1 α [61]. Hydrogen peroxide also facilitates the adhesion of neutrophils and monocytes to the extracellular matrix and endothelial cells by modulating the expression of leucocyte adhesion molecule and leucocyte endothelial adhesion molecules [19]. Adhesion also induces the expression of monocyte colony stimulating factor-1 (CSF-1) that supports monocyte and macrophage survival at the wound site [59]. Thus ROS and RNS have a significant role in potentiating the inflammatory phase.

Reepithelization

ROS also helps in the reepithelization of wounds by activating collagenase expression and mediate EGF signaling. H₂O₂ activates AP-1 that further induces collagenase (MMP-1) expression. Collagenase helps in the degradation of extracellular matrix that further helps in the migration of wound related cells [70]. One-two days after injury keratinocytes began to proliferate in order to support their migration across the wounds. H₂O₂ is also responsible for mediating signaling of epidermal growth factor (EGF) that promotes proliferation of keratinocytes [53].

Angiogenesis and matrix deposition

A significant angiogenesis and matrix deposition is observed about 4 days after injury. Angiogenesis involves the formation of new capillaries that give the tissue a granular appearance. FGF-2 and vascular endothelial growth factor (VEGF) released at the wound site promote angiogenesis. ROS enhances the affinity of FGF-2 to its receptor and also induces its expression. Sen et al. have found that micromolar concentrations of H₂O₂ induce VEGF expression in keratinocytes and during cutaneous wound healing [59].

A provisional matrix, formed by structural molecules produced by fibroblasts, provides a support for the formation of granulation tissue. This provisional matrix is further replaced by cross-linked collagen matrix synthesized by activated fibroblasts. H₂O₂ in-

duces collagen I, III, IV formation and their subsequent cross linking. As fibroblasts synthesize and remodel a collagen rich matrix, a portion of these fibroblasts transform into myofibroblasts that helps in wound contraction. ROS also mediate conversion of fibroblasts to myofibroblasts thus aiding in wound contraction. Wound contraction helps in faster reepithelization by bringing the edges of the wounds closer [59].

Negative impact of oxidative stress and nitroxidative stress in wound healing

Recent evidence implicates the direct involvement of ROS/RNS in chronic wounds. An increase in the allantoin uric acid percentage ratio (AUR), a biomarker for increased oxidative/nitroxidative stress has been reported in chronic wounds [30]. Isoprostanes are prostaglandin-like compounds generated by the free radical induced oxidation of unsaturated fatty acids in membrane phospholipids. They are also an important indicator of oxidative stress *in vivo*. Levels of 8-isoprostane were found to be higher in chronic venous ulcers than in acute wound fluid suggesting a greater oxidative activity in chronic wounds [75]. Increased iNOS activity is present in both chronic venous ulcers and diabetic foot ulcers [1, 33]. There is excess iron deposition in the skin of patients with venous ulceration that increases the chances of free radical production by Fenton reaction [3].

Various damaging effects of ROS/RNS can be seen in chronic wounds. An overproduction of ROS/RNS results in inactivation of epidermal enzymatic antioxidants, despite increased enzymatic antioxidant expression in the wound and significantly depletes nonenzymatic antioxidant levels in wound tissues. This results in sustained elevation and survival of ROS/RNS in chronic wounds [31]. Sustained oxidative and nitroxidative stress prolongs the inflammation in chronic wounds as both ROS and RNS stimulate neutrophil and macrophage chemotaxis and migration and also induce the expression of adhesion molecules in the capillaries. Direct cellular effects of ROS/RNS include impaired migratory, proliferative and extracellular matrix (ECM) synthetic properties of dermal fibroblasts and keratinocytes [48].

Nitroxidative stress also increased matrix degradation and apoptosis in ulcers. iNOS expression and arginase is increased in chronic venous and diabetic ulcers. Arginase is responsible for increased matrix deposition however due to high nitroxidative stress and subsequent proteolytic activity there is defective matrix deposition in ulcers. High levels of NO produced by iNOS interacts with oxygen free radicals derived from polymorphonuclear (PMN) and macrophages to produce peroxynitrite. Peroxynitrite induces apoptosis/necrosis depending on its concentration in the ulcer site [1].

Balanced protease activity plays an important role in wound healing. PMN migrate from blood to the wound where they release proteases. Leucocyte released proteases then degrade provisional matrix and remodel ECM components. Additionally, they also have important role in vasoconstriction, increasing membrane permeability, promoting coagulation, leucocyte adhesion, chemotaxis and migration, bacterial killing and removal of tissue debris and also modulate the inflammatory response and activity of growth factors. In acute wounds protease inhibitors regulate the activity of proteases. Thus, there is a delicate balance between the activity of proteases and antiproteases in a healing wound. An excess of oxidants in chronic wounds create an imbalance between the level of proteases and antiproteases by increasing protease/inhibiting antiprotease activity. An overexpression of syndecans in mice delays healing due to a subsequent increase in proteolytic activity in wounds [18]. Hydrogen peroxide increases expression of syndecan in rat ventricular myocytes. Thus oxidative stress can be implicated to increase protease activity by increasing syndecan expression [37]. NO₂ and ONOO⁻, but not NO, activate the latent form of human neutrophil procollagenase (protease) at micromolar concentrations [52]. Continuous influx of neutrophils generates ROS/RNS that disturbs the protease-antiprotease balance by decreasing the levels of protease inhibitors. Chloramines and HOCl oxidize both α₂ macroglobulin and α₁ antiprotease thus reducing the levels of inhibitors [8, 73]. Resulting excess protease activity causes more than required degradation of ECM components of the skin e.g. collagen, proteoglycans and hyaluronan resulting in delayed healing [68].

Fibroblasts are important in wound repair as they synthesize and remodel extracellular matrix molecules such as collagen and also produce mitogens for keratinocytes, endothelial cells and fibroblasts. In the

chronic wounds the fibroblasts are larger with abnormal morphology similar to senescent fibroblasts. One study showed that when neonatal fibroblasts were incubated with venous ulcer wound fluid they reduced the proliferative capacity of neonatal fibroblasts and induced senescence like morphological characteristics. This suggests that the venous ulcer microenvironment affects fibroblast function, at least in part, by inducing fibroblast senescence. Oxidative stress has been linked to cause stress induced premature senescence in human diploid fibroblasts. This suggests that high oxidative stress in chronic wounds might be a causative factor for inducing senescence in wound fibroblasts. Senescent fibroblasts are unable to replicate but remain metabolically active with altered cell functions; are less motile; can accumulate in tissue due to their resistance to apoptosis; and produce a different array of proteins, including elevated levels of matrix metalloproteases and pro-inflammatory cytokines – all of which affect tissue integrity and normal healing [4, 47, 67]. Oxidative stress also impairs fibroblast contraction when grown in hyperglycemic media [15].

High oxidative stress can inhibit the migration and proliferation of keratinocytes, especially hydrogen peroxide when given in micromolar concentrations which has been shown to inhibit these processes [51]. Oxidative stress is proposed as an important pathogenic factor in diabetic wound complications. Oxidative stress induces apoptosis in keratinocytes when these cells were cultured in hyperglycemic media [15].

Positive effects of nitric oxide in wound healing

L-arginine, a semiessential amino acid during normal physiological processes, was found out to be essential during the process of wound healing [58] and its beneficial effects were attributed to it being a secretagogue for various growth factors. L-arginine can be metabolized in the wounds by two sets of enzymes that were expressed during different stages of wound healing. During initial stages of wound healing L-arginine was catalysed by oxidative L-arginine deiminase (OAD)/NOS to citrulline and reactive nitrogen species in early phases of wound healing and by arginase to ornithine in late phases of wound healing. These findings implicated the role of NO in wound

healing [6, 7]. However the effect of arginine may also be due to subsequent increase in production of the amino acid L-ornithine, a precursor of L-proline during collagen synthesis. A definitive role of NO in wound healing has still not been established. One study even showed that NO inhibits collagen synthesis in wounds [62]. On the other hand several studies have implicated that NO might play a vital role in all the phases of wound healing [57]. All three NOS isoforms are present in the skin and expressed during wound healing. nNOS is expressed in keratinocytes and melanocytes, whereas eNOS is present in keratinocytes of basal epidermal layer, dermal fibroblasts, endothelial cells and eccrine glands. iNOS is induced in keratinocytes, fibroblasts, Langerhans and endothelial cells [43].

Inflammation

NO has a vital role in the inflammatory process as it acts as a vasodilator, antimicrobial, prevents platelet aggregation and induces vascular permeability. NO is responsible for both the upregulation and downregulation of the inflammatory phase of wound healing. NO acts as proinflammatory by acting as a chemoattractant for various cytokines like IL-1, TGF- β 1, monocytes and neutrophils. High levels of NO may also be anti-inflammatory during the later phase of inflammation. NO was shown to suppress monocyte attracting cytokines RANTES (regulated upon activation, normal T-cell expressed and secreted) and macrophage chemoattractant protein (MCP-1) [12, 57].

Angiogenesis

Angiogenesis is the process of formation of new blood vessels and NO is important for enhancing angiogenesis. NO promotes angiogenesis by activating factors such as VEGF, bFGF and TGF- β . These growth factors then induce endothelial cell migration, adhesion and proliferation. VEGF in turn also induces eNOS expression [76].

Reepithelization

iNOS derived NO is important for the proliferation of keratinocytes as treatment with iNOS inhibitor delays reepithelization with atrophied hyperproliferative epithelium at the wound edge [64]. IL-1 is a potent modulator of keratinocyte proliferation, recruitment

and differentiation. Since NO acts as a chemoattractant of IL-1, it is indirectly responsible for reepithelization. NO also protects the keratinocytes from apoptosis as addition of NOS antagonists to irradiated keratinocytes increases apoptosis, an effect that is reversed by adding NO donor S-nitroso-N-acetylpenicillamine (SNAP) [57].

Matrix deposition and remodeling

Most *in vivo* studies show a relationship between increased NO levels (NO donors, L-arginine and iNOS overexpression *via* gene therapy) and increased collagen deposition in wounds. Also *in vitro* studies prove that NO increases collagen production in both wound derived fibroblasts and normal skin derived fibroblasts. NO also helps in the activation of fibroblasts by converting latent TGF- β 1 to an active form [50].

Antioxidants in the therapy of wound healing

In acute as well as in chronic wounds, though the expression of enzymatic antioxidants increases, their activity decreases due to high oxidative stress. Also high oxidative stress leads to depletion of nonenzymatic antioxidants [31, 63, 65]. This effect is more pronounced in chronic wounds than in acute wounds. Thus, supplementation of wounds with antioxidants should help to prevent oxidative damage of cells and enhance healing.

Skin ischemia provides a favorable environment for the generation of oxidative stress by activated leukocytes. Raxofelast, a hydrophilic vitamin E analog administered to diabetic wounds, was able to reduce oxidative stress by reducing lipid peroxidation and edema. Raxofelast subsequently stimulated reepithelization, neovascularization, proliferation of fibroblasts and synthesis and maturation of extracellular matrix [20]. Similarly taurine, as a chitosan gel formulation, has been used as an antioxidant to enhance reepithelization, tensile strength and collagen production in wounds. As a direct acting antioxidant, taurine significantly reduced lipid peroxidation and as an indirect antioxidant, it stabilized plasma membrane [14].

It has been suggested that supplementation of diabetic wounds with antioxidants can protect the diabetic wound cells from oxidative stress generated by high glucose levels. When fibroblasts were grown under high glucose medium they showed reduced contraction ability and resistance to growth factor induced proliferation whereas keratinocytes were more susceptible to apoptosis. Addition of glutathione restored the ability of fibroblasts to contract and protected the keratinocytes from apoptosis. Also addition of ascorbic acid, selenite, vitamin E, carotenoids and Q10 reversed the high glucose induced growth factor resistance in human fibroblasts [15, 23].

Several other antioxidants such as ascorbic acid, catalase, combination of antioxidants and trace minerals have also been used to improve healing. Ascorbic acid has a role in both the formation and maintenance of collagen in healing wounds of man and guinea pigs, as well as in the prevention of hemorrhage from the vascular components of connective tissue. Ascorbic acid can also be used to ameliorate radiation induced delay in wound healing [29]. Catalase detoxifies hydrogen peroxide which can otherwise inflict severe damage to regenerating cells. Topical application of catalase improved healing of dental pulp tissue after direct pulp capping [5]. A combination of antioxidants comprising vitamin E, sodium pyruvate and fatty acids have been employed for enhancing healing in normal, laser resurfacing and immunocompromised wounds. While sodium pyruvate and vitamin E act as antioxidants, unsaturated fatty acids act as a replacement source for damaged membrane fatty acids [44]. Zinc also acts as an antioxidant in the skin and has beneficial effects in wound healing. Its antioxidant effects have been proposed to be associated with replacement of redox reactive metals such as iron and copper at cellular and extracellular sites thus preventing generation of hydroxyl radical and also to induce metallothionein synthesis that acts as a free radical scavenger [54].

Nitric oxide in the therapy of wound healing

There is much evidence to support that NO plays a major role in wound healing. Administration of NOS inhibitors results in delayed reepithelization and

collagen formation [56, 64]. Also iNOS/eNOS KO mice show delayed healing of excisional wounds [41, 74]. Moreover impaired wound healing in diabetics has been associated with reduced NO synthesis. Thus high level of bioavailable NO in wounds is important for enhancing the healing process. Various studies have been done focusing on increasing NO levels in wounds either by delivering NO donors, NOS substrate arginine and NO complexes. L-arginine, NOS substrate has been known to be an essential amino acid in wound healing. Animals treated with L-arginine show increased wound breaking strength and collagen deposition as compared to the control [58].

In situ NO generating systems and NO complexes have been developed to deliver NO to wounds. A chemical system utilizing sodium nitrite and ascorbate has been developed to generate and supply NO to skin. In order to prevent damage to the skin because of the acidic nature of the mixture a selectively permeable, hydrophilic, polyester co-polymer membrane was placed between the mixture and the skin. This system was effective in producing vasodilatation in the human forearm skin and had antimicrobial properties. This system can be used to treat skin ulceration and chronic wound infection [22]. NO has been immobilized to various polymers to release NO in a controlled fashion. Polyethyleneimine cellulose diazeniumdiolates (NONOate) polymer, a nonsoluble, non-toxic, polymer-based NO donor (one of a new class of compounds that spontaneously release NO in a controlled fashion in aqueous media) were developed. Polyethyleneimine cellulose NONOate polymer was applied topically on dermal wounds of rats. Polymer treated wounds had increased percent wound closure at days 7, 10 and 17 as compared to control wounds [60]. Hydrogel dressing made by crosslinking poly vinyl alcohol (PVA) with NO donor have also been used as a carrier for NO release to wounds. These hydrogel dressings when applied to fibroblasts *in vitro* increase the production of extracellular matrix from fibroblasts and when applied to wounds, show increased granulation tissue and scar tissue thickness [46]. Another NO delivery complex developed is the NO-naproxen complex, a NO releasing derivative of naproxen. This complex when applied to incision wounds showed 62% increase in collagen deposition [49].

NO donors have been used to treat infectious and diabetic wounds. SNAP has been applied in the form of cream to skin wounds infected with leishmaniasis. Treatment with the cream healed the infected ulcer by

day 30 whereas there was no improvement in the wound treated with vehicle alone [42].

NO donor molsidomine (N-ethoxycarbonyl-3-morpholinyl-sidnonimine) was administered to incisional diabetic wounds. It was found out that NO donor improved collagen deposition and wound breaking strength both in control and diabetic wounds [72].

Sildenafil, a phosphodiesterase inhibitor, can be considered a new class of compound that prolongs the effect of NO by inhibiting hydrolysis of 3',5'-cyclic guanosine monophosphate (cGMP). Sildenafil citrate has been shown to enhance angiogenesis and vasodilatation in wounded dogs [66].

Acknowledgments:

This work was supported by GEMI grant from Linde Pharmaceuticals and Bionanotechnology Program at Ohio University. This investigation was conducted in a facility constructed with support from Research Facilities Improvement Program Grant COG-RR-14575-01 from the National Center for Research Resources, NIH.

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Received:
August 24, 2005.