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Reactive Oxygen Species& Its Role in Periodontal Disease

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I. Introduction

Free radicals have been defined as any species capable of independent existence that contain one or more unpaired electrons. (Halliwell B, 1991)

Free radicals are a family of highly reactive and diverse species capable of extracting electrons and thereby oxidizing a variety of biomolecules vital to cell and tissue function, which not only includes oxygen free radicals, but also nitrogen and chlorine species

Reactive oxygen species (ROS) encompasses other reactive species which are not true radicals but are nevertheless capable of radical formation in the intra- and extracellular environments.

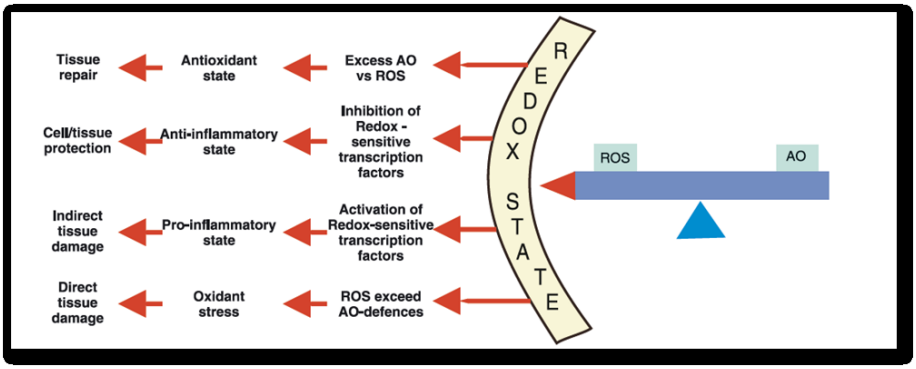
Antioxidants are defined as those substances which when present at low concentrations, compared to those of an oxidizable substrate, will significantly delay or inhibit oxidation of that substrate. (Halliwell B, 1989)

Oxidative stress is defined as a disturbance in the pro-oxidant/antioxidant balance in favour of the former, leading to potential damage. (Sies H, 1991)

Periodontitis is a term used to describe an inflammatory process, initiated by the plaque biofilm, that leads to loss of periodontal attachment to the root surface and adjacent alveolar bone and which ultimately results in tooth loss.Primary etiological agent of periodontitis is specific, predominantly gram-negative anaerobic or facultative bacteria within the subgingival biofilm.

The majority of periodontal tissue destruction is caused by an inappropriate host response to those microorganisms and their products.More specifically, a loss of homeostatic balance between proteolytic enzymes (e.g. neutrophil elastase) and their inhibitors (e.g. a 1-anti-trypsin) and reactive oxygen species (ROS) and the antioxidant defensesystems that protect and repair vital tissue, cell, and molecular components is believed to be responsible. The basis for such dysregulation is in part genetic (38–82 %) and in part theresult of environmental factors (e.g. smoking).

The smaller, more subtle, changes in intracellular redox-state trigger gene transcription events, lead to tissue damage secondary to the induction of a pro-inflammatory state.The larger upward shifts in the pro-oxidant/ antioxidant ratio causes intracellularly direct damage to vital biomolecules and structures, cell membrane damage and dysfunction, and cell death (by necrosis or accelerated apoptosis). Extracellularly, direct connective tissue damage (both mineralized and unmineralized) and damage to extracellular matrices and their components.Fig(1).



Oxidative killing mechanism of neutrophils and other phagocytes involves the formation of reactive oxygen species (ROS).

True radical and reactive oxygen species (ROS) and their symbols(Table 1)

The biological effects of small and large shifts in the balance of activity between reactive oxygen species (ROS)

True radicals	Radical symbol	ROS	ROS symbols
Superoxide	$O_2^{\bullet-}$	Hydrogen peroxide	H_2O_2
Hydroxyl	$^{\bullet}OH$	Hypochlorous acid	HOCl
Perhydroxyl	$HO_2^{\bullet}$		
Hydroperoxyl	$HOO^{\bullet}$	Singlet oxygen	$^1O_2$
Alkoxyl	$RO^{\bullet}$	Ozone	O_3
Aryloxyl	$ArO^{\bullet}$		
Arylperoxyl	$ArOO^{\bullet}$		
Peroxyl	$ROO^{\bullet-}$		
Acyloxyl	$RCOO^{\bullet}$		
Acylperoxyl	$RCOOO^{\bullet}$		

Within the gingival crevice/pocket, a low redox potential is regarded as essential for the growth and survival of subgingival anaerobes, whereas within cells and tissues a reducing environment (low redox potential) is protective against oxidative stress.So there is an apparent conflict in developing future therapeutic strategies for periodontitis which are based on redox biology, because maintaining low redox status (i.e reducing environment) to protect host cells and tissues from oxidative stress is conducive to encouraging growth and survival of anaerobes.Bacteria are not intracellular pathogens (unlike viruses) and therefore maintaining a low redox state within a cell may not have relevance to a high redox state within the periodontal pocket/gingival crevice.

Origin and formation of ROS and oxygen radicals

1. Exogenous sources include:

a. Heat, trauma, ultrasound, ultraviolet light, ozone, smoking, exhaust fumes, radiation, infection, excessive exercise, and therapeutic drugs.

2. Endogenous sources are primarily:

a. By-products of metabolic pathways – electron leakage from mitochondrial electron transport systems forming superoxide;

b. Functional generation by host defence cells (phagocytes) and cells of the connective tissues (osteoclasts and fibroblasts).

Mechanisms of Tissue Damage

Reactive oxygen species may cause damage to various cellular and extracellular tissues by causing:

1. Protein damage
2. Lipid peroxidation
3. DNA damage

II. Protein damage

The effects of ROS on proteins are adapted from Dean et al.

1. Protein folding or unfolding;
2. Protein fragmentation and polymerization reactions;
3. Protease degradation of the modified protein;
4. Formation of protein radicals;
5. Formation of protein-bound ROS;
6. Formation of stable end products e.g. carbonyl compounds such as oxoacids or aldehydes (e.g. alanine to acetaldehyde).

III. Lipid peroxidation

Lipid peroxidation is one of the most important reactions of free radical species.Most effective at activating this process is the hydroxyl radical and also peroxynitrite anion (ONOO).

Krinsky describes six stages, but Halliwell simplifies the reaction to three major stages:

1. Initiation;
2. Propagation;
3. Termination.

Products of lipid peroxidation include a variety of bioactive molecules:

1. Conjugated dienes;
2. Lipid peroxides;

3. Aldehydes, e.g. malondialdehyde, which is an example of a thiobarbituric acid reactive substance;
4. Acrolein;
5. Isoprostanes, e.g. F2-isoprostanes from arachidonic acid (8-iso-PGF2) ;
6. Neuroprostanes (F4-isoprostanes);
7. Volatile hydrocarbons, e.g. pentane, ethane

IV. DNA damage

Mechanisms of DNA damage by peroxynitrite and hydroxyl radicals include:

1. Strand breaks;
2. Base pair mutations (purine and pyrimidine bases);
3. Conversion of guanine to 8-hydroxyguanine, which is measured as a marker of DNA damage as the nucleoside 8-hydroxydeoxyguanosine;
4. Deletions;
5. Insertions;
6. Nicking;
7. Sequence amplification.

Evidence for the presence and role of ROS in periodontal tissue damage

The idea that ROS are associated with the pathogenesis of a variety of inflammatory diseases and have a role (direct or indirect) in tissue damage has become a major area of research over the last decade as demonstrated by electronic searches of the literature. However, supporting evidence for their role in tissue damage is often indirect and circumstantial.Indeed, few reports fulfill any, or all, of Halliwell's Postulates, those being the criteria required to be fulfilled before ROS can be concluded to be key mediators of tissue injury in a given disease.

The four criteria proposed byHalliwell, similar to those proposed by Robert Kochin 1884 to establish a causal relationship between an organism and a disease, are:

1. ROS or oxidative damage caused must be present at site of injury;
2. Time course of ROS formation or the oxidative damage caused should occur before or at the same time as tissue injury;
3. Direct application of ROS relevant time course to tissues at concentrations found in vivo should reproduce damage similar to that observed in diseased tissue;
4. Removing or inhibiting ROS formation should decrease tissue damage to an extent related to their antioxidant action in vivo.

ROS production by neutrophils and other cells in periodontal disease

Priming effect:

Products of some bacteria leads to altered PMN function modifying their response to second stimulus. This action of preparing PMN for stimulation is referred to as priming.For example, LPS extracted from Porphyromonasgingivalis was found to prime the neutrophils for enhanced stimulated superoxide production.

In vivo conditions required for ROS production by neutrophils

Significant ROS generation by neutrophils requires a minimum oxygen tension of about 1% and a pH of 7.0–7.5. Both these conditions are found within periodontal pockets, indicating that chronic or excess ROS production is possible at this important site of periodontal tissue damage.

Oxidation products produced locally by neutrophil ROS (e.g. oxidized low-density lipoprotein) could further increase neutrophil ROS generation directly as well as up-regulating adhesion molecules.Factors present at high levels at diseased sites may enhance ROS production by neutrophils locally. For example, polyamines are found at high levels within the diseased periodontiumand have the capacity to enhance ROS generation by neutrophils.

Neutrophils are able to function and initiate respiratory burst activity in the presence of sulfide at the toxic levels found at diseased sites.

In vitro ROS generation by neutrophils in periodontal health and disease

Overall there is no agreement as to whether ROS generation is altered in periodontal disease. Most of the early studies (1984–1992) investigated juvenile periodontitis patients and used bacteria or zymosan, with and without opsonization with autologous or heterologous serum.

Most data supported the view that neutrophil ROS generation was associated with disease but one study suggested that the effect was the result of opsonic activity of the patient's serum rather than a function of the cells themselves. (Henry CA et al. 1984).Neutrophils in both chronic and juvenile periodontitis show a hyperreactive phenotype with respect to luminol-dependent chemiluminescence after FcγR stimulation using immunoglobulin G-opsonized bacteria.

The underlying basis for the hyperreactive phenotype of peripheral neutrophils in respect of FcγR-stimulated ROS generation seen in periodontitis is unclear.Thus, the most consistent finding from studies on peripheral neutrophils in periodontitis is that disease is associated with a heightened ROS response to FcγR stimulation. Preliminary studies also indicate that peripheral neutrophils from patients with chronic periodontitis exhibit a low level of extracellular ROS production that is significantly higher than that of controls.
Treatment appeared to increase both baseline ROS production and response to phorbolmyristate acetate, restoring a phenotype similar to that found peripherally.

These data could indicate that neutrophils isolated from diseased sites are either inhibited from responding or that in vivo activation and ROS generation have reduced their ability to respond in vitro.

Effects of ROS on periodontal tissues and components

1. Gingival cells
2. Bone
3. Ground substance
4. Collagen
5. DNA

Gingival cells:

Direct damaging effects of ROS on gingival cells have received little attention. ROS generated using a neutrophil myeloperoxidase, chloride, glucose, and glucose oxidase system caused lysis of epithelial targets that could be inhibited by azide and catalase. (Altman et al. 1992).This observation has important implications for disease pathogenesis but evidence that in vivo levels of ROS production by neutrophils in periodontitis cause such an effect are lacking.

There are no studies comparing ROS-mediated damage to cells or extracellular matrix components by neutrophils isolated from patients and periodontally healthy controls, despite the growing evidence base that neutrophils in periodontitis exhibit a hyperactive/reactive phenotype in respect of ROS production.

Bone resorption:

Although the effects of ROS on bone resorption have not been studied in periodontal disease it has been shown that certain ROS (superoxide and hydrogen peroxide) activate osteoclasts (Bax et al.1992, Hall et al. 1995) and promote osteoclast formation (Garrett et al. 1990). Osteoclasts produce ROS at the ruffle border/bone interface, suggesting a more direct role in resorption.(Key et al. 1994).Such a direct role in bone resorption in periodontitis is supported by the finding that hydroxyl radicals and hydrogen peroxide can degrade alveolar bone proteoglycans in vitro. (Moseley et al. 1998)

Ground substance degradation:

More recent studies by Moseley and co-workers in 1998 have investigated the effects of ROS on glycosaminoglycans and proteoglycans present in the soft and calcified tissues of the periodontium.All glycosaminoglycans undergo a variable degree of chain depolymerization and residue modification (especially in the presence of hydroxyl radicals).

Sulfatedglycosaminoglycans were more resistant to ROS degradation than the non-sulfated glycosaminoglycan hyaluronan.Chondroitin sulfate proteoglycans from alveolar bone were particularly susceptible to damage by hydroxyl radicals, which caused degradation of both the core proteins and glycosaminoglycan chains.By contrast, hydrogen peroxide caused more selective damage with core proteins being more susceptible than glycosaminoglycan chains.

A similar pattern of ROS damage is said to occur with proteoglycans isolated from gingival soft tissue. (Waddington et al. 2000)

Evidence suggests that ROS at physiological levels can selectively damage proteoglycans associated with both the soft periodontal tissues and alveolar bone. These extracellular matrix components are degraded in periodontal disease is supported by data from a large number of studies based on the analysis of gingival crevicular fluid and tissue extracts for their degradation products. (Embery G et al. 2000, Waddington et al. 2000)

What is less clear is whether the degradation of proteoglycans in periodontal disease is, at least in part, the result of oxidative damage.

Pattern of proteoglycan and glycosaminoglycan degradation seen in periodontitis reflects the in vitro data on the effects of ROS and is consistent with a role for oxidative damage to non-collagenous components of both the hard and soft tissues of the periodontium.

Collagen:

ROS have a variety of effects on type I collagen in vitro including direct fragmentation and polymerization as well as producing oxidative modifications, rendering the molecule more prone to proteolysis.The structure of collagen, with its high proline/ hydroxyproline content, is particularly susceptible to damage by ROS.

Superoxide anions and hydroxyl radicals are able to cleave collagen into small peptides at proline and hydroxyproline residues, liberating hydroxyproline-containing peptides. (Monboisse et al. 1998)

Although periodontal disease is associated with increased levels of superoxide dismutase-1 (found in the cytoplasm and nuclei of cells) in gingival extracts, there are no studies of the extracellular isoenzyme (superoxide dismutase-3) in periodontitis.

Modification of collagen and serum proteins indirectly by ROS, via interaction with lipid peroxidation products such as malondialdehyde, can significantly alter fibroblast functions such as adhesion, proliferation, and longevity. (Rittie et al. 2002)Such alterations of in vivo fibroblast function are expected in periodontal disease because of increase in lipid peroxidation within the gingival tissues.While the presence of the collagen metabolites in gingival crevicular fluid is likely to be the result of a combination of proteolysis by host and bacterial collagenases, oxidative damage may make a direct or indirect contribution to their production.

Oxidation-dependent changes in collagen within the periodontal connective tissues could retard neutrophil migration through the tissues and increase their potential to produce ROS, two factors that may be important in the pathogenesis of periodontal disease.Superoxide in vitro can modify a chloroform-extractable factor bound to serum albumin rendering plasma, and its chromatographically purified albumin, highly chemotactic to neutrophils in vitro and in vivo. ROS-mediated modification of tissue fluid albumin within the periodontal tissues could thus contribute to the influx of neutrophils seen in disease.

The imbalance of metalloproteinases and their tissue inhibitors in gingival crevicular fluid and in tissues associated with disease could be the result of a direct damage of tissue inhibitor of matrix metalloproteinases by ROS. ROS-induced alterations in metalloproteinases and tissue inhibitor of matrix metalloproteinase expression by cells in periodontium.

DNA damage

There appears to be only one published report investigating DNA damage in gingival tissues in periodontal health and disease.(Sugano et al. 2000).PCR found deletions within mitochondrial DNA only in samples from periodontitis patients.

Once damaged, oxidative stress within the cell can be amplified because of decreased expression of proteins critical for electron transport, leading to cell death.

Redox-sensitive signaling pathways and periodontal disease

ROS at high levels, or chronically produced, can cause oxidative stress within tissues and result in direct damage to cells and the extracellular matrix.Products of this oxidative damage like advanced glycation end products and lipid peroxide-modified proteins, can lead to further ROS-induced damage by their priming and chemotactic actions on neutrophils.

There are two redox-sensitive transcription factors of potential importance in the pathogenesis of periodontal disease, namely nuclear factor-κB and activator protein.

They can be activated by a variety of stimuli, including bacterial products, viral proteins, cytokines, growth factors, radiation, ischemia/reperfusion, and oxidative stress.

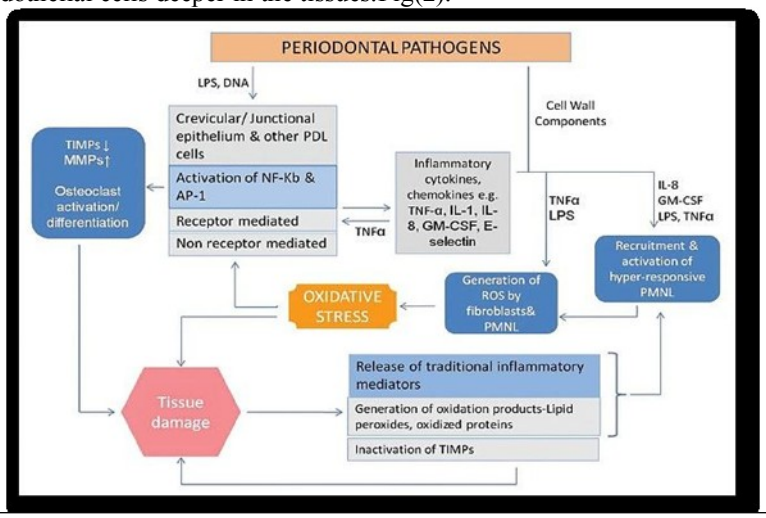
Activator protein-1 and nuclear factor- κB

Nuclear factor- κB affects several genes linked to overall inflammatory process such as those encoding interleukins (1,6, 8), MHC class I antigens, TNF-α, NF-κB. H_2O_2 is an inducer of nuclear factor- κB activity. There is only one report on the presence and distribution of nuclear factor-κB in gingival tissues in health and disease. This immunohistological study indicates a higher incidence of nuclear nuclear factor-κB (staining in the suprabasal layers of epithelium in the gingiva from patients (87.5 % positive) compared to controls (17.5% positive)

Periodontitis is associated with epithelial activation of nuclear factor-κB.

Activation of activating protein-1 and nuclear factor-κB can be the result of a variety of stimuli, including products whose genes are controlled by the transcription factors themselves (e.g. IL-1, TNF-α).Thus,

cytokines produced by overlying epithelium in response to bacteria, might be important in stimulating cells such as fibroblasts and endothelial cells deeper in the tissues.Fig(2).



Central role of ROS in generating chronic inflammation and tissue damage in response to periodontal pathogens.

Local presence of ROS in periodontal disease

There are no published studies investigating directly the presence and levels of ROS in periodontal tissues, gingival crevicular fluid, saliva or blood in periodontal health and disease.Possibilities do exist for local detection of ROS using endogenous molecular spin traps such as urate.

Allantoin is one of urate's oxidation products that have been shown to be elevated in conditions associated with oxidative stress and periodontal disease such as diabetes (Benzie et al. 1999), lung disease in pre-term infants, rheumatoid arthritis, and chronic heart failure.A second potential avenue of enquiry is the direct detection of hydrogen peroxide, a relatively stable ROS, by sampling the air within the oral cavity.Studies have shown that hydrogen peroxide can be detected in exhaled air and breath condensate, and that levels appear to correlate with inflammation.

None of the biomarkers is absolutely specific for ROS damage (i.e. they can be generated by means other than reacting with ROS) or specific to the periodontal tissues or periodontitis.

Measuring ROS and oxidative stress damage in biological samples

Free radicals and other reactive species have extremely short half-lives in vivo (10^6–10^9 s) and simply cannot be measured directly.In vitro systems called spin traps are used to measure radical species but there are currently no suitable spin traps/probes available for in vivo measurement of ROS production in the human, because of their unknown toxicity.

Ex vivo spin traps can be used and these would include:

- Ascorbic acid
- Aromatic traps – such as salicylates and phenylanine
- Urate

Main sources of biomarkers of ROS activity are:

1. Lipid peroxidation;
2. Protein/amino acid oxidation;
3. Carbohydrate damage;
4. DNA damage.

Antioxidants

Ascorbic acid (vitamin C):

1. Scavenging water-soluble peroxyl radicals;
2. Scavenging superoxide and perhydroxyl radicals;
3. Prevention of damage mediated by hydroxyl radicals on uric acid;
4. Scavenger of hypochlorous acid;
5. Decreases heme breakdown and subsequent Fe 2+ release thereby preventing Fenton reactions;
6. Scavenger of singlet oxygen and hydroxyl radicals

7. Reforms α-tocopherol from its radical;
8. Protects against ROS-release from cigarette smoke.
9. Ability to reduce C-reactive-protein-mediated expression of monocyte adhesion molecules and the ability to decrease pro-inflammatory gene expression via effects on the nuclear factor-κB transcription factor
10. Gingival crevicular fluid levels are reported to be three-fold higher than plasma levels and vitamin C has been shown to prevent activation of neutrophil collagenase.

Vitamin E

1. Generally regarded as the most important and effective lipid-soluble antioxidant in vivo , vital to maintaining cell membrane integrity against lipid peroxidationbyperoxyl radical scavenging
2. Its antioxidant behavior is the result of a single phenolic OH group, which when oxidized gives rise to the vitamin E (tocopheryl) radical.
3. Possesses anti-inflammatory as well as antioxidant properties and these were reviewed by Brock:
4. Inhibition of protein kinase C and subsequent platelet aggregation;
5. Inhibition of nitric oxide production by vascular endothelium;

Carotenoids

1. Lycopene;
2. α-carotene;
3. ß-carotene;
4. Lutein;
5. Cryptoxanthine;
6. Retinol (vitamin A)
7. Dehydroretinol (vitamin A)

ß-carotene is efficient at scavenging singlet oxygen (O_2) and other carotenoid antioxidant activities include the scavenging of peroxyl radicals. Vitamin A is controversial as an antioxidant because its behaviour depends upon the oxygen tension of the immediate environment.

At the low partial oxygen pressures found in most tissues ß-carotene acts as an antioxidant but this initial activity is followed by pro-oxidant behavior at higher oxygen tensions, associated with substantial detrimental effects upon the surrounding tissues

Co-Enzyme Q10

It exists in an oxidized form (ubiquinone or CoQ) and a reduced form (ubiquinol or CoQH, both of which possess anti-oxidant activity. Co Q10 is also regarded as a pro-oxidant molecule in response to various pathophysiological events. Co-enzyme Q10 deficiency has been demonstrated in the gingival tissues of periodontitis subjects but there is currently a lack of intervention studies in human periodontitis to substantiate clinical therapeutic benefit; these are needed.

Uric acid

Uric acid is one of the major radical scavengers within plasma, urine, and saliva.Its antioxidant activities include:

1. Scavenger of singlet oxygen
2. Scavenger of hydroxyl radicals
3. Scavenger of hypochlorous acid ;
4. Protection of a antitrypsin when combined with ascorbate;

Polyphenols

Polyphenols function by:

1. Radical scavenging;
2. Terminating lipid peroxidation;
3. Iron chelation;
4. Sparing vitamin E;
5. Restoration of vitamin C.

Glutathione

Glutathione plays a major role in maintaining the intracellular redox balance and thus regulating signaling pathways which are affected by oxidative stress.

Lazaroids

They are a newly identified family of compounds which are derived from glucocorticoids, but lack both glucocorticoid and mineralocorticoid activities. These compounds scavenge lipid peroxyl radicals and inhibit iron-dependent lipid peroxidation by a mechanism similar to that of vitamin E.

AO Pro Products Antioxidants for the Professional Advantage

AO ProRinseis a flavorful mouth rinse using novel, patent pending combination antioxidants as well as Green Tea catechins. The alcohol-free formula is optimized with non-cariogenic xylitol and essential oils all designed to deactivate odor-causing compounds in the mouth.

AO ProVantage Dental Gel is an aqueous gel using our novel, patent pending antioxidants, xylitol and essential oils that are specifically formulated for use in the mouth.

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