The Influence of Iron and Free Radicals on Chronic Leg Ulceration

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Summary

It has been well established that ambulatory venous hypertension and accompanying chronic inflammation cause a major alteration in tissue and ultimately skin ulceration. In spite of considerable advances made in venous ulcer research, the underlying pathophysiology of chronic venous leg ulceration and the failure to heal is not completely understood. Currently there is considerable clinical and scientific research into the role of inflammatory and tissue cells and their mediators in wound repair. Few studies have addressed the possible contribution of free radical production to venous ulceration and little has been done to examine the role of iron in initiating and/or perpetuating tissue damage in venous ulceration. Interest in the role of iron in venous ulceration has come with increasing evidence implicating free radical generation by iron in other chronic inflammatory and iron–overload diseases. Conditions such as chronic inflammation, tissue ischaemia, activation of neutrophils and high levels of localised iron in the tissues are conducive to the generation of free radicals in venous disease. It is possible that iron may participate in tissue damage in a similar manner in which it does in other inflammatory disorders. However, this remains to be elucidated.

Introduction

Wound healing in the skin is a complex biological process. The ability of a wound to heal involves an interaction between the cellular, molecular, biochemical and micro–environmental factors at both local and systemic levels. The complexities of wound restoration means the stages of healing from the initial blood clot through to inflammation, cellular proliferation, angiogenesis and lymphatic formation and reconstitution of the extracellular matrix must be regulated in a highly organised and controlled manner to ensure optimal repair [1]. When the regulation of repair is disrupted at any one of these stages, healing can be impaired, thus resulting in chronic non–healing wounds.

By definition, chronic wounds are wounds resulting from repeated trauma, occurring in poorly perfused tissue or wounds contaminated by bacteria and which, as a consequence, do not heal within the expected period of time.

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An example of a chronic wound with an underlying healing defect is the chronic venous leg ulcer. This is a significant problem because it impacts severely on the quality of the individual's life causing discomfort, inconvenience, restricting mobility and, in some cases, may lead to loss of employment and feelings of social isolation [2, 3]. Since the condition is characterised by chronicity and ulcer recurrences, prolonged outpatient treatment or lengthy hospitalisation present a substantial cost to the health care system. There is a need for greater understanding of the mechanisms of tissue damage and impairment of healing in order to be able to better prevent and treat venous ulceration.

Cause of venous ulceration

It is generally agreed that the cause of venous ulceration is impairment of the calf muscle pump, due to damaged venous valves in the deep, perforating or superficial veins resulting in venous insufficiency. This is followed by sequential dermatological changes over time resulting in skin ulceration [4].

The underlying physiological problems causing venous disease have been well characterised, but little is known about the cellular and biochemical abnormalities that result in skin ulceration. Considerable advances have been made in venous ulcer research, emphasising that the impairment in healing of

these ulcers may be attributed to the elevated and sustained levels of inflammatory cytokines and proteases in the wounds [5].

A less well–studied area in the field of venous ulceration is the influence of iron and free radicals on wound repair. This review will address the issue of reactive oxygen species (ROS) and the influence of iron in tissue damage during inflammation, with particular emphasis on wound healing and tissue damage in chronic venous leg ulceration.

Free radicals

Free radical research is receiving more attention in recent times; there is increasingly more evidence to support the involvement of free radical reactions in the pathogenesis of a vast number of pathological disorders/diseases affecting various systems of the human body [6, 7]. Diseases known to be linked to excessive ROS production include those that are degenerative, ischaemic, cardiovascular, inflammatory, immunological, gastro–intestinal, chemically induced, respiratory and some skin conditions [7]. It has been perceived that there is hardly a pathological process in which free radicals are not involved. Whether free radical reactions are a major cause of tissue injury or an accompaniment to such injury is not fully understood due to the limitations of techniques that are used to detect and measure them, both *in vivo* and in the clinical situation.

The term 'radical' is derivative from the Latin word *radix*, meaning root [8]. A free radical can be viewed as fragments of molecules or atoms, which contain one or more unpaired electrons. They can have cationic, anionic or neutral characteristics, are extremely reactive and will combine with any other entity to stabilise the unpaired electron. This results in the formation of a paired electron system together with the generation of a fresh radical [8, 9]. In trying to gain stability by snatching electrons from neighbouring entities, they initiate a chain of oxidation reaction, which moves from molecule to molecule, cell to cell, organelle to organelle, causing damage along the way [10].

Free radicals are difficult to identify, detect, quantitate and study because of their reactivity, having an average rate constant of 10^5–$10^{10}M^{-1}sec^{-1}$, with the hydroxyl radical being the most reactive with a rate constant of 10^9–$10^{10}M^{-1}sec^{-1}$ [8, 11].

Not only are they extremely reactive, free radicals are also short lived. They are produced *in vivo* and react at or close to their source of formation. Consequently, free radical activity is measured by indirect techniques such as measuring end products resulting from free radical reactions with cellular components such as lipids, proteins and DNA [12]. Many of these products of free radical reactions possess biological activity and are themselves reactive and able to cause further damage.

Free radicals can be ROS and reactive nitrogen species (RNS) and both are important in the pathogenesis of human diseases. However, for this review more emphasis will be on the ROS. ROS include superoxide ($O_2^{\bullet}$), hydroxyl radical ($OH^{\bullet}$), peroxyl ($RO_2^{\bullet}$), alkoxyl ($RO^{\bullet}$), hydroperoxyl ($HO_2^{\bullet}$) and derivatives of oxygen which do not contain unpaired electrons, such as hydrogen peroxide (H_2O_2), singlet $O_2(^1\Delta g)$, hypochlorous acid (HOCl), and ozone (O_3) [11, 13]. The formation of $O_2^{\bullet}$ and H_2O_2 can occur either deliberately or accidentally and can originate from endogenous (produced during cellular metabolism) or exogenous (external) sources. Oxygen metabolism, the fundamental process for aerobic life, can cause the formation of partially reduced oxygen species when molecular oxygen is being reduced to water: $O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$.

The cytochrome P450 and oxidase–catalyse reaction involves the transfer of four electrons to oxygen producing partially reduced oxygen species. This accidental 'leakage' of electrons from the electron transport chains in mitochondria and endoplasmic reticulum onto oxygen can produce ROS. Prostaglandin synthesis, enzymic activity (xanthine oxidase, NADPH oxidase) and flavin enzymes all have the ability to generate reduced oxygen species. Auto-oxidation reactions involving catecholamines, ascorbic acid and reduced flavins are all alleged to react with oxygen to form $O_2^{\bullet}$ [10, 14]. Although these oxygen utilising processes are tightly coupled to avoid partial reduction of oxygen, $O_2^{\bullet}$ generation is unavoidable in the biological system. A one electron reduction (addition of an electron) of oxygen produces $O_2^{\bullet}$ and two electron reduction produces H_2O_2. Further addition of electrons by cleaving the bond between oxygen atoms will produce the highly reactive $OH^{\bullet}$ in a reaction that is catalysed by iron (Fenton reaction), which will be discussed in detail later. Free radicals can therefore be produced in a biological system where the transfer of one electron can lead to a cascade of reactions that can form multiple ROS [15].

Endogenous free radical production

Endogenous or deliberate generation of $O_2^{\bullet}$, H_2O_2 and HOCl by polymorphonuclear leukocytes (PMN), monocytes, macrophages, mast cells or basophils and eosinophils occurs during phagocytosis and killing of bacteria and fungi. Such cells utilise an NADPH oxidase enzymic system to generate $O_2^{\bullet}$ and direct it against invading micro–organisms [16].

Cells other than phagocytes that are known to produce $O_2^{\bullet}$ include lymphocytes, fibroblasts and vascular endothelial cells [11]. Deliberate generation of ROS as a normal protective mechanism against foreign organisms is essential. However, in chronic inflammation, this process may be damaging because excessive phagocytic activation causes an over production of free radicals and the surrounding tissues are directly exposed to high levels of ROS and their metabolites [11, 13, 16].

Exogenous ROS

Exogenous or environmental sources of ROS include electromagnetic radiation (radon and cosmic) and man–made sources (pesticides, air pollutants and tobacco smoke). Low wavelength electromagnetic radiation such as gamma rays can split water in the body to produce $OH^{\bullet}$ [17]. Toxic chemicals (paraquat) and drugs (adriamycin and bleomycin) are also known sources of ROS.

Antioxidant defences

Oxygen free radicals are capable of damaging all biological compounds such as nucleic acids, protein, free amino acids, lipids, lipoproteins, carbohydrates and connective tissue macromolecules [9]. These may impact on cellular activities, affecting membrane function, metabolism and gene expression [6]. It is now well established that there is a continuous production of free radicals *in vivo* [18]. In response to this, aerobic cells have evolved elaborate defence mechanisms to detoxify these radicals and these are known as antioxidants.

Antioxidant defences fall into two categories, those whose role is to prevent the generation of free radicals and those that intercept them as they are generated [19]. These exist in both the aqueous and membrane compartment of cells and can be enzymatic (superoxide dismutase, glutathione peroxidase, catalase) or non–enzymatic (vitamin E, C, uric acid, bilirubin, albumin).

In spite of the huge armoury of antioxidants *in vivo*, some free radicals still escape and cause damage. If the rate of free radical formation outweighs the scavenging capacity of the antioxidants, oxidative stress will occur. Tissues can respond by making extra antioxidant defences but if they fail, cell injury and death may occur; this can proceed to necrosis or apoptosis [17]. Both $O2^{\bullet}$ and H_2O_2 can be detoxified by appropriate enzymes. For example, excess $O_2^{\bullet}$ can be removed by superoxide dismutase and H_2O_2 can be removed by glutathione peroxidase and catalase [20].

Iron and free radical reactions

Although $O_2^{\bullet}$ and H_2O_2 are reactive on their own and have a number of biologic targets, their toxicity lies in their ability to react with one another to produce $OH^{\bullet}$. Several mechanisms have been proposed to explain their formation *in vivo* such as interaction of $O_2^{\bullet}$ with nitric oxide ($NO^{\bullet}$) and the involvement of transition metal ions in catalysing the reaction between $O_2^{\bullet}$and H_2O_2.

Several transition metals (able to transit between two different states on the basis of electron transfer) such as iron, copper and zinc can react with H_2O_2 to form $OH^{\bullet}$. Amongst these, iron has been shown to be the most likely precursor of $OH^{\bullet}$ *in vivo*, through the Fenton or iron–catalysed Haber–Weiss reaction. In this reaction, $O_2^{\bullet}$ dismutates to form H_2O_2 and also acts as a reducing agent for iron, reducing iron from the ferric to the ferrous state which subsequently becomes oxidised when reacting with H_2O_2 to generate $OH^{\bullet}$ [21].

$Fe^{3+} + O2^{\bullet} \rightarrow Fe^{2+}$

$Fe^{2+} + H_2O_2 \rightarrow OH^{\bullet} + Fe^{3+} + OH^{-}$ [20].

Other than promoting the generation of $OH^{\bullet}$, iron facilitates the decomposition of lipid peroxides to produce products such as cytotoxic aldehydes and hydrocarbon gases [6]. Iron is also involved in the generation of $O_2^{\bullet}$ during autoxidation [22].

$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet}$.

The availability of iron to catalyse $OH^{\bullet}$ formation during inflammation has been proposed in the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis, haemochromatosis, psoriasis and ischaemic tissue injury to list just a few [23]. Iron must therefore be tightly regulated and sequestered to prevent harmful generation of toxic radical.

Iron is the most abundant transition metal in the human body and is an essential element for cellular metabolism, growth and differentiation [24, 25]. However, in iron overload situations the normal regulation of iron acquisition and storage may be disrupted, resulting in the formation of iron–catalysed toxic oxidant species, especially $OH^{\bullet}$ [26, 27].

As a protective measure against the toxicity of free iron in the biological system, iron within the body is located in heme–containing proteins and iron–binding proteins (transferrin, lactoferrin, ferritin and haemosiderin) and is unreactive in this form unless reduced and mobilised, rendering it available for redox reactions [28–30]. Transferrin has a high affinity for iron while ferritin has a high capacity of iron storage. Ferritin is regarded as the main storage form of iron, whereas haemosiderin is considered to be the storage of pathological iron overload. In general, tissues which have a high haemosiderin content contain large amounts of ferritin [31].

The mobilisation of iron from its iron–binding protein is not completely understood under physiological conditions. However, there is evidence suggesting that mobilisation of iron from ferritin can occur in the presence of xanthine oxidase and $O_2^{\bullet}$ [23, 32]. Under acidic conditions (pH 4.5) iron can be spontaneously released from haemosiderin indicating that pH is an important factor involved in the release of iron. In an inflammatory situation, hypoxia and low pH are common features and iron release from storage may induce or accelerate tissue damage [33, 34].

Iron, ROS and chronic venous leg ulceration

With increasing evidence implicating the involvement of iron in toxic free radical formation during inflammation, the role of iron in free radical damage in venous ulcers should be examined.

The concept of free radical involvement in the pathogenesis of venous ulceration has been receiving more attention in recent times. A number of theories put forward on the underlying physiological problem causing chronic venous disease have suggested the involvement of ROS in tissue damage. It has been proposed that venous hypertension results in the accumulation of white cells in vessels that have reduced venous flow and may cause capillary occlusion [35, 36]. These leucocytes are sequestered and activated which results in the generation of free radicals, proteases, histamine, neutrophil chemoattractants and complement [37–40]. The generation of these substances can damage the endothelium and its basement membrane, increasing vascular permeability [41, 42]. Studies have shown that patients with venous hypertension exhibit an increase in free radical production in leg neutrophils which is signalled via an amplified calcium–dependent pathway [43].

More recently, a study has demonstrated that more leucocytes become 'trapped' in the limbs of patients with chronic venous disease and that more activated neutrophils and monocytes are sequestered during venous hypertension [44]. The 'trapped' neutrophils occurred within dependent limbs and, on elevation, reperfusion of ischaemic tissue may promote the release of free radicals. Glutathione peroxidase levels were also found to be reduced during dependency and this may reduce protection against free radical damage in the limb [45]. Activated leucocytes can interact with platelets, erythrocytes and components of the coagulation–fibrinolysis pathway giving rise to thrombosis [46, 47].

The deposition of fibrin cuffs around capillaries has been proposed to impede the diffusion of oxygen and nutrients into the tissues thereby causing local ischaemia and subsequently leading to ulceration [48]. Ischaemia–reperfusion mechanisms are another source of free radicals, by producing $O_2^{\bullet}$ via the xanthine oxidase/dehydrogenase enzymatic pathways [49].

Ischaemia–reperfusion has been described within the limbs of patients with chronic venous disease [50]. Others have shown relative ischemia and slow reperfusion in limbs with venous leg ulcers and with venous effluent containing high concentrations of mediators that are associated with reperfusion injury. However, the authors have suggested that the induction of free radical production by macrophages and neutrophils was by cytokine–mediated pathways or by infection rather than by reperfusion [51]. Whether the severity of ischaemia–reperfusion observed in venous disease is sufficient to cause excessive production of free radicals and resultant tissue damage requires further investigation.

Iron in venous ulcers

Increased vascular permeability resulting from endothelial cell

damage may cause the leakage of serous fluid containing water, large protein molecules and red blood cells into the tissues [52]. The presence of erythrocytes in the tissues may have a significant impact on tissue damage since haemoglobin is a potential source of iron for catalysing free radical reactions. Haemoglobin is known to stimulate lipid peroxidation and generate $OH^{\bullet}$. The iron from haemoglobin can be released by H_2O_2 and lipid peroxides and can participate in haemoglobin-stimulated free radical reactions [53, 54].

The possibility of iron involvement in venous ulceration was suggested as early as 1965 when Meyer noted that "stasis" pigmentation, which is a common feature surrounding venous ulcers, results from the deposition of haemosiderin in the skin, which is assumed to derive from disintegrated red blood cells [55] (Figure 1). Ackerman *et al.* reported that the brown pigmentation is caused by increased dermal iron deposition and may have an active role in perpetuating tissue damage in venous ulceration by augmenting local inflammation, collagen deposition and fibrosis [56]. Histological examination of tissue biopsies from venous ulcers have shown decreased haemosiderin levels as ulcers heal [4].

The mobilisation of iron from its iron–binding protein is not completely understood under physiological conditions. However, there is increasing evidence to suggest that mobilisation of iron from ferritin can occur in the presence of xanthine oxidase and superoxide ($O_2^{\bullet}$) [23, 32, 57]. Nitric oxide also has the capability to mobilise iron from ferritin, thereby disrupting intracellular iron homeostasis and increasing the level of ROS [58]. With increasing evidence supporting an over production of $O_2^{\bullet}$ and H_2O_2 by phagocytes at the site of chronic inflammation, the iron released from ferritin and haemosiderin may play an active role in promoting $OH^{\bullet}$ reactions.

The effect of free radicals on tissues

The increased $OH^{\bullet}$ from the catalytic action of iron formation during inflammation exacerbates tissue damage through peroxidation of membrane phospholipids and denaturation of macromolecules, proteins and DNA [8, 35] (Figure 2). The availability of iron during inflammation has been extensively demonstrated and implicated in the pathogenesis of numerous chronic inflammatory disorders [23] and it is extremely likely that the existence of an iron pool observed in the tissue of leg ulcers may also participate in the process of tissue damage (Figure 3). This damage may involve direct damage by lipid peroxidation processes involving the cell membrane which contain oxidisable unsaturated fatty acids (phospholipids, glycolipids, glycerides and sterols) and transmembrane proteins and amino acids [59, 60]. Membranes affected by peroxidation can become rigid and can lose selective permeability, hence affecting ion gradients, secretory functions, metabolic functions and cellular integrity.

An increase in cytosolic calcium (Ca^{2+}) can activate a number of enzymes such as phopholipases and proteases which can break down membrane and cytoskeletal proteins [61]. Lipid peroxidation also releases arachidonic acid (AA) which is the substrate for the synthesis of eicosanoids. The oxidised lipid bilayers are a substrate for phospholipase enzymes (phospholipase A_2) which are responsible for releasing AA metabolites [62]. The eicosanoid mediators have many physiological and chemical

Figure 1. Picture of pigmentation around a venous leg ulcer

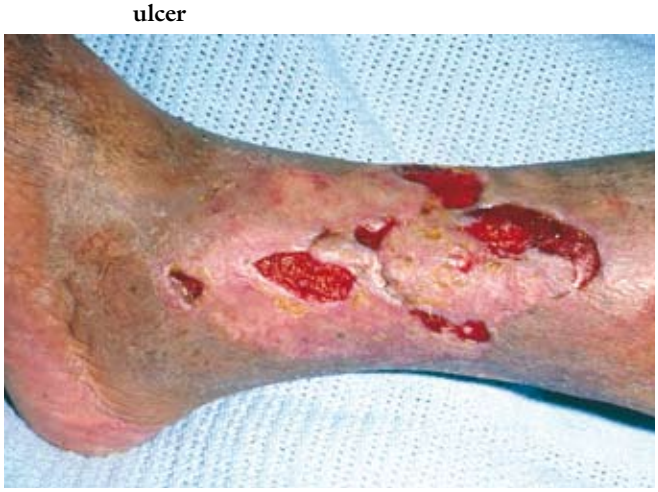


Figure 2. Immunohistochemistry staining for ferritin in leg ulcer biopsy

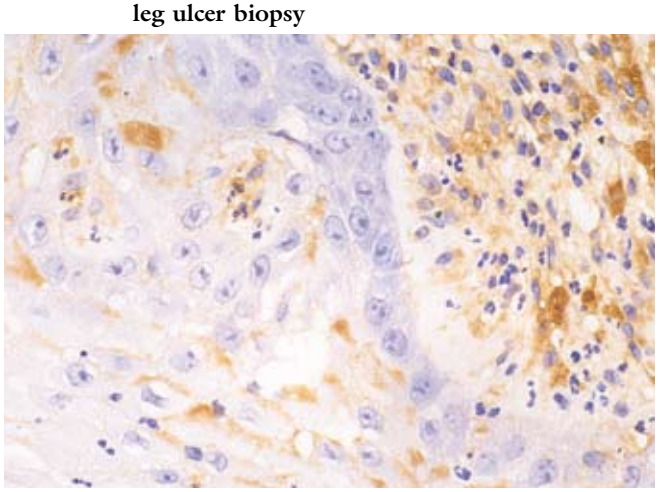
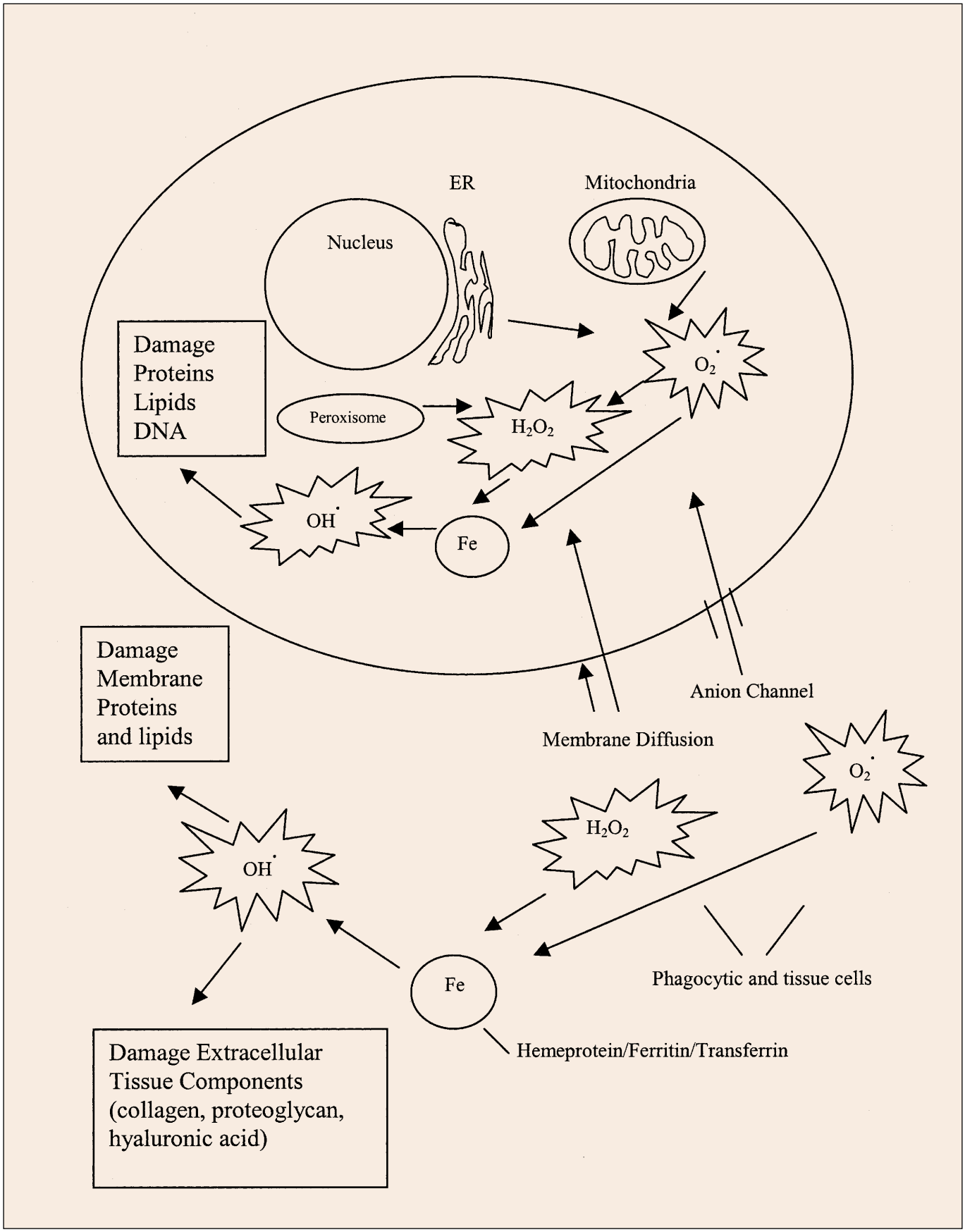


Figure 3. Intracellular and extracellular damage by reactive oxygen species.



roles in inflammation and tissue destruction.

The inactivation of antiproteases and the activation of metalloproteinases by reactive free radicals can lead to increased destruction of extracellular matrix. Extracellular tissue components such as collagen, proteoglycans and hyaluronic acid are therefore at risk of inflammatory cell mediated free radical damage as seen in inflammatory osteoarthritis [63]. *In vitro* studies have demonstrated that $O_2^{\bullet}$ prevents collagen gelation; since collagen is a major macromolecular constituent of connective tissue, alteration in its biochemistry can affect the structural integrity of the tissue [64].

Iron–generated ROS can exert a secondary effect by the activation of several genes. An increase in intracellular ROS (H_2O_2) has been shown to induce interstitial collagenase, matrix–metalloproteinase^{-1} (MMP–1) mRNA and protein, tissue inhibitor of MMPs (TIMP–1), and IL–1α and β mRNA steady–state levels in human dermal fibroblasts [65]. The iron driven Fenton reaction and lipid peroxidation may play an important role in signal transduction leading to increased MMP–1 and stromelysin–1 (MMP–3) mRNA in cultured cells [65]. Appropriate regulation of MMPs and their inhibitors are very important during the wound healing processes, since elevated and persistent levels of MMPs can lead to extensive tissue destruction and impaired wound healing.

Reactive oxygen intermediates and lipid peroxidation products are also known to activate transcription factor NF-κB which in turn can activate the expression of genes involved in inflammatory, immune and acute phase responses [66, 67]. *In vitro* studies have demonstrated the activation of NF-κB by H_2O_2 and it has been suggested that the local concentration of H_2O_2 in extracellular fluids during inflammatory processes are sufficient to activate NF-κB in tissue and blood cells [68].

Ischaemia/reperfusion is another mechanism involved in the activation of the transcription factor NF-κB [69]. A recent study has demonstrated the generation of intracellular H_2O_2 and $OH^{\bullet}$ in human endothelial cells following ischaemia and reperfusion, which may have initiated NF-κB activation [70]. NF-κB activation in wounded human keratinocytes has recently been demonstrated in cell culture system [71]. Activation of NF-κB therefore plays an important role in wound healing by regulating cytokine expression (TNFα, IL–6 and IFNβ), by stimulating keratinocyte cell migration, by stimulating the synthesis of matrix repair related proteins [71] and by stimulating the expression of urokinase type plasminogen activator, which is essential for keratinocyte migration, matrix infiltration and wound surface re–epithelialisation [72–74].

Conclusion

The exact role of iron–mediated ROS in wound healing needs further investigation but it is likely that the continued generation of free radicals will maintain the chronicity of the disease process. Persistent inflammation, repeated trauma, and inappropriate activation of neutrophils and macrophages will ultimately lead to tissue destruction. Microbleeding in venous disease resulting in an increased iron deposition and mobilisation in tissue may have additional deleterious consequences.

There is a greater need to understand the role of iron in initiating and/or exacerbating tissue damage during the pathogenesis of chronic venous leg ulceration to enable the development of therapeutic measures that will improve wound healing.

References

1. Levenson SM, Demetriou AA. Metabolic Factors. In: Cohen IK, Diegelmann RF, Lindblad WJ, editors. Wound Healing: Biochemical and Clinical Aspects. 1st ed. Philadelphia: W.B. Saunders Company, 1992; 40–61.

2. Callam M, Harper D, Dale J, Ruckley C. Chronic leg ulceration: socio–economic aspects. Scott Med J 1988; **33**:358–360.

3. Ruckley CV. Socio-economic impact of chronic venous insufficiency and leg ulcers. Angiology The Journal of Vascular Diseases 1997; **48**(1):67–69.

4. Herrick SE, Sloan P, McGurk M, Freak CN, Ferguson MWJ. Sequential changes in histologic pattern and extracellular matrix deposition during the healing of chronic venous ulcers. Am J Pathol 1992; **141**(5):1085–1095.

5. Trengove NJ, Stacey MC, Macauley S, Bennett N *et al.* Analysis of the acute and chronic wound environments: the role of proteases and their inhibitors. Wound Rep and Reg 1999; **7**:442–452.

6. Cross CE, Halliwell B, Borish ET, Pryor WA, Ames BN, Saul RL *et al.* Oxygen radicals and human disease [clinical conference]. Ann Intern Med 1987; **107**(4):526–45.

7. Ansari KU. Free radicals, their relation to disease and pharmacologic interventions. Indian Pract 1991; **XLVI**(4):261–272.

8. Lunec J. Free radicals: their involvement in disease processes. Ann Clin Biochem 1990; **27**:173–182.

9. Dean R, Simpson J. Free radicals: the good, the bad. Today's Life Science 1989; September:28–34.

10. Ansari KN. The free radicals – the hidden culprits – an update. Indian J Med Sci 1997; **51**(9):319–336.

11. Halliwell B, Cross CE. Oxygen–derived species: the relation to human disease and environmental stress. Environ Health Perspect 1994; **102** (Suppl 102): 5–12.

12. Holley AE, Cheeseman KH. Measuring free radical reactions *in vivo*. Br Med Bull 1993; **49**(3):494–505.

13. Halliwell B. Oxidative stress, nutrition and health. Experimental strategies for optimization of nutritional antioxidant intake in humans. Free Radic Res 1996; **25**(1):57–74.

14. Fridovich I. Superoxide dismutases. An adaptation to a paramagnetic gas. J Biol Chem 1989; **264**:7761–7764.

15. Halliwell B, Gutteridge JMC, Cross CE. Free radicals, antioxidants, and human disease: where are we now? J Lab Clin Med 1992; **119**(6):598–620.

16. Babior BM. Activation of the respiratory burst oxidase. Environ Health Perspect 1994; **102** (Suppl):53–56.

17. Halliwell B. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? Lancet 1994; **344**:721–724.

18. Halliwell B. The role of oxygen radicals in human disease, with particular reference to the vascular system. Haemostasis 1993; **23** (Suppl 1):118–126.

19. Cotgreave I, Moldeus P, Orrenius S. Host biochemical defense mechanism against prooxidants. Ann Rheum Pharm Toxicol 1988; **28**:189–212.

20. McCord JM. Human disease, free radicals, and the oxidant/antioxidant balance. Clin Biochem 1993; **26**(5):351–357.

21. Trenam CW, Blake DR, Morris CJ. Skin inflammation: reactive oxygen species and the role of iron. J Invest Dermatol 1992; **99**(6):675–682.

22. Cheeseman KH, Slater TF. An introduction to free radical biochemistry. Br Med Bull 1993; **49**(3):481–493.23. Biemond P, Swaak AJG, Van Eijk HG, Koster JF. Superoxide dependent iron release from ferritin in inflammatory diseases. Free Radic Biol Med 1988; **4**:185–198.

24. Okada S. Iron–induced tissue damage and cancer: The role of reactive oxygen species–free radicals. Pathol Int 1996; **46**:311–332.

25. Fontecave M, Pierre JL. Iron: metabolism, toxicity and therapy. Biochimie 1993; **75**(9):767–73.

26. Olakanmi O, Stokes JB, Britigan BE. Acquisition of iron bound to low molecular weight chelates by human monocyte–derived macrophages. J Immunol 1994; **153**:2691–2703.

27. Lesnefsky EJ. Tissue iron overload and mechanisms of iron–catalysed oxidative injury. In: Armstrong D, editor. Free Radic Diag Med. New York, 1994; pp.129–146.

28. Ward RJ, O'Connell MJ, Dickson DPE, Reid NMK, Wade VJ, Mann S *et al.* Biochemical studies of the iron cores and polypeptide shells of haemosiderin isolated from patients with primary or secondary haemochromatosis. Biochim Biophys Acta 1989; **993**:131–133.

29. Ward PA, Warren JS, Till GO, Varani J, Johnson KJ. Modification of disease by preventing free radical formation: a new concept in pharmacological intervention. Baillieres Clin Haematol 1989; **2**(2):391–402.

30. McCord JM. Effects of positive iron status at a cellular level. Nutr Rev 1996; **54**(3):85–88.

31. Worwood M. Ferritin. Blood Rev 1990; **4**:259–269.

32. Bolann BJ, Ulvik RJ. Release of iron from ferritin by xanthine oxidase. Biochem J 1987; **243**:55–59.

33. O'Connell MJ, Ward RJ, Baum H, Peters TJ. The role of iron in ferritin and haemosiderin mediated lipid peroxidation in liposomes. Biochem J 1985; **229**:135–139.

34. Ozaki M, Kawabata T, Awai M. Iron release from haemosiderin and production of iron–catalysed hydroxyl radicals in vitro. Biochem J 1988; **250**:589–595.

35. Bradbury AW, Murie JA, Ruckley CV. Role of the leucocyte in the pathogenesis of vascular disease. Br J Surg 1993; **80**:1503–1512.

36. Thomas P, Nash G, Dormandy D. White cell accumulation in dependent legs of patients with venous hypertension: a possible mechanism for trophic changes in the skin. Br Med J 1988; **296**:1693–1695.

37. Belch JF, Chopra M, Hutchinson S *et al.* Free radical pathology in chronic arterial disease. Free Radic Biol Med 1989; **6**:375–378.

38. Gill DS, Barradas MA, Fonseca VA, Gracey L, Dandona P. Increased histamine content in leukocytes and platelets with peripheral vascular disease. Am J Clin Pathol 1988; **89**:622–626.

39. Cambria RA, Anderson RJ, Dikdan G, Lysz TW, Hobson RW. The influence of arachidonic acid metabolites on leucocyte activation and skeletal muscle injury after ischaemia and reperfusion. J Vasc Surg 1991; **14**:549–556.

40. Bengston A, Holmberg P, Heidman M. The ischaemic leg as a source of complement. Br J Surg 1987; **14**:697–700.

41. Smedley LA, Tonneson MG, Sandhaus RA *et al.* Neutrophil mediated injury to endothelial cells: enhancement by endotoxin and the essential role of neutrophil elastase. J Clin Invest 1986; 77:1233–1243.

42. Weiss SJ. Tissue destruction by neutrophils. N Engl J Med 1989; **320**:365–376.

43. Whiston RJ, Hallett MB, Davies EV, Harding KG, Lane IF. Inappropriate neutrophil activation in venous disease. Br J Surg 1994;81:695–698.44. Smith PD. Neutrophil activation and mediators of inflammation in chronic venous insufficiency. J Vasc Res 1999; **36** (Suppl 1):24–36.

45. Edwards AT, Herrick SE, Suarez–Mendez VJ, McCollum CN. Oxidants, antioxidants and venous ulceration. Br J Surg 1992; **79**(5):443.

46. De Gaetano G, Evangelista V, Rajtar G *et al.* Activated polymorphonuclear leucocytes stimulate platelet function. Thromb Res 1990; **XI** (Suppl):25–32.

47. Lowe G, Rumley A. The biochemistry of critical limb ischaemia. Critical Ischaemia 1991; **1**:11–23.

48. Browse NL, Burnand KG. The cause of venous ulceration. Lancet 1982; **2**:243–245.

49. McCord JM. Oxygen–derived free radicals in postischemic tissue injury. N Engl J Med 1985; **312**(3):159–163.

50. Blalock A. Oxygen content of blood in patient with varicose veins. Arch Surg 1929; **19**:898–905.

51. He C, Cherry GW, Arnold F. Postural vasoregulation and mediators of reperfusion injury in venous ulceration. J Vasc Surg 1997; **25**:647–653.

52. Cullum N, Roe B. Leg Ulcers: Nursing Management: A Research Based Guide. 1st ed. London: Scutari Press, 1995.

53. Gutteridge JMC. Antioxidant properties of the proteins caeruloplasmin, albumin and transferrin. A study of their activity in serum and synovial fluid from patients with rheumatoid arthritis. Biochim Biophys Acta 1986; **869**:119–127.

54. Puppo A, Halliwell B. Formation of hydroxyl radicals from hydrogen peroxide in the presence of iron. Biochem J 1988; **249**:185–190.

55. Myers HL. Topical chelation therapy for varicose pigmentation. Am Coll Angiol 1965; **11th annual meeting**:66–68.

56. Ackerman Z, Seidenbaum M, Loewenthal E, Rubinow A. Overload of iron in the skin of patients with varicose ulcers. Arch Dermatol 1988; **124**:1376–1378.

57. Halliwell B. Superoxide, iron, vascular endothelium, and reperfusion injury. Free Radic Res Commun 1989; **5**:315–318.

58. Reif DW, Simmons RD. Nitric oxide mediates iron release from ferritin. Arch Biochem Biophys 1990; **283**(2):537–541.

59. Freeman BA, Crapo JD. Biology of disease: free radicals and tissue injury. Lab Invest 1982; **47**(5):412–426.

60. Mylonas C, Kouretas D. Lipid peroxidation and tissue damage. *In vivo* 1999; **13**:295–310.

61. Cotran RS, Kumar V, Robbins SL. Robbins Pathologic Basis of Disease. Philadelphia: W.B. Saunders Company, 1994.

62. Angel MF, Ramasastry SS, Swartz WM, Basford RE, Futrell JW. The causes of skin ulcerations associated with venous insufficiency: a unifying hypothesis. Plast Reconstr Surg 1987; **79**(2):289–297.

63. Greenwald RA. Effect of oxygen–derived free radicals on connective tissue macromolecules. In: Bannister WH, editor. In: Development in Biochemistry; 1980. pp.160–169.

64. Greenwald RA, Moy WW. Inhibition of collagen gelation by action of the superoxide radical. Arthritis Rheum 1979; **22**(3):251–259.

65. Brenneisen P, Briviba K, Wlaschek M, Wenk J, Scharffetter–Kochanek K. Hydrogen peroxide (H_2O_2) increases the steady–state mRNA levels of collagenase/MMP–1 in human dermal fibroblasts. Free Radic Biol Med 1997; **22**(3):515–524.

66. Baeuerle PA, Baltimore D. Molecular aspects of cellular regulation, hormonal control regulation of gene transcription. In: Cohen PaF, JG, editor. Amsterdam: Elsevier/North Holland Biomedical Press, 1991. pp.409–432.

67. Maziere C, Auclair M *et al.* Oxidized low density lipoprotein induces activation of the transcription factor NF–kB in fibroblasts, endothelial and smooth muscle cells. Biochem Mol Biol Int 1996; **39**(6):1201–1207.

68. Schreck R, Rieber P, Baeuerle PA. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF–kB transcription and HIV–1. Embo J 1991; **10**(8):2247–2258.

69. Rupec RA, Baeuerle PA. The genomic response of tumor cells to hypoxia and reoxygenation. Differential activation of transcription factors AP–1 and NF–kB. Eur J Biochem 1995; **234**:632–640.

70. Collard CD, Agah A, Stahl GL. Complement activation following reoxygenation of hypoxic human endothelial cells: role of intracellular reactive oxygen species, NF–kB and new protein synthesis. Immunopharmacology 1998; **39**(1):39–50.

71. Haas AF, Wong JW, Iwahashi CK *et al.* Redox regulation of wound healing? NF–kB activation in cultured human keratinocytes upon wounding and the effect of low energy HeNe irradiation. Free Radical Biology and Medicine 1998; **25**(9):998–1005.

72. Novak U, Cocks BG, Hamilton JA. A labile repressor acts through the NFk–B–like binding sites of the human urokinase gene. Nucleic Acids Res 1991; **19**:3389–3393.

73. Morioka S, Lazarus G, Baird J, Jenson P. Migrating keratinocytes express urokinase–type plasminogen activator. J Invest Dermatol 1987; **88**:418–423.

74. Kramer MD, Schaefer B, Reinartz J. Plasminogen activation by human keratinocytes: molecular pathways and cell–biological consequences. Biol Chem Hoppe Seyler 1995; **376**:131–141.