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REVIEW

Reactive Oxygen Species and Human Inflammatory Periodontal Diseases

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Abstract—Reactive oxygen species (ROS) have emerged as important signaling molecules in the regulation of various cellular processes. They can be generated by the mitochondrial electron transport chain in mitochondria and activation of polymorphonuclear leukocytes (PMN) during inflammatory conditions. Excessive generation of ROS may result in attack of and damage to most intracellular and extracellular components in a living organism. Moreover, ROS can directly induce and/or regulate apoptotic and necrotic cell death. Periodontal pathologies are inflammatory and degenerative diseases. Several forms of periodontal diseases are associated with activated PMN. Damage of tissues in inflammatory periodontal pathologies can be mediated by ROS resulting from the physiological activity of PMN during the phagocytosis of periodontopathic bacteria.

Key words: reactive oxygen species (ROS), polymorphonuclear leukocytes (PMN), mitochondrial electron transport chain, cell death, periodontal pathologies

Reactive oxygen species (ROS) include a number of chemically reactive molecules derived from molecular oxygen. Reactive oxygen species have also emerged as important signaling molecules in the regulation of various cellular processes. The most important effect of ROS in living cells is damage to cellular biomolecules (including DNA) taking place under oxidative stress conditions [1, 2]. ROS are generated by a wide variety of sources. It is clear that virtually all mammalians cells produce ROS [3]. The external environment can also generate ROS from many sources including heat, UV light, therapeutic drugs, and χ- and γ-radiation [4-7]. However, the inherent ROS are generated from cellular functions including mitochondrial metabolism [8, 9], polymorphonuclear leukocyte (PMN) activations [10, 11], and oxidation pathways. Also behavioral activities (e.g. smoking, chronic excessive exercise) can contribute to oxidative damage by producing ROS [1].

ROS that cause oxidative damage can be divided into two categories: free oxygen radicals and non-radical molecules. Free oxygen radicals can be defined as any chemical species capable of independent existence that contains one or more unpaired electrons. It is important that, when free radicals react with non-radicals, a new radical can occur, which may result in chain reactions of free radical formation.

The most common free oxygen radicals include hydroxyl radical (OH), nitric oxide (NO), and superoxide (O_2^-). Non-radical ROS include any molecules such as hydrogen peroxide (H_2O_2). Most of these molecules can damage cellular macromolecules including DNA by reacting with them.

Evidences implicate PMN as the primary mediators of the host response against proliferating pathogenic microorganisms during inflammatory diseases, such as periodontal pathologies [12]. PMN produce a range of antimicrobial factors, which include ROS.

The human periodontal diseases are inflammatory disorders that give rise to tissue damage and loss, as a result of the complex interaction between pathogenic bacteria and the host immune response. During the development and progression of the diseases, there is increased colonization in the dental plaque of microorganisms termed periodontopathogens. Pathogens such as Gram-negative species, mobile rods, and spirochetes may have the ability to invade gingival tissues.

This change in bacterial flora and interaction between pathogenic bacteria and the host immune response are accompanied by an increase in cytokine expression and immunological activity in gingival tissues.

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As a result of stimulation of cytokine activity and immunological activity by bacterial antigens, PMN produce large amount of ROS. Tissue destruction in periodontal diseases is caused by increased level of ROS in inflammatory periodontal tissues [13, 14].

In this review, we discuss sources of ROS, production of ROS by PMN during the phagocytosis of periodontopathogens in inflammatory periodontal diseases, and also the relationship between ROS and tissue destruction in inflammatory periodontal diseases.

SOURCES OF REACTIVE OXYGEN SPECIES

All ROS are related with oxygen metabolism. Oxygen is ubiquitous in aerobic organisms and hence, reactive oxygen species may come from a variety of different sources. ROS are formed in all living organisms as byproducts of normal metabolism (endogenous sources) and as a consequence of exposure to environmental compounds (exogenous sources). Especially mammalian cells can generate ROS by different biological mechanisms such as the mitochondrial respiratory chain and PMN activation in inflammation.

On the other hand, external environmental factors may generate ROS. For example, ionizing radiation, uremic milieu, pollutants such as automobile emissions and chemical oxidants in air, water, and food chains also contribute to the oxidant challenge, as do behavioral activities such as smoking and using areca nut and lime.

Here we will list significant sources of ROS that come from biological mechanisms.

Mitochondria as a source of ROS. Mitochondria are unique organelles as they are the main sites of oxygen metabolism, accounting for approximately 85-90% of the oxygen consumed by the cell [8, 15]. Mitochondria constantly metabolize oxygen thereby producing ROS as a byproduct. The respiratory chain in mitochondria is a powerful source of ROS, primarily superoxide radical (O_2^-) and consequently hydrogen peroxide (H_2O_2) as a product of superoxide dismutase [8, 16, 17].

ROS may lead to the oxidative damage of virtually any biomolecules. Mitochondria are particularly susceptible to this damage because ROS are generated continuously by the mitochondrial electron transport chain [18, 19]. Mitochondria are also a major site for the accumulation of low molecular weight Fe^{2+} complexes, which promote the oxidative damage of membrane lipids, proteins, and mitochondrial DNA. Increase in ROS level in mitochondria and accumulation of low molecular weight Fe^{2+} complexes cause mitochondrial dysfunction by damaging mitochondrial structures. Recently, a large number of studies have associated mitochondrial dysfunction caused by ROS to both accidental cell death (necrosis) and programmed cell death (apoptosis) [20, 21].

Mitochondria have their own ROS scavenging mechanisms that are required for cell survival. It has been shown, however, that mitochondria produce ROS at a rate higher than their scavenging capacity, resulting in the incomplete metabolism of 1-3% of the consumed oxygen [16, 18].

The primary product of oxygen metabolism in the mitochondrial respiratory chain is superoxide radical (O_2^-). The formation of O_2^- occurs via the transfer of one electron to molecular oxygen. This reaction occurs at specific sites of the electron transport chain, which resides in the inner mitochondrial membrane. Electron transport chain complexes I (NADH dehydrogenase) and III (ubisemiquinone) produce most of the superoxide radical [22, 23]. After producing this radical, it is scavenged by the mitochondrial enzyme manganese superoxide dismutase (MnSOD) to produce hydrogen peroxide (H_2O_2).

A disadvantage concerning the mitochondrial scavenging system is that mitochondria do not contain catalase. These organelles only have the enzyme glutathione peroxidase (GSPx) neutralizing the potentially toxic hydrogen peroxide (H_2O_2). Glutathione peroxidase requires a coenzyme, which is reduced glutathione (GSH). GSPx can completely convert hydrogen peroxide (H_2O_2) to water. This reaction results in detoxifying of ROS [24]. Despite the ROS-detoxifying mechanism, in the presence of reduced transition metals such as Fe^{2+}, hydrogen peroxide can produce the highly reactive hydroxyl radical. Hydroxyl radical is the most reactive radical and can cause extensive damage to proteins, lipids, and especially DNA molecules [25].

Another important radical is nitric oxide (NO). Nitric oxide is a free radical produced from L-arginine through the action functioning isoenzyme NO synthases (NOS) [26]. Recent data has demonstrated that mitochondria have their own NO synthase, called mtNOS, and produce nitric oxide. Furthermore, a large amount of nitric oxide can come from exogenous sources in this organelle. Because nitric oxide is a free gas, it easily penetrates through biological membranes [24].

On the other hand, superoxide radical has a high affinity toward NO. Reaction between nitric oxide and superoxide radical produces a new molecule, which is called peroxynitrite ($ONOO^-$). Peroxynitrite is as highly reactive a molecule as hydroxyl radical and can cause extensive damage to proteins, lipids, and especially DNA molecules. However the main mitochondrial targets of peroxynitrite are electron transport chain complexes I, II, IV, and V, mitochondrial membranes, mitochondrial DNA, and some enzymes such as aconitase, creatine kinase, and superoxide dismutase. Damage to these molecules may induce mitochondrial swelling, depolarization, calcium released, and the permeability transition [24, 25].

Inflammation as a source of ROS. Another significant source of ROS is inflammatory reactions, especially chronic inflammation. Inflammation cells such as acti-

vated macrophages and neutrophils release various ROS (H_2O_2, NO, O_2^-, $OH^\cdot$) and hypochlorite (HOCl) [27, 28].

PMN have been demonstrated to produce a range of antimicrobial factors, which include ROS. Whilst ROS production by PMN in inflammatory condition provides a host protective role, evidences also suggest that ROS production in inflammatory diseases can lead to the destruction of extracellular matrix components and to connective tissue damage incurred during inflammatory diseases [29, 30].

Hydrogen peroxide, nitric oxide, superoxide radical, and hydroxyl radical are produced by mitochondria leading to elevated numbers and activity of PMN. In addition to hydrogen peroxide, nitric oxide, superoxide radical, and hydroxyl radical, another reactive molecule is hypochlorite (HOCl), which can be produced by PMN during inflammatory conditions. Hypochlorite is formed by the action of phagocyte (not macrophage) myeloperoxidase upon hydrogen peroxide and is released extracellularly [31]. Instead of being neutralized to water, hydrogen peroxide can also be metabolized by the enzyme myeloperoxidase to form the potent chlorinating as well as oxidizing agent hypochlorite. This reaction is specifically considered to be relevant in inflammatory conditions, as the hemoprotein myeloperoxidase is one of the most abundant proteins in phagocytes.

Myeloperoxidase is believed to be present in human macrophages as well. When activated, neutrophils can secrete myeloperoxidase extracellularly [31]. Hypochlorite is 100-1000 times more toxic than superoxide radical or hydrogen peroxide and it has distinct biochemical targets such as inactivating essential enzymes [32], oxidizing membrane bound protein [33], disrupting certain cellular membrane functions [34], and decreasing the adhesive properties of some extracellular matrix components [35]. In addition, exposure to hypochlorite seems to increase endothelial permeability through the mobilization of cellular Zn^{2+} molecules [36]. There is no specific hypochlorite-neutralizing enzyme in mammals, but hypochlorite can be removed by reaction with albumin and ascorbic acid [37].

ROS MEDIATED DAMAGE

Excessive generation of ROS can result in attack upon and damage to all cellular and extracellular components. However, it is important that ROS have high reactivity and generally produce high damage in close to the site of their generation. So, oxidative damage can be extreme in mitochondria and in regions of inflammation.

Cellular targets of ROS. *Lipids of biological membranes.* The polyunsaturated fatty acids located within biological cell membranes such as cytoplasmic cell membranes and mitochondrial membranes are major targets of ROS [38, 39]. Lipid peroxidation is probably the most explored area of research when it comes to ROS. Polyunsaturated fatty acids are, because of their multiple double bonds, excellent targets for reactive oxygen species. Cell membranes are particularly effectively attacked by hydroxyl radical and polyunsaturated fatty acids located within biological cell membranes can be damaged by hydroxyl radical. Lipid peroxidation (LPO) continues as a chain reaction to generate lipid hydroperoxides and aldehydes, and a single oxidative event can thus affect many lipid molecules. The accumulation of hydroperoxides in cell membranes has a profound effect on their fluidity, affecting the activity of transmembrane enzymes, transporters, receptors, and other membrane proteins. As a result, lipid peroxidation causes changes in membrane permeability and selectivity [40, 41].

LPO accelerates only when the cellular detoxification system has failed to remove the precursors of hydroxyl radical, in particular hydrogen peroxide. LPO is most successfully combated by antioxidants such as vitamin E, although it has also been shown that nitric oxide can act as an antioxidant against lipid peroxidation [42]. This reaction reflects a protective effect of nitric oxide against oxidative damage.

Proteins. Proteins are the most abundant cell constituents, which make them important ROS targets [43]. Moreover, a relatively minor structural modification of a single protein molecule can lead to a marked change in its biological activity.

ROS have been shown to react with several amino acids, generating anything from modified and less active enzymes to denatured, nonfunctioning proteins [44, 45]. Hydroxyl radical seems to be most effective in inducing oxidative protein damage [43]. The process of protein oxidation introduces new functional groups, such as hydroxyls and carbonyls, which contribute to altered function, turnover, and degradation. Secondary effects of ROS on proteins include protein fragmentation, cross-linking, and unfolding [46].

Another structural modification of protein molecules is their nitrosylation, and peroxynitrite is also responsible for such modification [47, 48]. Tyrosine is an important amino acid involved in phosphorylation reactions and signal transduction pathways [43]. Tyrosine nitration may not only compromise protein function, but may also have serious consequences in cellular regulation. Such enzymes as SOD have been identified as specific protein targets of nitrosylation. Nitrosylation of cytoplasmic SOD has been reported to occur without loss of enzymatic activity [49]. However, tyrosine nitrosylation of the mitochondrial enzyme (MnSOD) is associated with the loss of enzyme function [50].

DNA molecules. All living organisms, for example mammals, are constantly exposed to oxidative stress from environmental agents and from endogenous metabolic processes. Oxidative modifications can occur in DNA molecules by the effect of reactive oxygen species [51]. A

large number of DNA modifications caused by oxidative stress have been detected in various mammalian cells [52]. Both nuclear and mitochondrial DNA are known targets of ROS attack [53, 54]. The most common modification is base hydroxylations and strand cleavage, leading to ATP depletion and gene mutations, which can in turn result in malignant transformation or cell death. Superoxide radical is relatively unreactive with DNA molecules, and other ROS implicated in DNA damage are nitric oxide and peroxynitrite, which can directly damage chromatin [55-58].

The most substantial portion of DNA modifications is thought to involve hydroxyl radical as the attacking species [59, 60]. When hydrogen peroxide escapes from its cytosolic neutralizing enzymes and reaches the cell nucleus, it will react with chromatin-bound iron (or copper released from oxidatively damaged Cu/Zn-SOD enzymes) [61, 62] producing hydroxyl radical, which in turn will attack nearby DNA molecules [63].

Modifications of DNA molecules in mammalian cells occur in both the nucleus and mitochondria. However, the mitochondrial DNA has a much higher modification (mutation) rate than the nuclear genome. Since mitochondrial DNA is a small molecule and extremely susceptible to oxidative damage, being located in mitochondrial matrix, it is close to the major source of ROS; moreover, lacking introns and being devoid of histones and other DNA-associated proteins, the probably of oxidative modification of a coding region of mitochondrial DNA is very high [64]. ROS were shown to induce extensive fragmentation and deletions in mitochondrial DNA. Up to 187 different deletions were listed by Ozawa [65]. Increased content of mitochondrial DNA defects has been reported in ageing and age-associated diseases [64].

Another significant damage to DNA molecules is indirectly associated with the p53 gene. Suppressor gene (p53) encodes a transcription factor that has been termed the "guardian of the genome". Oxidative stress can affect nuclear DNA, then p53 gene activations can occur, which triggers transcription of p53-related genes. These genes play a central role in the regulation of cell cycle arrest and apoptosis. Expression of p53, at the level of the protein, can be stimulated under conditions of oxidative stress, as can its DNA-binding properties [66]. However, it is far from clear that oxidative conditions always promote p53 activity. The p53 gene activation by oxidative stress may result from ROS-induced DNA damage. Detection of such damage promotes p53 protein phosphorylation in a process that appears to be regulated by a DNA-dependent protein kinase [67]. Also, cellular detection of DNA damage may reduce the rate of p53 degradation in a process regulated though its interaction with the MDM2 protein [68, 69].

In addition to regulation by DNA damage, p53 gene activation can also be modulated directly by redox conditions. Oxidation of the p53 gene impairs the DNA binding capacity of the corresponding protein via a cysteine-dependent mechanism [70]. In fact, multiple cysteine residues within p53 are involved in the redox regulation of its activity. The redox regulator Ref-1 can reactivate DNA binding of oxidized p53 and, as for AP-1, may play an important role in p53 activation following cellular oxidative challenge.

Extracellular targets of ROS. Excess of ROS causes indiscriminate damage to cellular constituents, such as DNA molecules, biological membrane lipids and proteins, as well as destruction to extracellular matrix components and further alteration of metabolic reactions responsible for synthesis of extracellular matrix components. While production of ROS by polymorphonuclear leukocytes provides a host protective role, evidence also exists that ROS lead to the destruction of extracellular matrix components and to connective tissue damage incurred during inflammatory diseases [29, 30].

In vitro studies have shown that ROS are capable of degrading a number of extracellular matrix components including proteoglycans (PG) and their constituent glycosaminoglycans (GAG), which are important extracellular matrix components within connective tissue. Because of the polyanionic nature of the GAG chains, they have been implicated in binding to calcium and hydroxyapatite crystals, thereby providing a role in regulating mineralization [71, 72] and in binding growth factors, such as transforming growth factor β, thus regulating their action on cellular functions [73].

Mineralized connective tissues, such as bone, contain predominantly small chondroitin PG species, decorin and biglycan while soft connective tissues, such as skin and gingiva contain small dermatan sulfate PGs together with the larger chondroitin sulfate PG [74-76]. All connective tissues also contain in varying amounts the non-sulfated GAG hyaluronan, which has been identified to interact with larger PG species and has been proposed roles in the control of local calcium concentration and remodeling of mineralized tissues.

Degradation of PG by ROS involves the modification of amino acid functional groups and leads to fragmentation of the core protein, while the constituent GAG chains undergo limited depolymerization. There is evidence that non-sulfated GAG such as hyaluronan and chondroitin in connective tissue are the most susceptible to degradation in the presence of ROS. However, the highly sulfated GAG heparin showed minimal depolymerization when exposed to ROS [77]. The presence of sulfate on the GAG chain can apparently protect the molecule against ROS attack. Chemical modification of GAG, however, may affect PG function and may be of importance in considering connective tissue destruction in a variety of pathological situations.

In addition to connective tissue damage, ROS have been demonstrated to be capable of degrading bone proteoglycans *in vitro*. In the presence of hydrogen peroxide,

only core proteins were more susceptible to degradation than the GAG chains, although both the core proteins and the GAG chains were extensively degraded in the presence of hydroxyl radical [78].

Another important molecule in the extracellular matrix is collagen, which is also effected by ROS attacks [79]. Collagen is one of the most abundant proteins in nature, occurring in variety of tissue in most multicellular systems including mammals [80, 81]. The prototypical collagen molecule is collagen type I, where three α-polypeptide chains, each of which contains approximately 1000 amino acids, form a triple helix. This characteristic triple helical structure renders collagen rather resistant towards unspecific proteolytic attack. ROS are believed to play an important role, both by directly attacking to the collagen and rendering it more susceptible to the normal enzymatic breakdown by the enzyme collagenase, which in itself is also believed to be activated by reactive oxygen species [79].

Role of ROS in cell death. Cell death can follow two distinct pathways, necrosis, or apoptosis [82]. Apoptosis (or controlled cell death) differs from necrosis (chaotic cell death due to overt injury) in distinct morphological and biochemical features. Histologically, apoptosis is associated with cell shrinkage, membrane blebbing, chromatin condensation, and the formation of fragmented chromatin into apoptotic bodies, while necrosis is associated with cell swelling and cell lysis with loss of cell content into the surrounding tissue. Although the early biochemical events that initiate the mode of cell death are still unclear, several lines of evidence implicate ROS as modulators of apoptosis [83]. *In vitro* exposure to low doses of ROS, or a depletion of cellular antioxidants, has been shown to result in apoptosis [84, 85], and, conversely, apoptosis can be blocked by the addition of antioxidant compounds [86, 87].

The processes underlying the controlled removal of cells via apoptosis are energy-dependent and thus require ATP. In contrast, necrosis is a passive process with cell swelling reflecting loss of osmotic control via such molecules as Na/K-ATPase [88]. During inflammatory reactions, ROS mediated signaling may modulate the expression of pro-apoptotic cell surface changes such as the upregulation of fas (Apo1) and the appearance of phosphatidylserine on the outer cell membrane [89]. These markers signal for the cell to be removed by macrophages and other phagocytic cells. Alternatively, an excessive inflammatory response can lead to the release of high levels of H_2O_2, through respiratory burst activity, and then lead to cell swelling and cell lysis with further release of cytolytic compounds leading to widespread necrotic cell death [90].

Intracellular glutathione (GSH) concentration appears to play an important part in the induction of apoptosis, but the exact role is unclear [91]. In some systems, prevention of GSH efflux appears to prevent apoptosis [92, 93] while other groups see GSH efflux as a bystander event in cell death [94]. Second, the artificial lowering of cellular GSH pools does not seem to induce apoptosis, and may indeed prevent apoptosis and possibly encourage necrosis in some systems [95, 96] while in others GSH removal has been reported to sensitize cells and increase apoptosis [97].

Two major pathways for the induction of apoptosis are known. In both, the redox state of the cell plays an important effector or regulatory role. A well-recognized reason for the induction of apoptosis is seen as a response to DNA damage, including oxidative damage. Detection of DNA damage is mediated through p53, and in the absence of wild-type p53 there is a significantly higher risk of clonal expansion of damaged cells, with the potential to form carcinomas. The mechanism by which p53 detects damage and signals the induction of apoptosis is not fully elucidated.

ROS MEDIATE DAMAGE IN INFLAMMATORY PERIODONTAL PATHOLOGIES

Human periodontal diseases are inflammatory disorders that give rise to tissue damage and loss as a result of complex interactions between pathogenic bacteria and the host's immune response. Several lines of evidence implicate PMN as the primary mediators of host response against pathogenic microbes during inflammatory periodontal diseases. Recent studies demonstrate that PMN produce a range of antimicrobial factors, which include ROS, during phagocytosis of periodontopathic bacteria in inflammatory periodontal diseases [98].

Reactive oxygen species have been implicated in the pathogenesis of over 100 conditions [99] including rheumatoid arthritis [100], acute respiratory distress syndrome [101], and, as recently established, inflammatory periodontal diseases [78, 102-115]. It has been suggested that as a result of stimulation by bacterial antigens, PMN produce and release a large quantity of ROS, culminating in heightened oxidative damage to gingival tissue, periodontal ligament, and alveolar bone [116].

Reactive oxygen species are active in depolymerization of extracellular matrix components [29, 78], lipid peroxidation (LPO) [111], oxidation of enzymes such as anti-proteases [111], increased apoptosis in deepest area of the sulcular pocket [117], induction of pro-inflammatory cytokines, and DNA damage [111].

Lipid peroxidation. Intracellular production and extracellular release of ROS can occur via PMN stimulated by periodontopathic bacteria such as Fusobacteria. Especially when neutrophils are stimulated by periodontopathic bacteria, extracellular release of ROS increases.

The potential extracellular damage to lipids that might be caused by ROS was recently estimated by Sheikhi et al. [118]. To assess the damage that might be

caused by ROS from PMN stimulated by *F. nucleatum*, they measured LPO in the presence of normal and stimulated neutrophils. It was shown that bacterium-stimulated neutrophils release extracellularly ROS, which cause intensive oxidative damage of lipid molecules.

Also peroxynitrite is capable of oxidizing or nitrating many biomolecules in periodontal tissues. The stable end products are nitrate and nitrite, which are excreted in the urine. Numerous investigators have suggested that peroxynitrite, by its ability to directly oxidize DNA bases, catalyze iron-independent membrane lipid peroxidation [119-121]. Sheikhi et al. studied whether vitamin E, an antioxidant, could prevent the production of ROS and lipid peroxidation [122]. Their results indicated that superoxide production of the neutrophils was reduced in a concentration-dependent way after the addition of vitamin E in the presence but not in the absence of plasma. On the other hand, saliva is also rich in antioxidants, mainly uric acid, with lesser contributions from albumin, ascorbate, and glutathione [123-125], and it has been demonstrated that saliva has a role in suppressing lipid peroxidation [126].

Protein damage. ROS, which are produced by PMN under inflammatory conditions, have been demonstrated to be capable of *in vitro* degradation of the major extracellular matrix components in periodontal tissues, including collagen, proteoglycans, and glycosaminoglycans, such as hyaluronan, using cell-free systems [29, 78]. Studies have indicated that PMN isolated from patients with early-onset periodontitis generate more elevated levels of O_2^- than PMN from healthy control group [107, 127]. Elevated O_2^- productions have also been reported during chronic apical periodontitis [128] and adult periodontitis [129]. *In vivo*, O_2^- is capable of reacting further with other ROS, such as hydrogen peroxide, in the presence of transition metal ions such as Fe^{2+}, producing the highly reactive hydroxyl radical [130].

Hyaluronan is a high molecular weight (104-107 kD), non-sulfated glycosaminoglycan component of the extracellular matrix present in many tissues, such as skin, synovial joints, and periodontal tissues, consisting of repeating disaccharide units of N-acetylglucosamine and D-glucuronic acid. Hyaluronan has many structural, rheological, and physiological functions within tissues [131]. Hyaluronan is also a key component of chronic wounds during each stage of the wound healing process, including the inflammatory, granulation, and re-epithelization stages [132]. Hyaluronan plays numerous roles in the activation and modulation of the inflammatory response, such as scavenging of ROS derived from PMN and other inflammatory cells [133]. All known ROS including superoxide radical species, hydrogen peroxide, and the hydroxyl radical are now strongly implicated in the pathogenesis of chronic wounds [134].

Such a property is of significance to the wound healing process, as despite the beneficial role that ROS play in killing invading microbial pathogens, excessive production of ROS can be detrimental to the host tissues [135]. Excess of ROS cause destruction to extracellular matrix components, such as collagen, proteoglycans, and hyaluronan [112], and further alter the metabolism of cells responsible for dermal extracellular matrix synthesis [136].

During the course of gingival inflammation, bacterial components and sometimes whole bacteria penetrate into the connective tissue and interact with phagocytic cells and fibroblasts [137]. Reactive oxygen species have been shown to depolymerize gingival hyaluronic acid and proteoglycans [29], and also hydrogen peroxide has especially cytotoxic effect to fibroblasts [138].

In addition to PMNs, monocytes, eosinophils, lymphocytes, and platelets, ROS production has also been demonstrated in fibroblasts. Fibroblasts release superoxide anion at approximately three times higher levels than unstimulated granulocytes when stimulated by phorbol myristate acetate or Ca^{2+} ionophore [139].

ROS are also produced by osteoclasts, which are responsible for bone destruction, and they may play a role in remodeling of alveolar bone in health and diseases. Some studies demonstrated that ROS are capable of degrading alveolar bone proteoglycans *in vitro* [78]. The degradation effects observed included cleavage of protein core and the glycosaminoglycan chains. Hydroxyl radical species result in greater modification than hydrogen peroxide. The core proteins have more susceptibility to degradation than the glycosaminoglycan chains in the presence of hydrogen peroxide alone. But both core proteins and glycosaminoglycan chains are extensively degraded in the presence of hydroxyl radical in inflammatory periodontal diseases.

Another target of ROS in periodontal tissues is anticollagenase enzyme. This enzyme plays a very important role against the collagenase enzyme and protects collagen structures [140]. The other important finding is that peroxynitrite inhibits SOD enzymatic activity by nitration and oxidation of critical tyrosine residues. However, there is no information about inhibition of intracellular MnSOD activation in periodontal tissue cells.

Effects of pro-inflammatory cytokines. In addition to direct intracellular and extracellular injury, ROS can affect cell injury indirectly by enhancing pro-inflammatory gene expression. These include cytokines (e.g. TNFα, interleukin (IL)-1), chemokines (e.g. IL-8), and cellular adhesion molecules. Moreover, ROS can induce stress genes such as the gene of hemoxygenase-1, which may protect against inflammatory tissue injury. ROS are thought to modulate the activity of nuclear factor-κB (NF-κB) and activating protein-1 (AP-1) [141], which regulate genes such as the hemoxygenase-1 gene. Activation of NF-κB can also be caused by bacterial endotoxins, IL-1 and TNFα.

The level of NF-κB increases in the cytoplasm of most inflammatory cells affected by ROS in periodontal

inflammation. The free NF-κB diffuses from the cytoplasm, across the nuclear membrane, and binds to DNA, stimulating the transcription of mRNA for the various pro-inflammatory cytokines such as IL-2, IL-2 receptor, IL-6, IL-8, TNFα, and β-interferon. The role of ROS could be control of IκB (NF-κB inhibitor) phosphorylation, which causes release of free active NF-κB (from its inactive complex with IκB) into cytoplasm [111].

Increased apoptosis. In many cell types such as macrophages, pancreatic islets, neurons, and thymocytes, nitric oxide, which is a form of ROS, activates apoptosis [142]. Also induction of apoptosis may be seen as a response to oxidative DNA damage, this damage can occurred by ROS, and especially by nitric oxide. Both in experimental models and human samples of periodontal diseases, an increase in local NO production has been reported [143]. The induction of NO synthesis expression may also inhibit fibroblast proliferation and induce apoptosis, contributing to the imbalance of tissue destruction with tissue repair that is characteristic of periodontitis. A study of the relationship between apoptosis and destructive inflammatory periodontal disease showed that there is more proliferation than apoptosis in the sulcular portion of periodontal epithelium except in the deepest part of the sulcular epithelium [117]. More keratinocytes undergo apoptosis in the deepest area of the sulcular pocket closest to the junctional epithelium in destructive form of inflammatory periodontal tissues, which is called periodontitis.

On the other hand, apoptosis and immunolocalization of p53 gene have similar topographical distribution patterns in gingival epithelium, which is an indicator of p53 gene mediated apoptosis in this tissue. Also detection of oxidative DNA damage, which is responsible for induction of apoptosis, is mediated through the p53 gene.

Evidences of ROS-mediated DNA damage in individuals with periodontal disease. ROS, which are derived predominantly from PMN in the pathogenesis of periodontal tissue destruction, can cause extreme DNA damage in periodontal tissue cells [111]. However, there is little information about ROS mediated DNA damage in inflammatory periodontal diseases. The DNA damage in inflammatory periodontal diseases has been estimated only in a few studies. Also, ROS generation can occur together with reactive oxygen species mediated DNA damage in a number of disorders, including cancer, neurodegenerative diseases, diabetes, and chronic inflammatory conditions [144, 145].

The concentration of products from oxidatively damaged macromolecules serve as an indicator of ROS mediated DNA damage. Several different DNA base adducts have been identified after exposure of mammalian chromatin to free radicals. 8-Hydroxy-deoxyguanosine is the most common stable product of ROS mediated DNA damage [146, 147]. Increases in 8-hydroxy-deoxyguanosine levels have been found in infectious diseases such as *Helicobacter pylori* infections, papilloma virus infections, and recently periodontitis, which is a form of destructive inflammatory periodontal disease. Takane et al. measured 8-hydroxy-deoxyguanosine levels in human whole saliva using enzyme immunoassay [148]. Their studies showed that the 8-hydroxy-deoxyguanosine levels were significantly higher in the whole saliva of subjects with periodontitis than in subjects with healthy periodontium.

Mitochondrial DNA is the most susceptible molecule to attacks of ROS. In mammals, mitochondrial DNA exists as a double stranded closed circular molecule and the size of mitochondrial DNA is 16,569 bp in humans. Common information about mitochondrial DNA is that deletions of mitochondrial DNA that are caused by effects of ROS are associated with aging and several chronic diseases [149]. It is well known that periodontal diseases, especially chronic periodontitis, are chronic, degenerative, and inflammatory diseases. Also, the incidence and severity of periodontal diseases are affected by age. Sugano et al. detected mitochondrial DNA deletions in human gingival tissues [150]. Gingival tissue samples were collected during flap surgery from patients with chronic adult periodontitis conditions and these samples were analyzed via the polymerase chain reaction technique. As a result, the presence of 5 kb mitochondrial DNA deletions was found in gingival tissues. This mitochondrial DNA deletion was not found in gingival tissue of periodontally healthy individuals.

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