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Beneficial and Deleterious Roles of Oxygen and Reactive Oxygen Species in Healing of Cutaneous Wounds

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ABSTRACT

Wound healing is a complex yet well-orchestrated physiological response initiated to re-establish the structural integrity of wounded tissue. This occurs through four distinct yet overlapping phases viz., haemostasis, inflammation, proliferation and remodelling. Oxygen is an essential nutrient required for meeting high energy demand of healing tissue. It is also responsible for production of reactive oxygen species – ROS, which not only regulate various sub-cellular and molecular events during wound healing but also curtail the growth of microorganisms at wound site. Along with theses beneficial roles, ROS on the other hand, cause deleterious effects by increasing oxidative stress at the wounded site and delay the process of healing, eventually leading to chronic wounds. This review presents the contrasting roles of oxygen and ROS in wound healing essential for understanding the physiology of healing tissue which in turn is essential for encountering pathophysiology of chronic wounds. The review also summarises the intricate mechanism employed by the tissue to overcome these deleterious effects created by reactive oxygen species.

INTRODUCTION

Skin being the largest and most widespread organ, plays a vital role as physical barrier, in protecting the internal environment of the body from harmful external factors such as pathogens, UV radiation and oxidative stress(1). Any injury to the structural integrity of the skin causes depletion of the front-line defence system. This paves the way for entry of pathogens into the internal environment, which makes the host more susceptible to microbial attack(2). Hence, an intricate set of complex physiological responses are immediately initiated, that eventually lead to re-establishment of continuum in the connective tissue of skin(3). These physiological responses, collective know as the process of wound healing comprises of precisely synchronised several cellular, sub-cellular and molecular events(4). The entire series of events can be broadly divided into four phases, viz., hemostasis phase, inflammatory phase, proliferative phase and remodelling phase(5).

Haemostatic phase, characterised by formation of insoluble fibrin clot and platelet plug, sets in immediately after the injury and gets accomplished within few minutes. The former is initiated by damaged skin (extrinsic system), wherein, various zymogens are converted into their active form by the action of proteases.

Table 1: Normal physiology of wound healing: an overview.

Phase	Duration	Important events
Haemostasis	Immediate	Formation of fibrin clot (thrombus) and platelet plug, Vasoconstriction.
Inflammation	1 day – 2 weeks	Leucocyte recruitment, neutrophil infiltration, secretion of pro-inflammatory cytokines, growth factors and reactive oxygen species (ROS).
Proliferation	2 days – 3 weeks	Fibroblast proliferation, collagen synthesis, angiogenesis and formation of granulation tissue.
Remodelling	3 weeks – 2 years	Re-epithelialisation, formation of myofibroblasts, collagen remodelling and scar tissue formation

Fibrinogen being the ultimate substrate is acted upon by thrombin to form fibrin. The latter is initiated by exposed collagen (intrinsic system), leading to activation of thrombocytes that eventually get aggregated. These two events, along with short spanned local vasoconstriction, collectively prevent excessive loss of blood from wound site (Table1) (6).

Within 24 hours of wound formation, haemostatic phase is followed by inflammatory phase which is characterised by recruitment of immune cells and inflammatory cells to the wound site that include leucocyte and neutrophils. Leucocytes along with platelets secrete cytokines and growth factors that perform various functions significant all along the later stages of healing cascade. (Table 2) (7). Neutrophils on the other hand secrete mediator molecules such as, tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6, which are not only responsible for amplification of inflammatory response but also stimulate Vascular endothelial growth factor (VEGF) and IL-8 that ensure sufficient repair (Table1) (8). Neutrophils along with other inflammatory cells, also produce large amounts of reactive oxygen species (ROS), which protect the body from microbial infection but, at the same time, in excess amounts cause oxidative damage to the surrounding tissue, thereby delaying the process of wound healing (Table1) (9). To keep a check on this, within a few days of wound formation neutrophils receive apoptotic signals and the levels of pro-inflammatory cytokines are reduced to ensure normal process of wound healing. At this time, keratinocytes, fibroblasts, and endothelial cells start to migrate towards wound site that later produce various growth factors. These events set apart the phase of proliferation from inflammatory phase spanning from day 3 to a few months. Crucial events of this phase include fibroblast proliferation, collagen synthesis, angiogenesis and formation of granulation tissue(10). These events are followed by remodelling-the final phase of wound healing characterised by development of new epithelial layer. This is achieved by differentiation of fibroblasts into myofibroblasts which decrease proliferation but increase collagen synthesis and deposition responsible for contraction of wound. This phase lasts up to one or two years or sometimes even more, wherein the temporal extra-cellular matrix (ECM) is converted into a permanent mature scar (Table1) (11).

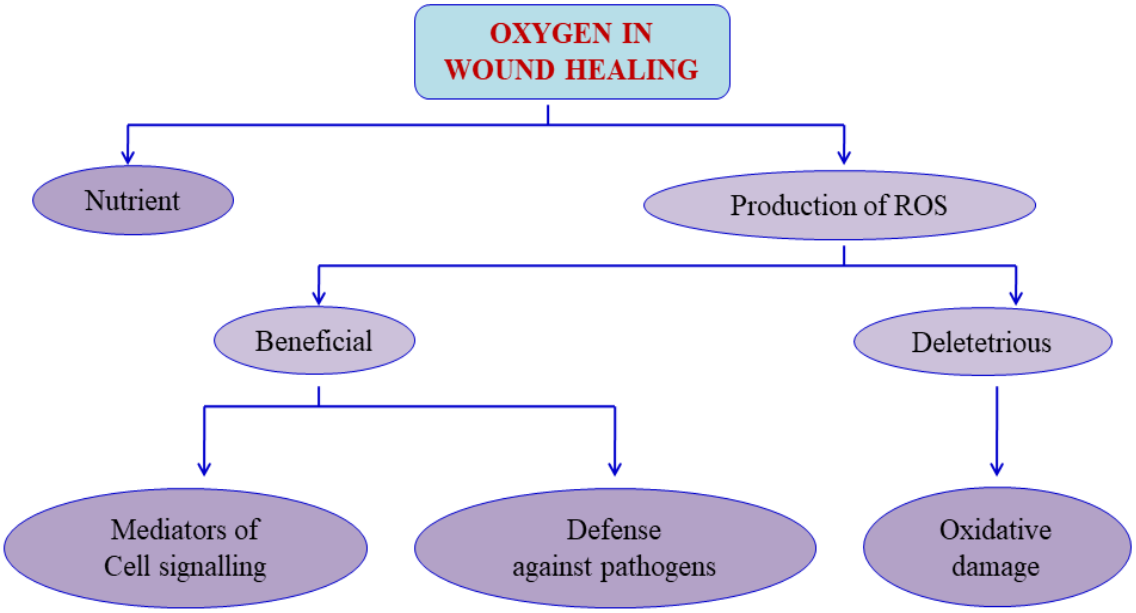

Fig. 1: Overview of roles of oxygen in wound healing emphasising beneficial and deleterious roles of ROS.

Functions of oxygen in wound healing:

Oxygen as nutrient

Various anabolic processes that takes place within cells are oxygen dependent. Transport of substances between cells and movement of several cell types, cell proliferation, synthesis of ECM are certain events that take place at elevated levels during wound repair. All these processes are powered by the co-enzyme adenosine-tri-phosphate (ATP) which is synthesized in mitochondria by oxidative phosphorylation. This primary process that fulfils energy needs of the cells is invariably oxygen dependent which virtually ceases in the absence of oxygen. Other catabolic processes such as aerobic glycolysis, β-oxidation of fatty acids, and the citric acid cycle associated with energy production are in turn linked to oxidative phosphorylation are oxygen dependent(12). However, the complete role oxygen in wound healing remains unclear till date.

During injury and successive process of healing, two additional enzymatic reactions set in which play significant role during inflammatory and remodelling phases respectively. First one being nicotinamide adenine dinucleotide phosphate(NADPH) -oxygenase catalysed reaction that uses enormous amount of oxygen to produce oxidants responsible to prevent

microbial growth(13). The second reaction is the one that converts pro-collagen to collagen which involves hydroxylation of proline and lysine residues. This is the most significant step in collagen synthesis without which stable triple helices cannot be formed(14). Evidence suggests that the process of wound healing is impaired during hypoxia(15).

Table 2: Pro-inflammatory cytokines and growth factors: Role in wound healing.

Cell type	Chemokine	Function
Thrombocytes and lymphocytes	1. IL-1α, IL-1β, IL-6 and TNF-α.	Activation of the inflammatory process.
	2. FGF-2, IGF-1, TGF-β.	Stimulate the collagen synthesis
	3. TGF-β	activate the transformation of fibroblasts to myofibroblasts
	4. FGF-2, VEGF-A, HIF-1α, TGF-β	initiate the angiogenesis.
	5. (EGF, FGF-2, IGF-1, TGF-α	Support re-epithelialization process.
Neutrophils	TNF-α, IL-1β and IL-6	amplify the inflammatory response and stimulate VEGF and IL-8 for an adequate repair response
Macrophages	TGF-β, TGF-α, basic FGF, PDGF and VEGF	which promote cell proliferation and the synthesis of ECM molecules by resident skin cells

Oxygen in production of reactive oxygen species (ROS):

During normal metabolism, generally, all cells of aerobic organisms constantly produce ROS and the production of same, shoots up during pathological conditions. During inflammatory phase, certain cell types, especially neutrophils and macrophages witness a phenomenon known as 'respiratory burst' wherein, high levels of NADPH oxidases are synthesised in their phagosomes following engulfment of invading pathogen. This multi-enzyme complex reduces the molecular oxygen (O_2), to highly reactive superoxide radical anion (O_2^-). This in turn produces hydrogen peroxide (H_2O_2) and water by the action of the enzyme superoxide dismutase (SOD). NADPH oxidases also reduce O_2 to H_2O_2, although not a free radicle as such, contributes in production of hydroxyl radicle in the presence of iron/copper ions (Fenton reaction) which are known to cause severe damage to the cells. These reactive free radicles along with H_2O_2 attack the invading pathogens and kill them. Several

micro/macromolecules mediated anti-oxidant response is initiated in response to increased ROS which thereby protects the cellular components from possible oxidative damages(16). Details of the same are discussed in separate section.

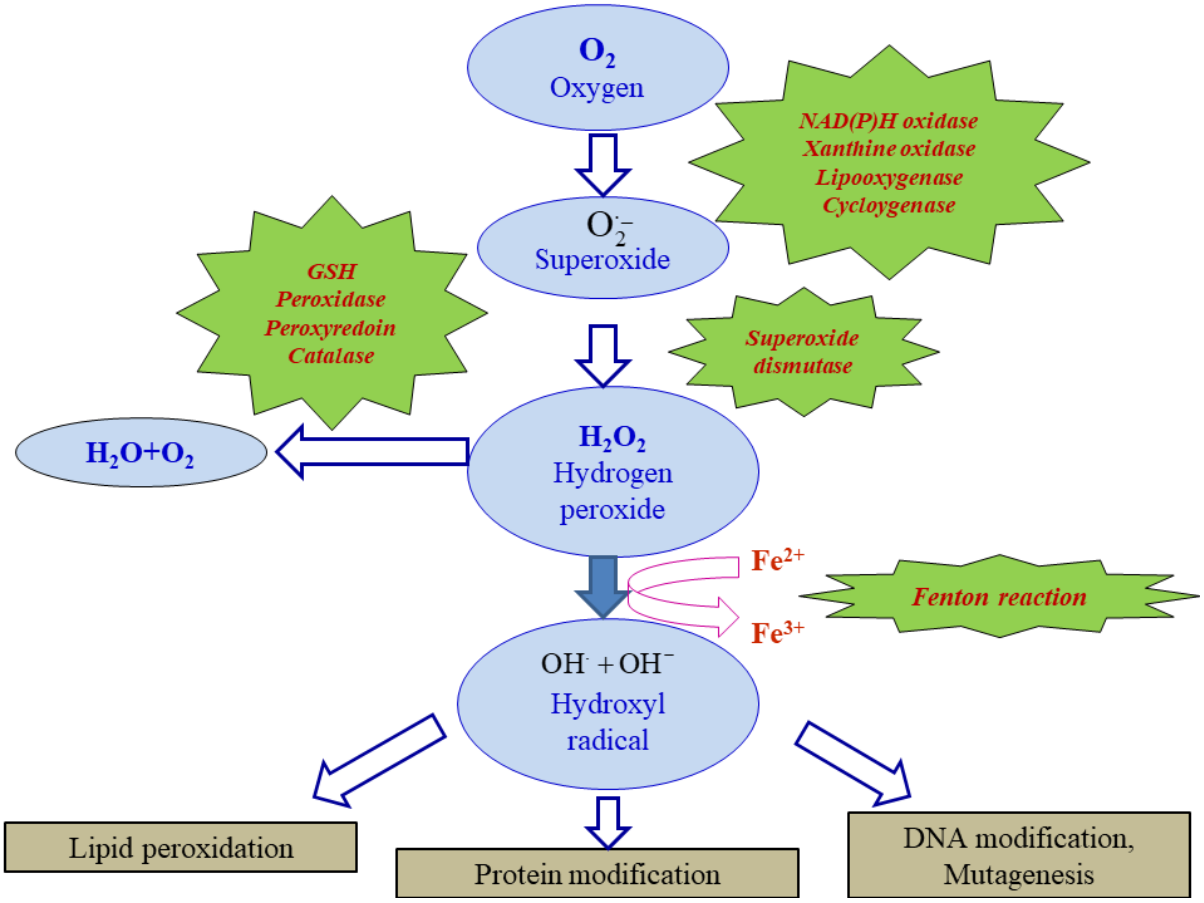

Fig. 2 Overview of production of ROS and their subsequent scavenging.

Beneficial roles of ROS

ROS as mediators cell signalling:

Evidence suggests that ROS produced in limited concentrations play paramount role in physiological cell signalling pathways, which are referred to as redox signalling. In addition to their roles in, several studies also report on the specific roles of ROS in cell signalling(17). During as early as coagulation phase, following vessel-wall injury, platelet recruitment occurs at wound site, coupled with sharp rise in ROS levels(18). Thrombin being the ultimate active enzyme of coagulation cascade not only mediates formation of fibrin clot, but also induces ROS generation through NADPH oxygenase (NOX) enzymes in vascular cells, which in turn trigger a thrombogenic cycle via upregulation of tissue factor

expression(18,19). Production ROS by platelets in NOX dependent manner is initiated when these cells come in contact with collagen, resulting in recruitment of more platelets (20). Platelets produce another important signalling mediator- platelet derived growth factor (PDGF), in an H_2O_2 dependent manner, which plays an important role in recruitment and proliferation of various cells in wound healing (21).

During inflammatory phase ROS though not directly, but through H_2O_2, induce nuclear factor-kappa B (NF-κB) signalling cascade which prevents infuriated deleterious inflammatory response, especially at lower concentration(21). In addition H_2O_2 in lower concentrations stimulates overexpression of thioredoxin, which in turn degrades ROS that suppress leukocyte recruitment to the wound site(22). Furthermore, ROS promote spreading of macrophages through extracellular kinase(23).

Studies show that H_2O_2 can induce, monocyte adhesion in vitro, responsible for their activation suggesting its role in monocyte activation, achieved through adherence to the ECM via specific integrin receptors(24). ROS also induce biosynthesis of cytokine, especially TNF-α and IL-6 that play several roles in wound that include migration of neutrophils and macrophages and facilitation of phagocytosis(25).

Angiogenesis is a complex process that requires several factors that include VEGF, fibroblast growth factors (FGFs), angiopoietin etc (26). Several studies report the role of H_2O_2 in VEGF expression in keratinocytes and macrophages in vitro(24,27).

Re-epithelialization an important event in wound healing is characterised by migration proliferation and differentiation of keratinocytes(28). In-vitro studies suggest role of ROS in keratinocyte migration at low concentration(29). Xanthine oxidoreductase has ability to produce both ROS and nitric oxide (NO) and has been reported to promote keratinocyte proliferation and angiogenesis in dermal wound repair of mice(30). Matrix metalloproteases (MMPs) are matrix degrading enzymes produced by keratinocytes are important in wound dissection. Among them, MMP-1 is produced by NOX mediated pathway in an ROS dependent manner(20). Inhibition of MMP-2 and MMP-9 are shown to exhibit keratinocyte migration(31).

ROS also modify fibrin in an oxidative manner and promote fibrinolysis(32). ROS and H_2O_2 upregulate production of epidermal growth factor (EGF) and keratinocyte growth factor

(KGF) respectively in keratinocytes that are responsible for their continuous proliferation (33,34).

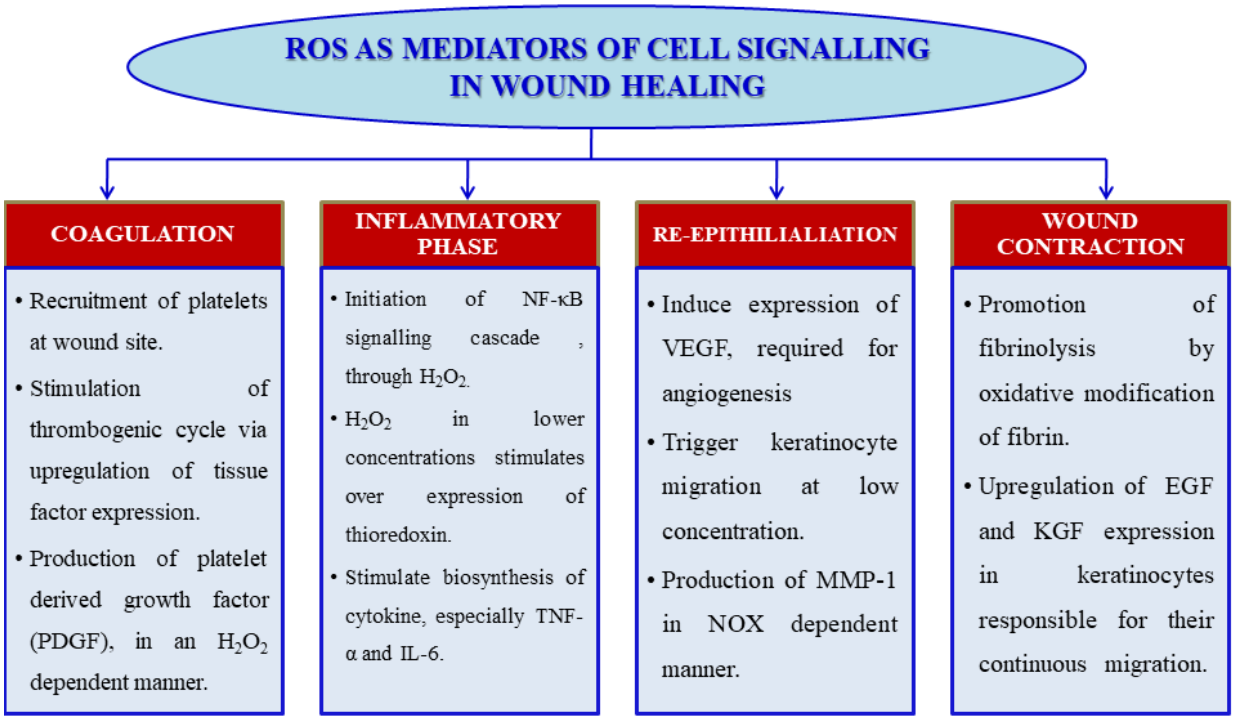

Fig. 3 Various roles exhibited as mediators of cell signalling pathways by ROS in different stages of wound healing.

ROS as germicidal agents:

Wound or an injury opens up the nutritional internal environment for vast variety of microorganisms harboured over skin and thus makes the underlying tissue more susceptible to infection. Microbial growth if not curtailed, not only causes severe damage for the wounded tissue but also delays the process of wound healing by transforming the same into chronic wound(35).

In order to achieve this task large amounts of highly reactive ROS are released (along with RNS- reactive nitrogen species), they destroy invading pathogens by irreversibly oxidizing their genetic material, although the actual mechanism remains to be clearly unveiled(36). Some studies reveal that ROS bring about oxidation of nitrogenous bases particularly guanine which proves to be mutagenic(37).

Besides superoxide and hydroxyl radicals, H_2O_2 possesses potent bacteriostatic potential at concentrations between 25 µM to 50 µM and bactericidal potential at concentrations >500 µM(38).

Deleterious roles of ROS

Along with the beneficial effects discussed thus far dysregulated overproduction of ROS leads to several harmful effects that include lipid peroxidation, oxidative damage to proteins, DNA mutations and activation of pro-apoptotic factors. Levels of ROS are maintained by means of several enzymatic and small molecular antioxidants. Imbalance between ROS production and detoxification leads to a condition known as oxidative stress resulting in molecular damage. Oxidative stress is implicated in various disease conditions including atherosclerosis, diabetes, carcinogenesis and tumour metastasis, neurodegeneration and ageing(39). Similar imbalanced condition created either by excessive secretion of ROS/ H_2O_2 or limited expression of antioxidant enzymes at healing tissue delays the process of healing and leads to pathological condition of chronic wound(40). Chronic condition of inflammatory phase is believed to be triggered by defective apoptosis of leukocytes and subsequent clearance of apoptotic cells by phagocytic macrophages(41).

Contrasting influences are seen to be executed by H_2O_2 on rates of wound healing depending on concentrations. Topical applications of H_2O_2 on murine excision wound healing model, 50 mM H_2O_2 enhanced wound healing and 980 mM H_2O_2 (3%) delayed healing(17).

Release of ROS is facilitated by NADPH dependent oxygenase in an oxygen dependent manner. The K_m value for the enzyme with oxygen as substrate ranges between a partial pressure (PO_2) of 40-80 mm Hg(42). Healthy wounds are characterised by PO_2 ranges of 30 – 50 mm Hg, while in chronic wounds PO_2 is reported to be in the range of 5 – 20 mm Hg(43).

Levels of oxygen also influence on bactericidal activities of neutrophils as the same depends on respiratory burst. At PO_2 levels less than 40 mm Hg, neutrophils are reported to lose their bactericidal potential in vitro(42). This bactericidal activity could be accredited to the diminished ROS production, contributing to prolonged prevalence of infection(44).

Defence against deleterious effects of ROS

In order to overcome the deleterious effects of ROS, several endogenous small molecules and enzymes, termed as antioxidants contribute significantly. Along with enzymatic and non-enzymatic antioxidants, the enzyme haem oxygenase (HO) plays indirectly to restrain ROS(45). Enzymatic antioxidants are advantageous over small molecular antioxidants as efficiency of later depends on quantity of dietary intake and conversion into active form. Stoichiometric interaction with ROS and subsequent conversion into inactive form is another limitation of small molecules(46).

Super Oxide Dismutase (SOD)

Primary superoxide anions produced from ROS are from molecular oxygen(47). SOD dismutates excessive ROS to avoid deleterious effects. Based on location three families of SOD have been distinguished, cytoplasmic SOD 1, mitochondrial transmembrane SOD 2 and extracellular matrix SOD 3. Role of SOD is reported to be crucial and useful in enhancing wound healing by several studies(48–50).

Peroxiredoxins (PRDX)

Peroxiredoxins bring about reduction of H_2O_2 in a thioredoxin dependent manner that acts as preferential electron donor(51). Based on cellular and tissue localisation, 6 families of PRDX have been identified(52). PRDX exerts positive effects in healing tissues by not on maintaining ROS levels but also regulate ROS signalling in finer manner, especially those involving activation of tissue kinase receptor(53).

Glutathione peroxidase (GPx)

The GPx family proteins not only catalyse the reduction of H_2O_2 but also some organic peroxidases which takes place in the presence of glutathione. In humans, eight distinct families of GPx gene transcripts are identified so far. GPx 1-4 are seleno-proteins. GPx 6 is another seleno-protein expressed exclusively in humans(54). Decreased glutathione levels in wound lesions are found to conceal GPx activity as the enzyme functions in glutathione dependent manner(46).

Catalase

Catalase catalyses the dismutation of H_2O_2 to molecular oxygen and water. The enzyme is explicitly localised in peroxisomes. During wound harling the levels of catalase mRNA, although remain unchanged but protein levels are found to be decreased(55). In mice adenovirus mediated overexpression of catalase is found to impair wound healing(17). This unusual observation can be correlated with high turnover number of the enzyme ($4x10^7$ s^{-1}) because of which ROS levels are reduced drastically not allowing them to exert positive roles in post inflammatory phases. During remodelling H_2O_2 induces expression of VEGF in keratinocytes(17,27). These observations suggest that a certain concentration of H_2O_2 is essential in wound healing.

Small molecular antioxidants

Several low molecular weight antioxidants are reported to play significant role in wound healing. Some of them are reported to play significant role in wound healing. Some of them are endogenous molecules such as glutathione, ubiquinone, uric acid and lipoic acid and certain other components of foods such as vitamin E and C, carotenoids and phenolic compounds(56). The following evidences suggest regarding their potent role in enhancing wound healing. Wounded skin of immunosuppressed rats was found to be associated with highly decreased levels of glutathione, vitamin E and ascorbic acid, compared to their immune potent counterparts(57). Healing wounds of diabetic mice were found to contain extremely lower levels of glutathione compared to non-diabetic mice(58). In wounded tissue of diabetic rats supplemented with vit E, the levels of lipid peroxides were found to be drastically reduced and healing process was found to be enhanced(59). Topical application of curcumin- a potent polyphenol, to full thickness excision wounds in rats is found to improve cellular proliferation and collagen synthesis at wound sites, with significantly faster healing. Treated mice also exhibited reduced levels lipid peroxides and enhanced expression of antioxidant enzymes(60).

CONCLUSION

Wound healing being a complex physiological phenomenon relies highly upon oxygen. It is essential as nutrient to fulfil energy demands by acting as ultimate electron acceptor in aerobic pathway. Oxygen also serves as substrate for production of ROS. These exhibit dual role in concentration dependent manner. ROS are involved in several cell signalling pathways

spanned over all stages of wound healing. ROS also provide protection against invading pathogens. I addition to these beneficial roles, ROS induce oxidative stress when not regulated in proper manner. To bring down the deleterious roles of ROS, several enzymatic and non-enzymatic antioxidants play vital role. Many pathways discussed in the review shed light on possible targets for therapeutic applications and pharmacological intervention to fight against pathophysiology of chronic wound.

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