Oxidative Stress in Applied Basic Research and Clinical Practice

Daisuke Ekuni
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Edward E. Putnins *Editors*

Studies on Periodontal Disease

Humana Press

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Donald Armstrong
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Preface

Oxidant stress plays an important role in a wide range of tissue pathologies, such as neurodegenerative disease, carcinogenesis, aging, and periodontal disease. Periodontal disease is one of the most prevalent diseases suffered by humans, although the reported prevalence varies according to diagnostic criteria. Periodontal disease is a chronic inflammatory disease characterized by gingival bleeding, periodontal pocket formation, connective tissue destruction, and alveolar bone resorption leading to tooth loss. Oxidative stress is involved in the initiation and progression of periodontal disease, and various studies have reported that levels of oxidative stress markers and oxidative damage in periodontal disease are greater than that in healthy controls. Furthermore, recent studies have demonstrated a relationship between periodontitis and systemic diseases, such as coronary heart disease, atherosclerosis, and diabetes. With regard to the mechanisms, oxidative stress and an altered inflammatory process are involved.

The chapters of this book widely cover the evidence for the relationship between oxidative stress and periodontal disease. Furthermore, the chapters provide possible mechanisms linking periodontal disease and systemic disease. The contributors are recognized experts in the field of oxidative stress and dentistry. We believe that this book will therefore prove to be useful for researchers in periodontology.

Finally, I would like to thank the coeditors and contributors for their kind assistance with the project.

Okayama, Japan — Daisuke Ekuni

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Part I
Oxidative Stress in Periodontal Diseases (Basic Science)

Chapter 1
Reactive Oxygen Species and Antioxidant Systems in Periodontal Disease

Masaichi-Chang-il Lee

1.1 Introduction

It is well known that free radicals, including reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot-}$) and hydroxyl radical (HO·), contribute to the development of various age-related diseases by causing oxidative stress [1]. Free radical reactions and oxidative stress induced by ROS are usually kept in check by antioxidant defense mechanisms; however, when an excessive amount of ROS are produced or defense mechanisms are impaired, oxidative stress leading to events such as lipid peroxidation may occur [1–3].

A role for oxidative stress in periodontal disease has been suggested by studies in which various ROS detection methods were used to demonstrate increased levels of oxidative stress markers in periodontal disease patients relative to healthy controls [4]. It is possible that increased oxidative stress generated by polymorphonuclear leukocytes (PMN) upon interactions with chemoattractants, endotoxin, cytokines, and adhesion molecules leads to cell injury in periodontal tissue [5, 6]. ROS-induced oxidative stress has been implicated as a potential contributor at various stages in the pathogenesis of periodontal disease. Various reports have suggested that dietary antioxidant deficiency is a contributing factor to oxidative damage at the tissue level associated with periodontal disease [7]. The assessment of potential antioxidant therapies for periodontal disease requires the use of appropriate animal models and direct methods for measuring ROS and oxidative stress in vivo [e.g., electron spin resonance (ESR)].

ESR is one of the most powerful techniques for the detection of ROS in biological tissues and cells. We have developed an ESR-based technique to detect ROS in

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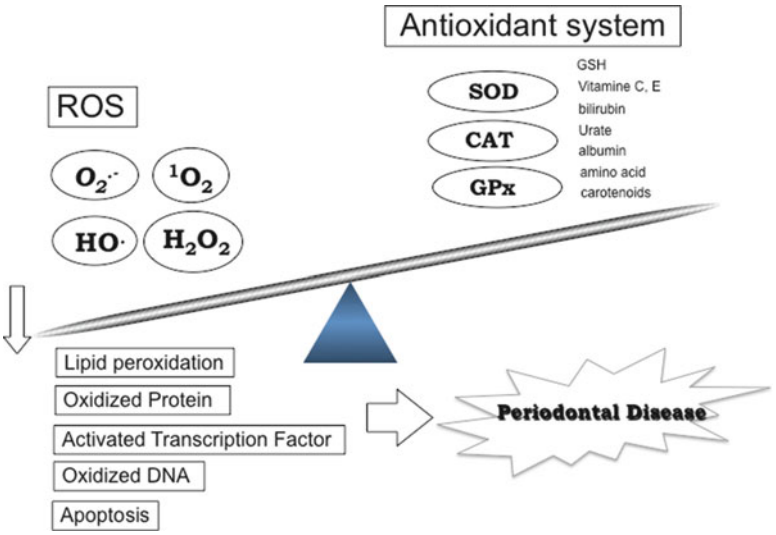

Fig. 1.1 Reactive oxygen species (ROS) and antioxidant system in periodontal disease. *SOD* superoxide dismutase, *CAT* catalase, *GPx* glutathione peroxidase. The disruption of redox balance between ROS and antioxidant system may lead to ROS-induced disease, including periodontal disease

biological systems in vitro and in vivo, including animal models of periodontitis [8–10]. This chapter focuses on the involvement of ROS-mediated oxidative stress in the pathogenesis of periodontal disease and, in particular, how disruption of the balance between oxidants and antioxidant defense systems contributes to the development of periodontal disease (Fig. 1.1). In addition, the use of ESR and other measurement techniques can be used to screen antioxidant compounds for potential use in the clinical treatment of human periodontal disease.

1.2 Reactive Oxygen Species

The biochemical characteristics of different ROS determine their ability to react with specific cellular substrates within the microenvironment in which they are produced. A free radical is defined as any atomic or molecular species capable of independent existence that contains one or more unpaired electrons. Molecular oxygen (O_2) itself qualifies as a free radical because it has two unpaired electrons. O_2 is capable of accepting electrons to its antibonding orbitals, becoming reduced in the process, and, therefore, functioning as a strong oxidizing agent. A one-electron reduction of O_2 results in the formation of superoxide ($O_2^{\cdot-}$) either by enzymatic catalysis or by electron leaks from various electron transfer reactions. $O_2^{\cdot-}$ chemistry in aqueous solution differs greatly from that in organic solvents. In contrast to its remarkable stability in many organic solvents, $O_2^{\cdot-}$ in aqueous solution is

short-lived. This instability in aqueous solutions is based on the rapid dismutation of $O_2^{\cdot-}$ to H_2O_2, a reaction facilitated by higher concentrations of the protonated form of $O_2^{\cdot-}$ ($HO_2\cdot$) in acidic pH conditions. Thus, the dismutation reaction has an overall rate constant of 5×10^5 M^{-1} s^{-1} at pH 7.0. The enzyme superoxide dismutase (SOD) speeds up this reaction almost tenfold (rate constant = 1.6×10^9 M^{-1} s^{-1}) [11]. This implies that any reaction of $O_2^{\cdot-}$ in aqueous solution will be in competition with SOD or, in its absence, with the spontaneous dismutation reaction itself. Nitric oxide (NO·) reacts with $O_2^{\cdot-}$ at near diffusion-limited rates and is, therefore, one of the few biomolecules that is able to outcompete SOD for $O_2^{\cdot-}$ [12]. Thus, in most biological systems, unless sufficiently high concentrations of NO· or other similarly reactive molecules are present, generation of $O_2^{\cdot-}$ usually results in the formation of H_2O_2.

Although dismutation of $O_2^{\cdot-}$ probably accounts for much of the H_2O_2 produced by eukaryotic cells, H_2O_2 can also be formed by direct two-electron reduction of O_2, a reaction mechanism shared by a number of flavoprotein oxidases [13]. Unlike $O_2^{\cdot-}$, H_2O_2 is not a free radical and is a much more stable molecule. H_2O_2 is able to diffuse across biological membranes, whereas $O_2^{\cdot-}$ does not, and H_2O_2 is a weaker oxidizing agent than $O_2^{\cdot-}$. However, in the presence of transition metals such as iron or copper, H_2O_2 can give rise to the indiscriminately reactive and toxic hydroxyl radical (OH·) by Fenton reaction [14].

ROS can rapidly modify cell constituents via oxidation of lipids, proteins, and DNA (Fig. 1.1). Oxidation of DNA by ROS can lead to activation of various genes, including those that encode for transcription factors (Fig. 1.1). $O_2^{\cdot-}$ is converted to H_2O_2 spontaneously or enzymatically, which occurs more than 1,000 times faster, by a process involving one of the three isoforms of SOD [14]. H_2O_2 can undergo one-electron reduction to the highly evanescent and reactive OH· in the presence of reducing metal ions or, through two-electron reduction, it can be converted to water by catalase (CAT). Intracellular ROS originate from multiple sites, including the mitochondrial electron transport chain, cytochrome *P*-450 oxygenase, xanthine oxidase (XO), lipoxygenase, cyclooxygenase, and uncoupled NO· synthase (NOS) [15]. The enzyme nicotinamide adenine dinucleotide phosphate oxidase (NOX) is a prominent source of ROS in vascular tissue and inflammatory cells. Several isoforms of NOX exist, localized to different sites within the cell. The catalytic subunit of NOX can be plasma lemmal bound and produce $O_2^{\cdot-}$ extracellularly or within the cytosol [16–18]. Extracellularly generated $O_2^{\cdot-}$ can reenter the cell through chloride ion channel-3 [19] or by conversion to H_2O_2 via extracellular SOD. NOX2 is most likely one of the key sources of ROS in periodontal tissues [20]. The NOX4 isoform is located in endosomes [21, 22], focal adhesions, and nuclei [23] and generates $O_2^{\cdot-}$ intracellularly. Other members of the NOX family include NOX1, which is found in various subcellular locations such as nuclei [24] and caveolae [23], and NOX3 and NOX5, both of which have been shown to colocalize with the plasma membrane [16, 25]. Thus, subcellular localization of NOX allows for stereospecific release of $O_2^{\cdot-}$, which is spontaneously or enzymatically (by SOD) converted to H_2O_2. As an uncharged molecule, H_2O_2 can traverse cell membranes, is rapidly inactivated by endogenous CAT and peroxiredoxins, and can reversibly alter enzyme function through oxidative modification of susceptible residues, including

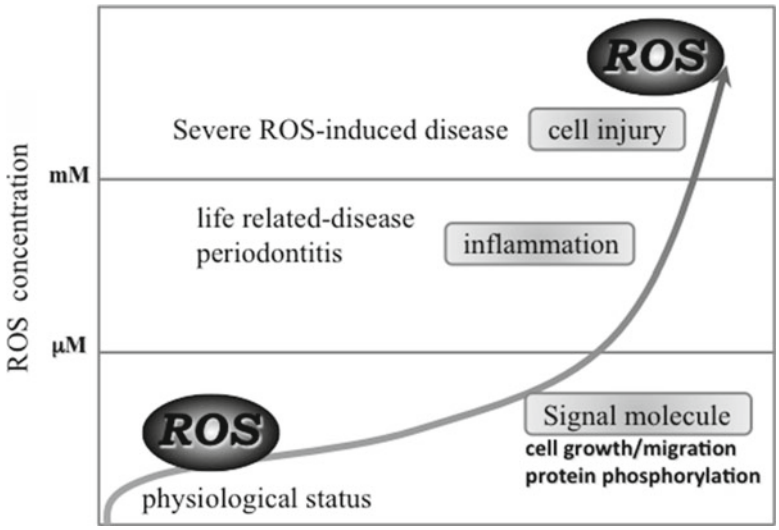

Fig. 1.2 Concentration-dependent effects of reactive oxygen species (ROS) on ROS-induced disease

arginine, cysteine, and histidine [26, 27]. These properties strongly support a signaling role for intermediate doses of H_2O_2. Signaling dose ranges for H_2O_2 have been established in human and animal models and vary from 1 μM to 10 mM (Fig. 1.2) [24, 28, 29].

ROS are implicated in the etiology of aging, angiogenesis, apoptosis, and a myriad of diseases including atherosclerosis, hypertension, hypercholesterolemia, obesity, cancer, diabetes mellitus, and neurodegenerative disorders [30–32]. The excessive production of ROS that occurs in certain pathological conditions promotes tissue inflammation and accelerates cell death and/or senescence. Under pathological conditions, ROS can reach high concentrations, often exceeding 500 μM for H_2O_2 at sites of inflammation or injury [33] (Fig. 1.2). $O_2^{\cdot-}$concentrations are tightly controlled by cytosolic Cu,Zn-SOD, which can rapidly lower $O_2^{\cdot-}$ concentrations from the nanomolar to the picomolar range [34]. Interestingly, pathological changes (including carcinogenesis) can also be evoked by excessively low levels of ROS, since they normally play a physiological role in proliferation, vasodilation, and host defense [35, 36]. The existence of a physiological role of ROS concentrations could explain some of the negative results from clinical trials in which large doses of exogenously administered antioxidants failed to improve outcomes of vascular disease [37]. There appears to be a physiological range of concentrations where intermediate levels of ROS can function as critical signaling molecules and mediate cellular growth, protein phosphorylation, and cell migration (Fig. 1.2) [38, 39]. This dose dependency should be considered when addressing the pathophysiological relevance of ROS, in diseases such as periodontal disease (Fig. 1.2).

1.3 Antioxidant Systems

The cellular response to oxidative stress involves the elimination of, protection against, and repair of damage caused by ROS. It has been shown in in vitro experiments that ROS induce lipid peroxidation, protein modification, enzyme inactivation, and DNA strand breakage/modification (Fig. 1.1). Scavenger antioxidant enzymes including SOD and CAT are responsible for the direct elimination of ROS, whereas systems that reconstitute antioxidants [e.g., glutathione (GSH) and GSH peroxidase (GPx)] can indirectly reduce ROS. Protection from oxidative damage can be achieved by a variety of mechanisms. Aerobic organisms are protected from oxidative stress by a well-organized defense system composed of various antioxidants with different functions. Hydroperoxides generate free radicals in the presence of transition metal ions such as iron and copper. Various proteins sequester such metal ions, while GPx reduces hydroperoxides and H_2O_2 to alcohols and water, respectively. CAT reduces H_2O_2 to water, while SOD quenches $O_2^{\cdot-}$ to yield water and O_2. These antioxidants function as the first line of defense against ROS [40]. Radical-scavenging antioxidants such as vitamin C, which is water soluble, and vitamin E, which is lipid soluble, function as the second line of defense against ROS [40]. Additionally, repair enzymes and de novo enzymes repair ROS-mediated damage, reconstitute tissues, and clear toxic waste materials [40]. Furthermore, there is an adaptation mechanism by which appropriate antioxidants are generated and transferred to the appropriate site when needed, and at the appropriate concentration. Molecules sensitive to oxidative stress can be surrounded by decoys that are preferentially oxidized, thereby protecting key cellular molecules (e.g., GSH, vitamin E, bilirubin, vitamin C, urate, albumin, and amino acids) [14]. Direct physical quenching of $O_2^{\cdot-}$ or other free radicals (e.g., by carotenoids) is another biological defense mechanism against oxidative stress [14]. Each of these antioxidant systems contributes to the local and global cellular redox balance, which has implications for cell signaling, cell growth, and cell death. Disruption of redox balance between ROS and antioxidant system may lead to ROS-induced disease, including periodontal disease (Fig. 1.1).

1.4 Oxidative Stress and Periodontal Disease

Periodontal diseases are common inflammatory conditions of the supporting apparatus of the teeth, which leads to early tooth loss. Various studies have reported that levels of markers of oxidative stress and oxidative damage are increased in periodontal disease cases relative to healthy controls [4]. PMN are the predominant leukocytes in blood and constitute the primary cellular host resistance factor against infection. In the oral cavity, following plaque accumulation and the development of clinical inflammation, there is an increase in the number of PMN in the gingival sulcus. However, a protective role of PMN in the pathophysiology of periodontal

disease has been suggested by the findings that severe periodontal disease occurs in patients with reduced PMN or impaired PMN function, and that individuals with early-onset or rapidly progressing forms of periodontal disease often exhibit relatively subtle neutrophil defects. However, most studies have not been able to demonstrate PMN defects in adult patients with various degrees of uncomplicated chronic periodontal disease [41]. NOX2 is most likely one of the key sources of ROS in periodontal tissues. The increased generation of ROS in periodontitis is thought to be caused by two factors: (1) genetically enhanced ROS generation and (2) oral pathogens that enhance NOX function [20]. Enhanced accumulation of PMN is associated with the upregulation of interleukin-8 (IL-8), intercellular adhesion molecule 1, interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) expression. In addition to macrophages, PMN and/or epithelial cells might also be important sources of IL-8, IL-1β, and TNF-α production in gingiva [6]. Gingival epithelial cells are likely to be highly susceptible to attack by PMN-derived oxidants [5]. PMN-mediated damage to human periodontal ligament-derived fibroblasts has been observed in PMN stimulated by f-met-leu-phe (FMLP) and endotoxins such as lipopolysaccharide (LPS) from periodontal pathogens. It is possible that increased oxidative stress generated from PMN stimulated by chemoattractants, endotoxin, cytokine, and/or adhesion molecules leads to cell injury in periodontal tissue.

Enhanced lipid peroxidation caused by oxidative stress may play a role in the inflammation and destruction of the periodontium in periodontal disease [42]. Previous reports indicate that it may be possible to measure oxidative stress markers such as salivary 8-hydroxydeoxyguanosine (8-OHdG) [43] and isoprostanes (IPs) [44] to reliably assess the degree of oxidative stress in periodontal patients. A study in a rat model of ligature-induced periodontal disease demonstrated increased plasma lipid peroxides, decreased hepatic GSH/GSSG ratio, and augmented ethanol-induced lipid peroxidation in the liver [45]. In addition, lipid peroxidation was increased in serum and aorta, as well as in periodontal tissue, and induction of atherosclerosis-related gene expression, histological changes, and lipid peroxidation occurred in the aorta [46]. Our laboratory reported that vascular endothelial function was decreased in animals with diabetes mellitus and/or periodontal disease due to increased oxidative stress in the gingival circulation [9]. Subsequent to the demonstration of a correlation between vascular disease (e.g., diabetes) and periodontal disease, this relationship and the role of oxidative stress have been further explored using animal disease models. It has recently been demonstrated that the core of this complex line of defense is a family of transcription factors, known as forkhead box O (FOXO), which can bind to β-catenin and initiate a transcriptional program regulating cell apoptosis, DNA repair, and neutralization of oxidative stress induced by ROS. An increase in ROS due to age or insulin resistance created a situation in which bone formation is impaired by activation of FOXO, and a decrease in Wnt signaling and bone resorption are promoted. The balance between FOXO and the Wnt pathway is finely tuned by systemic and local factors, creating a far-reaching mechanism that dictates the fate of mesenchymal progenitors and regulates the homeostasis of bone, providing a rationale for the impairment of

systemic and alveolar bone maintenance clinically observed with age and metabolic diseases [47]. Future research on the balance between FOXO and the Wnt pathway in relation to oxidative stress-induced bone loss and the pathophysiology of periodontal disease could be of interest.

1.5 Antioxidants and Periodontal Disease

In chronic periodontal (CP) disease, SOD activity is increased in gingival tissue but not in gingival crevicular fluid (GCF) [48]. In rats and humans, a significant correlation exists between salivary antioxidant activity towards $O_2^{\cdot-}$ and the severity of periodontal disease. In addition, the degree of salivary antioxidant activity depends on the level of SOD activity [10]. Systemic and local levels of malondialdehyde (MDA), a marker of lipid peroxidation, are increased in cigarette smokers with periodontal disease. The decreased local SOD, GSH-Px, and CAT activities observed in periodontal disease patients may increase with smoking [49].

The levels of vitamin C in serum might have a relatively weak but statistically significant relationship with periodontal disease in elderly individuals [50]. Studies using a rat periodontal disease model indicate that systemic administration of vitamin C can minimize atherosclerosis, 8-OHdG levels, and the reduced:oxidized GSH ratio by decreasing oxidative stress induced by periodontal disease. It is therefore likely that vitamin C could be clinically beneficial for minimizing periodontal disease-induced oxidative stress by down-regulating inflammatory gene expression [45, 51]. Several previous reports have demonstrated a lack of effect of vitamin E on periodontal disease, although other studies have reported contradictory findings [7]. It has recently been reported that adjunctive vitamin E supplementation and administration of SOD improves periodontal healing [52]; however, another study showed that vitamin E does not prevent bone loss and induces anxiety in rats with ligature-induced periodontal disease [53].

Several previous reports have suggested that dietary antioxidant deficiency is related to oxidative damage of tissues in periodontal disease [7]. Green tea catechin has been shown to have a bactericidal effect against black-pigment gram-negative anaerobic rods, and the combined use of mechanical treatment and administration of green tea catechin using a slow-release local-delivery system was effective in improving periodontal status [54]. Administration of tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl), a cell membrane-permeable ROS scavenger, was shown to decrease all parameters of inflammation in a rat model of periodontal disease [55]. *N*-acetylcysteine (NAC), a precursor for the powerful antioxidant GSH, exerts anti-inflammatory effects in LPS-stimulated gingival fibroblasts, functioning at least in part via down-regulation of the MAPK pathway [56]. Baicalin, which is a flavonoid compound purified from the medicinal plant *Scutellaria baicalensis Georgi* protects against tissue damage in ligature-induced periodontal disease in rats [21]. Quercetin, a flavonol that exhibits antioxidant properties, was shown to reduce LPS-induced osteoclast formation as well as ligature-enhanced

periodontal inflammation and bone loss [57]. Proanthocyanidin, a novel flavanoid extracted from grape seeds, has been shown to enhance host resistance as well as to inhibit the actions of biological and mechanical irritants involved in the onset of gingivitis and progression of periodontal disease [58].

1.6 Clinical Significance of the Measurement of Oxidative Stress in Periodontal Disease

There is convincing evidence that ROS-mediated oxidative stress is critically involved in periodontal disease. Compounds that inhibit lipid peroxidation or scavenge ROS can prevent pathophysiological changes associated with periodontal disease and promote functional recovery from periodontal disease, as indicated by studies in experimental in vitro systems and in vivo animal models, as well as studies conducted in humans. The ability of antioxidants such as tempol, NAC, vitamin C, vitamin E, and foods factors to minimize the symptoms of any specific disease depends on the extent to which ROS are involved in the pathophysiology of the disease [21, 55–59]. Thus, the protective properties of anti-periodontal disease agents could be associated with their antioxidant/ROS scavenging properties. Demonstrating such a link, however, is difficult due to a lack of appropriate animal models of periodontal disease and suitable techniques for directly measuring ROS in vivo.

We previously developed an ESR-based technique for the assessment of oxidative stress and antioxidant properties in biological systems [8]. Our research showed that the ESR technique could be useful for measuring oxidative stress in vivo in a rat model of periodontal disease [9, 10]. The ESR technique was successfully employed to demonstrate that periodontal disease is related to impaired salivary antioxidant activity. Additional in vitro and in vivo research using the ESR techniques is needed to further characterize the antioxidant properties of agents for the treatment and prevention of periodontal disease. The successful use of ESR to measure oxidative stress in saliva samples from periodontal disease patients, as described above, suggests that this method could also be used to assess the antioxidant properties of drugs and foods and their impact on oxidative stress in patients with periodontal disease (Fig. 1.3).

1.7 Conclusion

Oxidative stress induced by ROS appears to contribute to the pathogenesis of periodontal disease due to stimulation of signaling pathways involved in the inflammatory response. Cellular damage in periodontal tissue may arise when excessive oxidative stress causes endogenous antioxidant defense mechanisms to become overwhelmed. Infection by periodontal pathogens results in the production of ROS by PMNs that have migrated from the vascular system to periodontal tissue. The

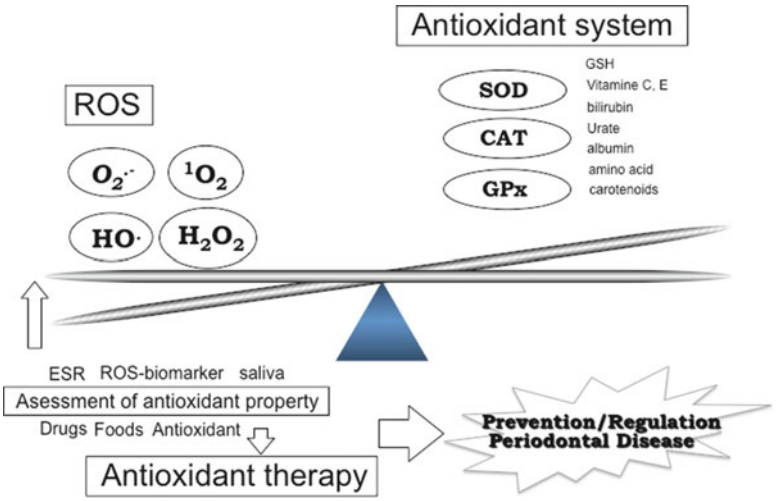

Fig. 1.3 Development of antioxidant therapies for periodontal disease. *ROS* reactive oxygen species, *ESR* electron spin resonance, *SOD* superoxide dismutase, *CAT* catalase, *GPx* glutathione peroxidase

pathophysiology of periodontitis is associated with oxidative stress induced by ROS generation in the vascular system and periodontal tissues, which results in the induction of various biochemical cascades leading to inflammatory damage. In addition, studies described in this chapter indicate that antioxidants can prevent pathophysiological changes associated with periodontal disease and promote functional recovery from the disease and recovery in animal models and in humans. Therapy with antioxidants may be effective for ROS-induced diseases such as periodontal disease. Additional research using direct ROS detection methods (e.g., ESR) in appropriate animal models is needed to identify potential therapeutic antioxidants for the management of periodontal disease.

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References

1. Halliwell B, Whiteman M (2004) Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? Br J Pharmacol 142:231–255
2. Sies H (1997) Oxidative stress: oxidants and antioxidants. Exp Physiol 82:291–295
3. Halliwell B (2009) The wanderings of a free radical. Free Radic Biol Med 46:531–542
4. Nibali L, Donos N (2013) Periodontitis and redox status: a review. Curr Pharm Des 19: 2687–2697

5. Altman LC, Baker C, Fleckman P et al (1992) Neutrophil-mediated damage to human gingival epithelial cells. J Periodontal Res 27:70–79
6. Liu RK, Cao CF, Meng HX et al (2001) Polymorphonuclear neutrophils and their mediators in gingival tissues from generalized aggressive periodontitis. J Periodontol 72:1545–1553
7. Battino M, Bullon P, Wilson M et al (1999) Oxidative injury and inflammatory periodontal diseases: the challenge of anti-oxidants to free radicals and reactive oxygen species. Crit Rev Oral Biol Med 10:458–476
8. Lee MC (2013) Assessment of oxidative stress and antioxidant property using electron spin resonance (ESR) spectroscopy. J Clin Biochem Nutr 52:1–8
9. Sugiyama S, Takahashi SS, Tokutomi FA et al (2012) Gingival vascular functions are altered in type 2 diabetes mellitus model and/or periodontitis model. J Clin Biochem Nutr 51:108–113
10. Yoshino F, Yoshida A, Wada-Takahashi S et al (2012) Assessments of salivary antioxidant activity using electron spin resonance spectroscopy. Arch Oral Biol 57:654–662
11. Fridovich I (1995) Superoxide radical and superoxide dismutases. Annu Rev Biochem 64:97–112
12. Huie RE, Padmaja S (1993) The reaction of no with superoxide. Free Radic Res 18:195–199
13. Massey V (1994) Activation of molecular oxygen by flavins and flavoproteins. J Biol Chem 269:22459–22462
14. Halliwell B, Gutteridge JMC (eds) (2007) Free radicals in biology and medicine, 4th edn. Oxford University Press, Oxford
15. Dikalov S, Griendling KK, Harrison DG (2007) Measurement of reactive oxygen species in cardiovascular studies. Hypertension 49:717–727
16. Serrander L, Jaquet V, Bedard K et al (2007) NOX5 is expressed at the plasma membrane and generates superoxide in response to protein kinase C activation. Biochimie 89:1159–1167
17. Frey RS, Ushio-Fukai M, Malik AB (2009) NADPH oxidase-dependent signaling in endothelial cells: role in physiology and pathophysiology. Antioxid Redox Signal 11:791–810
18. Gongora MC, Qin Z, Laude K et al (2006) Role of extracellular superoxide dismutase in hypertension. Hypertension 48:473–481
19. Hawkins BJ, Madesh M, Kirkpatrick CJ et al (2007) Superoxide flux in endothelial cells via the chloride channel-3 mediates intracellular signaling. Mol Biol Cell 18:2002–2012
20. Giannopoulou C, Krause KH, Muller F (2008) The NADPH oxidase NOX2 plays a role in periodontal pathologies. Semin Immunopathol 30:273–278
21. Cai X, Li C, Du G et al (2008) Protective effects of baicalin on ligature-induced periodontitis in rats. J Periodontal Res 43:14–21
22. Miller FJ Jr, Filali M, Huss GJ et al (2007) Cytokine activation of nuclear factor kappa B in vascular smooth muscle cells requires signaling endosomes containing Nox1 and ClC-3. Circ Res 101:663–671
23. Hilenski LL, Clempus RE, Quinn MT et al (2004) Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 24:677–683
24. Chamulitrat W, Schmidt R, Tomakidi P et al (2003) Association of gp91phox homolog Nox1 with anchorage-independent growth and MAP kinase-activation of transformed human keratinocytes. Oncogene 22:6045–6053
25. Nakano Y, Banfi B, Jesaitis AJ et al (2007) Critical roles for p22phox in the structural maturation and subcellular targeting of Nox3. Biochem J 403:97–108
26. Kuo LY, Hwang GY, Yang SL et al (2004) Inactivation of *Bacillus stearothermophilus* leucine aminopeptidase II by hydrogen peroxide and site-directed mutagenesis of methionine residues on the enzyme. Protein J 23:295–302
27. Stadtman ER, Berlett BS (1997) Reactive oxygen-mediated protein oxidation in aging and disease. Chem Res Toxicol 10:485–494
28. Kang LS, Reyes RA, Muller-Delp JM (2009) Aging impairs flow-induced dilation in coronary arterioles: role of NO and H_2O_2. Am J Physiol Heart Circ Physiol 297:H1087–H1095
29. Thengchaisri N, Kuo L (2003) Hydrogen peroxide induces endothelium-dependent and -independent coronary arteriolar dilation: role of cyclooxygenase and potassium channels. Am J Physiol Heart Circ Physiol 285:H2255–H2263

30. Csordas G, Hajnoczky G (2009) SR/ER-mitochondrial local communication: calcium and ROS. Biochim Biophys Acta 1787:1352–1362
31. Muller G, Morawietz H (2009) NAD(P)H oxidase and endothelial dysfunction. Horm Metab Res 41:152–158
32. Storz P (2006) Reactive oxygen species-mediated mitochondria-to-nucleus signaling: a key to aging and radical-caused diseases. Sci STKE 2006:re3
33. Hecquet CM, Ahmmed GU, Vogel SM et al (2008) Role of TRPM2 channel in mediating H_2O_2-induced Ca^{2+} entry and endothelial hyperpermeability. Circ Res 102:347–355
34. Wolin MS, Gupte SA, Oeckler RA (2002) Superoxide in the vascular system. J Vasc Res 39:191–207
35. Lu F (2007) Reactive oxygen species in cancer, too much or too little? Med Hypotheses 69:1293–1298
36. Vincent A, Crozatier M (2010) Neither too much nor too little: reactive oxygen species levels regulate *Drosophila* hematopoiesis. J Mol Cell Biol 2:74–75
37. Johansen JS, Harris AK, Rychly DJ et al (2005) Oxidative stress and the use of antioxidants in diabetes: linking basic science to clinical practice. Cardiovasc Diabetol 4:5
38. Rhee SG, Bae YS, Lee SR et al (2000) Hydrogen peroxide: a key messenger that modulates protein phosphorylation through cysteine oxidation. Sci STKE 2000:pe1
39. Ventura A, Pelicci PG (2002) Semaphorins: green light for redox signaling? Sci STKE 2002:pe44
40. Niki E (2010) Assessment of antioxidant capacity in vitro and in vivo. Free Radic Biol Med 49:503–515
41. Seymour GJ, Whyte GJ, Powell RN (1986) Chemiluminescence in the assessment of polymorphonuclear leukocyte function in chronic inflammatory periodontal disease. J Oral Pathol 15:125–131
42. Tsai CC, Chen HS, Chen SL et al (2005) Lipid peroxidation: a possible role in the induction and progression of chronic periodontitis. J Periodontal Res 40:378–384
43. Sawamoto Y, Sugano N, Tanaka H et al (2005) Detection of periodontopathic bacteria and an oxidative stress marker in saliva from periodontitis patients. Oral Microbiol Immunol 20:216–220
44. Wolfram RM, Budinsky AC, Eder A et al (2006) Salivary isoprostanes indicate increased oxidation injury in periodontitis with additional tobacco abuse. Biofactors 28:21–31
45. Tomofuji T, Ekuni D, Sanbe T et al (2009) Effects of vitamin C intake on gingival oxidative stress in rat periodontitis. Free Radic Biol Med 46:163–168
46. Ekuni D, Tomofuji T, Sanbe T et al (2009) Periodontitis-induced lipid peroxidation in rat descending aorta is involved in the initiation of atherosclerosis. J Periodontal Res 44:434–442
47. Galli C, Passeri G, Macaluso GM (2011) FoxOs, Wnts and oxidative stress-induced bone loss: new players in the periodontitis arena? J Periodontal Res 46:397–406
48. Akalin FA, Toklu E, Renda N (2005) Analysis of superoxide dismutase activity levels in gingiva and gingival crevicular fluid in patients with chronic periodontitis and periodontally healthy controls. J Clin Periodontol 32:238–243
49. Tonguc MO, Ozturk O, Sutcu R et al (2011) The impact of smoking status on antioxidant enzyme activity and malondialdehyde levels in chronic periodontitis. J Periodontol 82:1320–1328
50. Amarasena N, Ogawa H, Yoshihara A et al (2005) Serum vitamin C-periodontal relationship in community-dwelling elderly Japanese. J Clin Periodontol 32:93–97
51. Ekuni D, Tomofuji T, Sanbe T et al (2009) Vitamin C intake attenuates the degree of experimental atherosclerosis induced by periodontitis in the rat by decreasing oxidative stress. Arch Oral Biol 54:495–502
52. Singh N, Narula SC, Sharma RK et al (2013) Vitamin E supplementation, superoxide dismutase status and outcome of scaling and root planing in chronic periodontitis patients: a randomized clinical trial. J Periodontol DOI:10.1902/job2013.120727:1-10

53. de Carvalho Rde S, de Souza CM, Neves JC et al (2013) Vitamin E does not prevent bone loss and induced anxiety in rats with ligature-induced periodontitis. Arch Oral Biol 58:50–58
54. Hirasawa M, Takada K, Makimura M et al (2002) Improvement of periodontal status by green tea catechin using a local delivery system: a clinical pilot study. J Periodontal Res 37:4 33–438
55. Di Paola R, Mazzon E, Zito D et al (2005) Effects of Tempol, a membrane-permeable radical scavenger, in a rodent model periodontitis. J Clin Periodontol 32:1062–1068
56. Kim do Y, Jun JH, Lee HL et al (2007) N-acetylcysteine prevents LPS-induced pro-inflammatory cytokines and MMP2 production in gingival fibroblasts. Arch Pharm Res 30:1283–1292
57. Cheng WC, Huang RY, Chiang CY et al (2010) Ameliorative effect of quercetin on the destruction caused by experimental periodontitis in rats. J Periodontal Res 45:788–795
58. Govindaraj J, Emmadi P, Deepalakshmi et al (2010) Protective effect of proanthocyanidins on endotoxin induced experimental periodontitis in rats. Indian J Exp Biol 48:133–142
59. Kasuyama K, Tomofuji T, Ekuni D et al (2011) Hydrogen-rich water attenuates experimental periodontitis in a rat model. J Clin Periodontol 38:1085–1090

Chapter 2
The Role of Protein Oxidative Modification in Periodontal Diseases

Ryutaro Isoda and Kenji Matsushita

2.1 Introduction

As is explained elsewhere in this book, almost all biological macromolecules including DNAs, RNAs, lipids, carbohydrates, and proteins are oxidated. In most cases, oxidation means damage, but recent discoveries have shown that oxidation of biomolecules is playing important roles in fine-tuning of our body as well, for example, regulations of transcription factors and signal transductions. At the same time, it has also been becoming clear that imbalance of such fine-tuning can lead to complicated diseases.

In this regard, involvement of oxidative stress-induced post-translational modification (OPTM) in various disorders is drawing the interest in research. Different from other post-translational protein modifications such as glycosylations or phosphorylations, which are programmed to occur as a part of protein maturation processes in our body, OPTM implies alterations or degradation of matured proteins. OPTM of proteins is enhanced in aging and stressed cells and arises under physiological conditions [1, 2].

Target readers of this review article are those who are interested in periodontal disease (researchers in the field of periodontology and dental students) and would like to enter the field of oxidation research. For advanced readers, other excellent review articles [1, 3, 4] on oxidation of other biomolecules or effects of reactive oxidative species (ROS) are available and are highly recommended. The goal of this review article is to provide basic information and concept of oxidative protein modification to the newcomers of this field.

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2.2 Basic Knowledge About Protein Oxidation

2.2.1 Definition of the Oxidation

In our body, protein oxidation can occur both enzymatically or non-enzymatically. The former is specific catalytic function of many enzymes in substrate-specific manner and usually is a part of physiological functions of our body. The latter is rather nonspecific and can cause unwanted effects to our body leading to various disorders. In this review, therefore, we set aside the former and focus on the latter.

Protein oxidation can be defined as the covalent modification of protein induced either directly by ROS or indirectly by reaction with secondary by-products of "oxidative stress" [5]. ROS can be defined as "molecules and ions of oxygen that have an unpaired electron, thus rendering them extremely reactive." Oxidative stress is defined as "a disturbance in the pro-oxidant–antioxidant balance in favor of the former, leading to potential damage" [3]. In other words, oxidative stress occurs when the balance of formation of oxidants exceeds the ability of antioxidant systems to remove ROS, which occurs when inflammatory phagocytes (e.g., neutrophils and macrophages) are activated to undergo an oxidative burst by exposure to a foreign agent or when pro-oxidant xenobiotics are introduced into the body. Under these conditions, biomolecules become subjected to attack by excess ROS and significant molecular and physiological damage can occur.

Free radicals have been defined as "any species capable of independent existence that contain one or more unpaired electrons" [6]. The term "free radical" and "ROS" is almost the same, but "ROS" has become more popular because it encompasses other reactive species which are not true radicals but are nevertheless capable of radical formation in the intra- and extracellular environments. The primary free radical in most oxygenated biological systems is the superoxide radical ($O_2^{\cdot-}$), which is in equilibrium with its protonated form, the hydroperoxyl radical ($HO_2\cdot$). Although $O_2^{\cdot-}$ is relatively unreactive in comparison with many other radicals, biological systems can convert it into other more reactive species, such as peroxyl (ROO·), alkoxyl (RO·), and hydroxyl (HO·) radicals.

2.2.2 S-*Nitrosylation*

According to the above-mentioned definition, molecular oxygen (O_2) is not always necessary for oxidation, as long as the transfer of electrons occurs. Thus many different biochemical reactions, which include nitrosylation, peroxidization, are known to oxidate target molecules. Among them, *S*-nitrosylation is famous for its biological significance.

S-nitrosylation is the covalent attachment of a nitrogen monoxide group to the thiol side chain of cysteine. This has emerged as an important mechanism for dynamic, post-translational regulation of most or all main classes of protein.

S-nitrosylation thereby conveys a large part of the ubiquitous influence of nitric oxide (NO) on cellular signal transduction and provides a mechanism for redox-based physiological regulation [7].

2.2.3 Metal-Catalyzed Oxidation

Theoretically, any one of the pro-oxidant systems is able to oxidate proteins; however, there is reason to believe that under normal conditions the metal-catalyzed oxidation systems are the major source of oxidation. This follows from the fact that hydrogen peroxide and alkylperoxides are the most common end products of most ROS-generating systems. These peroxides by themselves are relatively unreactive compounds. However, in the presence of the transition metals, Fe(II) or Cu(I), they are converted to the highly reactive hydroxyl radical [reaction (2.1)] or alkoxyl radical [reaction (2.2)] which are capable of reacting with almost any organic substance.

$$\mathrm{H_2O_2} + Fe(II)/Cu(\mathrm{I}) \rightarrow HO\cdot + OH^- + Fe(III)/Cu(\mathrm{I}) \tag{2.1}$$

$$ROOH + Fe(II)/Cu(\mathrm{I}) \rightarrow RO\cdot + OH^- + Fe(III)/Cu(\mathrm{I}) \tag{2.2}$$

Indeed, virtually all kinds of amino acid residues of proteins are potential targets for oxidation by HO· generated by ionizing radiation or by high concentrations of H_2O_2 and Fe(II) [8, 9]. However, at the low concentrations of iron or copper ions and H_2O_2 present under most physiological conditions, protein damage is likely limited to the modification of amino acid residues at metal-binding sites on the protein, which effectively concentrate the ions. This consideration gave rise to the proposition that the oxidation of proteins under physiological conditions is a site-specific process in which the binding of Fe(II) or Cu(I) to metal-binding sites on the protein is followed by reaction with peroxides to generate reactive species (OH·, RO·, perferryl radical) that will react preferentially with amino acid residues at the metal-binding site [10–13]. Thus, the metal-binding site supports a biologically important "caged reaction." In the case of many enzymes, especially with those requiring a metal ion for activity, this will lead to loss of catalytic function [14].

2.2.4 Anti-oxidation System

Under normal circumstances, there is a well-organized balance between formation and neutralization of ROS so that there is minimal modification of biomolecules. Such balance is achieved by antioxidant systems which exist in our cells and tissues to scavenge or otherwise eliminate them. These include antioxidant enzymes such as catalase, superoxide dismutase, peroxiredoxins, and glutathione peroxidase as well as low-molecular-weight compounds such as vitamins C and E and reduced glutathione (GSH) [5].

2.2.5 Source of Pro-oxidants

Nonenzymatic oxidation of protein is caused by oxidative radicals and ROS which can be provided from exogenous and endogenous sources. Exogenous sources include heat, trauma, ultrasound, ultraviolet light, ozone, smoking, exhaust fumes, radiation, infection, excessive exercise, and therapeutic drugs [15–17]. Endogenous sources are primarily:

(1) By-products of metabolic pathways—electron leakage from mitochondrial electron transport systems forming superoxide;
(2) Functional generation by host defense cells (phagocytes) and cells of the connective tissues (osteoclasts and fibroblasts).

Cell metabolism involves the consumption of oxygen and its utilization via glycolysis to form pyruvate within the mitochondria. The amino acid cycle follows and ATP is generated. However, electrons leak from their transporters at a constant rate, reducing oxygen to the superoxide anion. The incomplete reduction of oxygen is estimated at 1–3 % of consumed oxygen [18, 19] and at a rate that exceeds the mitochondrial antioxidant scavenger's ability to remove superoxide. Superoxide dismutase 2 functions to remove the superoxide radicals that form. Nevertheless, mitochondrial DNA damage by ROS and reactive nitrogen species (RNS) still occurs and is a process believed to be important in certain chronic diseases and in aging [20].

Functional production of superoxide involves activation of the hexose-monophosphate (or NADPH-oxidase) shunt, which shunts glucose-6-phosphate from the glycolysis pathway and utilizes molecular oxygen and NADPH to form the superoxide radical anion (O_2^{-}). This process comprises the so-called respiratory burst within neutrophilic polymorphonuclear leukocytes (neutrophils) and is stimulated by a variety of mitogens/antigens/cytokines and other mediators such as granulocyte–macrophage colony-stimulating factor. Respiratory burst is known as an important killing mechanism in cells after phagocytosis of bacteria. Important activators are opsonized particles which activate Fcγ receptors (FcγR), bacterial DNA which can activate Toll-like receptors (e.g. TLR-4, TLR-9) [21], small peptides from bacteria, such as fMetLeuPhe, and protein kinase C agonists, such as phorbol myristate acetate. The NADPH-oxidase has a complex structure including cytosolic sub-units (e.g. p47phox, p40phox, p67phox), and sub-units which are bound within the lipid membrane (e.g. gp91phox, p22phox). The proximal pathways that link the cell surface receptors to the oxidase differ in temporal behavior and biochemical components, but the downstream pathways seem to converge at the cytosolic activation points of the NADPH-oxidase [22]. Therefore bacterial infection enhances production of superoxide mainly from infiltrated neutrophils leading to generation of oxidated proteins.

2.2.6 Biochemistry of Protein Oxidation

Oxidative modification on proteins can occur both in the backbone and the side chain of a protein. Oxidative modification in the backbone usually results in

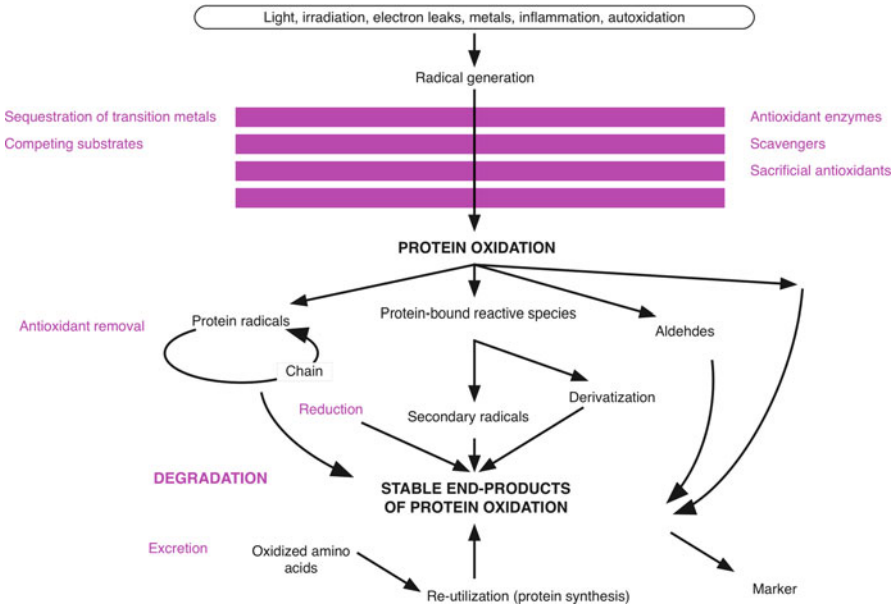

Fig. 2.1 Postulated mechanisms of protein modification in vivo. This figure is adapted from Dean et al. and shows postulated mechanisms of oxidative protein modifications in vivo. Processes depicted in color are those likely to result in the amelioration of protein damage in vivo

backbone fragmentation through primarily hydrogen atom abstraction at alpha carbon, while that in the side chain usually results in the formation of altered side-chains.

A variety of further consequences can occur after the initial oxidation of proteins. Usually the initial oxidation of protein caused by ROS leads to a formation of new reactive species (peroxides, DOPA) or further radicals which can oxidate sensitive biomolecules nearby. This reaction is repeated as a chain reaction until electrons of those ROS or radicals are absorbed by antioxidants. This process is often called propagation of oxidative effect.

The alteration of side chain of protein molecules can cause unfolding or conformational change. If this happens in multiple proteins simultaneously, dimerization or aggregation can also occur. Because structural conformation is critical for protein activities, those changes can affect in variety of functions of proteins. If this happens in enzymes or transcription factors, the functions of these molecules can be lost. This may affect numerous different cell functions including gene expressions, cell signaling, or induction of apoptosis and necrosis. Structural conformation is also important when proteins are recognized by protein processing systems in our body as well. Oxidative modifications of proteins can lead to alterations in cellular handling/turnover. This may further cause accumulation of abnormal proteins in cells which can be toxic [23, 24]. Above-mentioned consequences are summarized in a famous scheme (Fig. 2.1) created by Dean et al. [4].

2.2.7 Biological Characteristics of Protein Oxidation

For the most part, oxidatively modified proteins are not repaired and must be removed by proteolytic degradation, and a decrease in the efficiency of proteolysis will cause an increase in the cellular content of oxidatively modified proteins. The level of these modified molecules can be quantitated by measurement of the protein carbonyl content, which has been shown to increase in a variety of diseases and processes, most notably during aging. Accumulation of modified proteins disrupts cellular function either by loss of catalytic and structural integrity or by interruption of regulatory pathway.

2.2.8 Investigation

It is not easy to clarify the entire process in which protein modification affects. It is mainly because that protein oxidation is not a simple reaction. As mentioned above, oxidation often initiates multi-step chain reactions. Furthermore, intermediates in these reactions are highly unstable and have very short lives. Until recently, only possible way to detect the protein oxidation was measurements of protein carbonyls. Recently, increasing numbers of research tools have been introduced as exemplified by different monoclonal antibodies specific to oxidated motif such as malondialdehyde-oxidated-LDL, 4-hydroxy-2-nonenal (HNE), and hexanoyl-lysin (HEL). More and more developments of those useful tools are demanded.

2.2.9 The Involvement of OPTM in Periodontal Disease

One of the authors of this chapter has been exploring the involvement of OPTM in several different disorders. Several representative publications revealed that nitric oxide regulates exocytosis by *S*-nitrosylation or oxidation of Thiol-group on *N*-ethylmaleimide-sensitive factor (NSF) [25–29]. In another publication, it was demonstrated that innate immune response is controlled by the *S*-nitrosylation of MyD88 which is a key molecule of Toll-like receptor signal [30, 31]. Based on these experiences, it seems reasonable to think that OPTM is associated in the pathogeneses of PD as well as other chronic inflammatory diseases. Although numbers of papers have implied the involvement of oxidative stress in the pathogenesis of PD, those are mostly indirect and circumstantial.

In this review article, authors will focus on a new possible mechanism connecting OPTM and chronic inflammatory disease, which is breakdown of immunological tolerance. This mechanism is recently proposed [32, 33] and is implied to be involved in many chronic inflammatory diseases including atherosclerosis and autoimmune diseases. Authors would like to provide recent findings concerning this

mechanism and discuss about the possible involvement in the pathogenesis of both inflammatory bowel disease (IBD) and periodontal disease (PD).

2.3 The Role of Protein Oxidative Modification in Periodontal Diseases

2.3.1 Definition of the Target in This Discussion

As previously discussed, involvement of post-translational protein modification in various diseases has been proposed in recent years [1, 2]. In this chapter, we would like to discuss the possibility of its involvement in periodontal disease (PD). Before starting, we would like to define the target of our discussion. The term "periodontal disease" may involve a broad range of pathogeneses that arise in the area of periodontal tissue. In this review, we would like to use the term "periodontal disease" only to describe destructive chronic inflammation related to dental plaque, which consists of oral commensal bacteria and their biofilm. This category may include "chronic periodontitis", "aggressive periodontitis", and "periodontitis as a manifestation of systemic diseases" as they were identified in the most recent classification of periodontal disease by American Association of Periodontology [34].

2.3.2 The Changing Concept of the Pathogenicity of Periodontal Disease

In earlier days, when periodontal research was carried out mostly from the viewpoint of microbiology, periodontal disease was considered as an infection of specific causative bacteria and tissue destruction in this disease was thought to be caused by direct or indirect effects of specific pathogenic factors of those bacteria. Through the decades, however, this concept is changing. Nowadays, an increasing number of publications are suggesting that the direct effectors of the periodontal tissue destructions are components of the host immune system [35–41]. Although the old concept is still supported by many researchers, periodontopathic bacteria are now considered as a part of oral commensal bacteria rather than transient intruders.

All of the alimentary tract including oral cavity and colon lumen are covered by a heavy load of endogenous bacteria and are exposed to the flow of food. At the same time, mucosal surface of alimentary tract can be an entrance to foreign pathogenic bacteria. Therefore, it is currently accepted that antigen-specific immunological tolerance to those endogenous bacteria and food antigens exists so that systemic adaptive immune system would not overreact against those antigens, while at the same time maintaining reactivity to pathogenic bacteria [42].

It is still controversial if this "dogma" can be applied to oral commensal bacteria. For example, some researchers propose that immunological tolerance exists to oral commensal bacteria and breakdown of this tolerance may cause periodontal bone loss via RANKL-dependent manner [43]. Other researchers insist that *Porphyromonas gingivalis* (*P. gingivalis*), which is a representative periodontopathic oral bacteria, is not commensal bacteria, even though *P. gingivalis* can be isolated from healthy individuals. They explain that *P. gingivalis* is an opportunistic pathogen [44] rather than peaceful commensal bacteria. Thus, it may be fair to say that the recent concept regards periodontal disease as "chronic inflammatory disorder in which complex interaction of oral commensal bacteria and host immune system are involved."

2.3.3 IBD: Another Complicated Disorder in the Alimentary Tract

There is another complicated disorder in which aberrant host immune responses against endogenous bacteria are involved in the alimentary tract. This is known as IBD. IBD is a chronic inflammatory condition of the intestine, which is characterized by rectal bleeding, severe diarrhea, and weight loss. The two major types of IBD are ulcerative colitis (UC) and Crohn disease (CD). IBD used to be found mainly in Western countries, namely Europe and the USA, but the numbers of cases are increasing in other areas of the world. Accordingly, reliable etiological data from those recently emerging world regions has not yet been available. What we do know is that currently up to 1.4 million Americans have IBD, with ~30,000 new cases being diagnosed each year. At present, there is no cure for IBD. Over the long term, up to 75 % of patients with CD and 25–33 % of those with UC will require surgery. Although the clinical symptoms are similar, the pathological characteristics of these two diseases are quite different. UC is characterized by confluent inflammation of the colonic mucosa, extending to a variable extent from the rectum to the proximal colon. CD, on the other hand, is characterized by discontinuous transmural inflammation, involving any portion of the gastrointestinal tract, but most commonly the terminal ileum [45]. Even with these different characteristics, these two diseases are considered to share the similar mechanism of pathogenesis.

In 1995, Duchmann et al. [46] introduced the idea that immunological tolerance exists toward intestinal commensal bacteria and this tolerance was broken in active IBD. Before this paper, the relationship of commensal bacteria to host immune system was unknown or was thought to be ignored by the immune system: because alimentary tract is still outside of the body, i.e. beyond the area to be protected by the immune system. This paper evoked lively arguments concerning the pathogenesis of IBD, where at times their theory was vigorously scrutinized, but the principle concept of immunological tolerance exist toward intestinal commensal bacteria remained unaffected. The current general agreement is that IBD is the result of the interplay of at least four factors: genetic predisposition, an altered immune response, the microbial flora of the gut, and environmental factors that may act as a trigger of the disease manifestations [38].

For the genetic predisposition, specifically, large-scale genetic investigations have revealed that mutation in ATG16L1 (autophagy-related 16-like 1) gene has strong relation to the pathogenesis of CD [47, 48].

2.3.4 IBD and PD: Are These Share Similarities?

In 2001, Brandtzaeg pointed out the similarity of periodontal disease to IBD. In his review article [49], he described "both inflammatory disorders (IBD and PD) can apparently be explained by endogenous infection inducing hypersensitivity against commensal bacteria." His suggestion was, however, not well accepted in the dental research field. Investigations in line with this concept fell out of interest until 2010, when similarity of IBD and PD was finally revisited by Indriolo et al. [38].

One of the reasons why most researchers in periodontology field do not consider these two diseases to be similar may be due to the difference in their immunopathological characteristics. The periodontal disease lesion is characterized by the dominance of plasma cells and lymphocytes in the inflammatory infiltrate [50–57], while the majority of the infiltrates in IBD lesion are T cells. It should be noted that more detailed characteristics of the lesions of UC and CD are quite different as well. UC lesion is characterized by an atypical Th2 response, with elevated production of IL-13, while in CD predominates a Th1 type of immune response, dominated by overproduction of IFN-gamma. Even so, researchers in the field of gastroenterology still recognize them as the same pathogenesis. Therefore, it may be that periodontal researchers are simply overreacting to the pathological difference between IBD and PD. Perhaps periodontal researchers should set aside these differences and concentrate their effort on learning from the field of IBD research.

From the clinical point of view, both IBD and PD show similar natural history. In PD, the destruction of periodontal tissue does not occur at a constant rate: instead, there are cycles in which rapid tissue destruction occurs followed by slowing or even remission of the process. Socransky et al. [58] described this nature of PD and proposed a "random burst theory," which has been well accepted until now.

On the other hand, IBD is a refractory disease and frequent relapses occur after certain period of remissions [38]. It should be of note that the progresses of both diseases are not continuous. This may imply that the manifestations of both diseases arise from imbalance of delicate homeostasis. The homeostasis of immunological tolerance to endogenous flora may be disrupted at the onset of the diseases and can be returned to its balanced form when the cause disappears or at least becomes weak.

The above-mentioned "random burst theory" also comprises the site specificity. Even within a dentition in a patient, aggressive tissue destruction can occur in one site (i.e. periodontal pocket) while the adjacent site shows no sign of destruction. Similarly, CD is known to form discontinued lesions along the gastrointestinal tract in terminal ileum. This may mean that certain local factors are involved in the initiation or progress of both diseases, but so far, there are no good explanations why such site specificities exist in both diseases. Further research is necessary in order to elucidate the natural history of these two diseases.

As described so far, the similarity of IBD and PD is not well recognized in periodontal research filed. Indriolo et al. stated in their review paper, "To our knowledge, this paper is the first review evaluating the possible common pathogenic pathways between PD and IBD." The similarity of IBD and PD, however, seems more reasonable when the involvement of protein oxidation is taken into consideration.

2.3.5 Post-translational Protein Oxidization in IBD

As stated earlier, protein oxidation can be associated with aging and many chronic inflammatory diseases including atherosclerosis, rheumatoid arthritis, diabetes, and neurodegenerative diseases [59]. As new findings are accumulated year by year, several papers recently proposed that epitopes created through OPTM can induce a breakdown of immunological tolerance toward various antigens as well [32, 33, 60]. The underlying mechanism of this induction is molecular mimicry. Lipid and protein epitopes generated by lipid peroxidization (adducts) show cross-reactivities against DNA or epitopes expressed on cells undergoing apoptosis. Under normal conditions, antibodies against DNAs or apoptotic cells should not exist because they are self-reacting antibodies (Fig. 2.2). Oxidative modifications of biomolecules, however, may be able to generate new epitopes which have molecular similarity and may induce new antibodies which recognize self-antigens. These revelations expanded the possibility of OPTM involvement to chronic inflammatory diseases.

Some groups of researchers are also speculating the relationship between OPTM and IBD pathogenesis, but it has not been clearly demonstrated yet [45, 61].

Recent advancement in genetic research has provided supporting findings concerning OPTM involvement in IBD pathogenesis (Fig. 2.3). Etiological studies had implicated ATG16L1 protein to Crohn disease [48], thus suggesting that dysregulation of autophagy is one of the mechanisms of the pathogenesis of IBD. In 2008, Saito et al. clarified the mechanism of ATG16L1 in the pathogenesis of endotoxin-induced inflammatory diseases in mice. After that, involvement of more and more numbers of autophagy related genes was found in IBD patients.

It is currently considered that OPTM adducts are removed from our body by autophagy. If the function of autophagy is impaired in IBD due to other predisposed factors, it is plausible to think that accumulation of unprocessed OPTM adducts can occur and may sensitize the immune system leading to the production of harmful cross-reacting antibodies to both OPTM adducts and bacterial antigens. Candidates for cross-reacting bacterial epitopes which have been proposed so far are phosphocholine (PC) and cardiolipin. It can be possible that more cross-reacting antigens will be discovered in the future, however, given that specific immunological tolerance to PC and cardiolipin exists under usual circumstances and given that the breakdown of the tolerance to commensal bacteria is the principle pathogenesis of IBD, PC and cardiolipin are more than enough to be considered as the direct causative agents.

The regulation of immunological tolerance may not be so simple that the increase of the untreated OPTM adducts can easily breakdown the tolerance. It is also known,

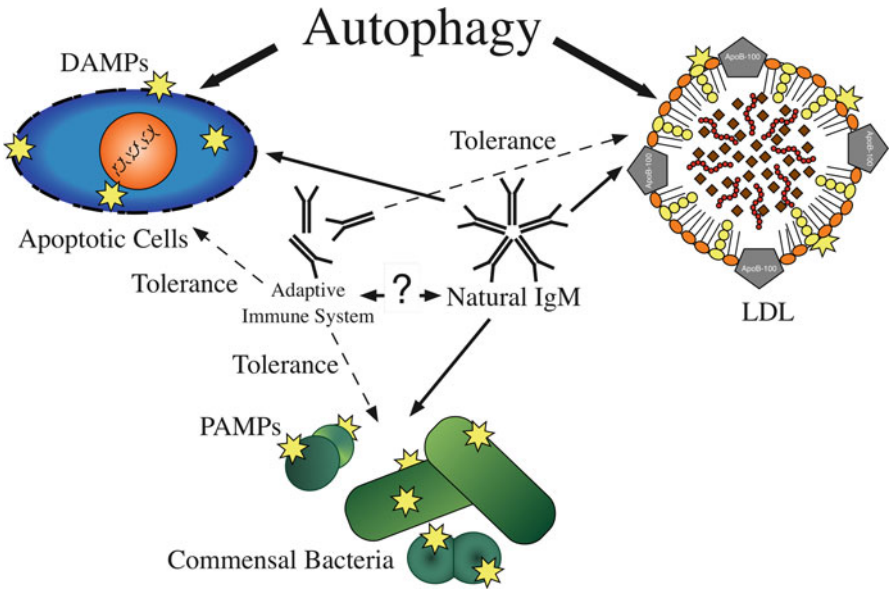

Fig. 2.2 Homeostasis of immune responses in healthy individual. This figure shows schematic expression of homeostasis of immune responses in healthy individual. Biomolecules (such as LDL) undergoes oxidative stress-induced post-translational modification (OPTM) and certain epitopes are converted to unusual forms. Such unusual epitopes (adduct) share molecular similarity with damage (Danger) associated molecular patterns (DMAPs) on apoptotic cells and pathogen associated molecular patterns (PAMPs) on commensal bacteria. This shared epitope is recognized by natural antibody whose subclass is IgM. Oxidatively modified proteins and apoptic cells are removed from cells and tissues by autophagy; therefore, the epitope does not evoke severe immune responses. At the same time, specific immunological tolerance exists towards commensal bacteria of our body

however, that both of those cross-reacting antigens, i.e. OPTM adducts and bacterial epitopes, are recognized by natural antibody IgM, which is one of the components of innate immunity. Therefore, there can be yet unknown regulatory mechanisms bridging innate and adaptive immune systems. This void in research further strengthens the need for future investigations.

2.3.6 Post-translational Protein Oxidization in PD

In case of PD, similar mechanisms which is underlying in the pathogenesis of IBD can be involved and this can be the reason why these two diseases show resembling natural history as described above (Fig. 2.4). Additionally, a noteworthy paper was published this year on the involvement of OPTM in PD. Turunen et al. [62] demonstrated that natural antibody IgM that is specific to one of the OPTM adducts,

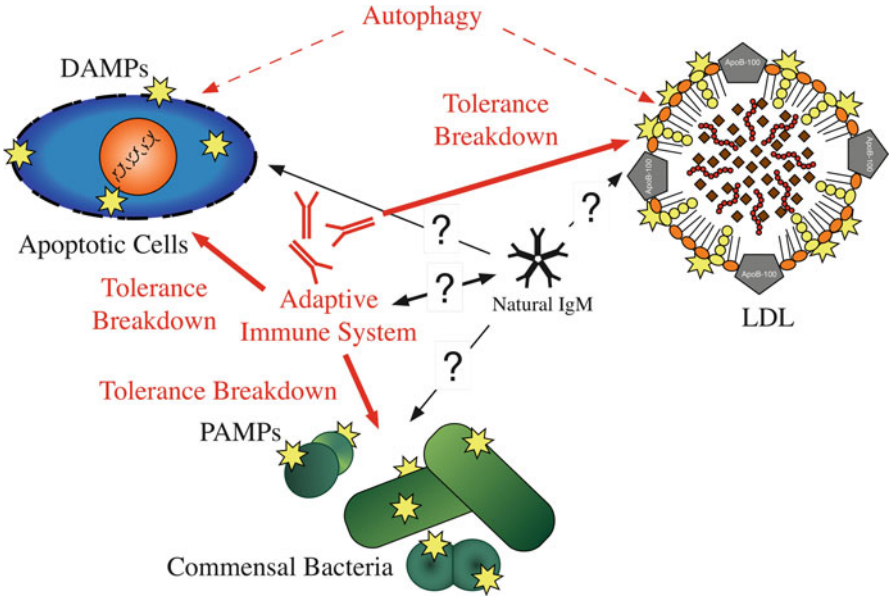

Fig. 2.3 Hypothetic involvement of OPTM in the pathogenesis of IBD. This figure shows schematic expression of the involvement of OPTM in IBD. Due to the predisposed factors, the function of autophagy declines in susceptible individuals. This leads to the accumulations of untreated modified proteins and apoptotic cells which further result in increase of shared epitope. This increase alters systemic immunological status and induce strong systemic immune response specific to the shared epitope. This immune response shows cross-reactivity with PAMPs on commensal bacteria and causes breakdown of immunological tolerance against commensal bacteria which is a proposed pathogenesis of IBD

malondialdehyde-oxidated molecule (MDA molecule) on oxidated LDL (OxLDL) has cross-reactivity against gingipain on *P. gingivalis* which is a major causative microbial agent of PD.

Although the aim of the authors was to suggest the potential roles of a natural antibody specific to this shared epitope in both atherosclerosis and PD, this paper provided a lot of implications to the pathogenesis of PD.

First of all, their data demonstrated that immunization with killed *P. gingivalis* whole cell or various fragments of gingipain was protective to atherogenesis, probably through diminishing uptake of OxLDL by macrophages. This result went against many previous publications reporting the positive relationship between atherosclerosis and *P. gingivalis* infection, because specific antibodies against major pathogenic factors are usually induced after infections. It is of note that the antibody subclass induced by *P. gingivalis* immunization was IgM class and gingipain-specific IgG antibody was not developed in either wild-type C57BL/6 or LDLR−/− mice. This finding is conflicting against the previous papers demonstrating that IgG class antibody could be induced by the immunization of gingipain [63–66]; however, it must be noted that either incomplete or complete Freund's adjuvant was used in the immunization in each of those papers, while adjuvant was not used in Turunen

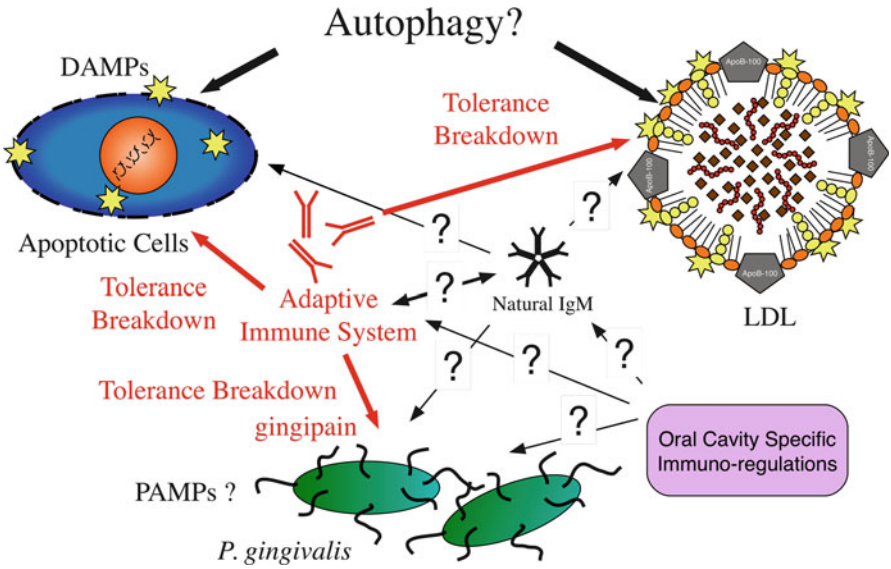

Fig. 2.4 Hypothetic involvement of OPTM in the pathogenesis of PD. This figure shows schematic expression of the involvement of OPTM in PD. In the case of PD, predisposed factors which lead to accumulation of oxidatively modified adducts have not yet firmly determined. Increase of OPTM is, however, reported to occur in various conditions including aging and inflammations. Gingipain, which is one of the major pathogenic factors of *P. gingivalis*, shares molecular similarity with one of the common adducts on oxidated LDL and increase of this adduct can breakdown immunological tolerance against gingipain. Involvement of other cross-reacting antigens (PAMPs) is still unknown. Oral cavity-specific immune-regulations may exist, but relationship with this regulation and natural IgM antibodies or adaptive immune systems is still under investigation

et al.'s experiment. Many researchers in periodontal research area insist that gingipain is such a powerful protease that can degrade immunoglobulins. This could be the reason for the failure of IgG Ab induction, but such a powerful protease has never been reported in any other bacteria. It is also difficult to explain why IgM subclass antibody was induced while IgG subclass was not. It may be more plausible to think that there is an yet identified regulatory mechanisms which is repressing the induction of gingipain-specific IgG antibodies. Furthermore, such regulatory mechanism can be canceled out by the usage of adjuvants.

It is also intriguing that immunizations with killed whole *P. gingivalis* or different fragments of gingipain were atheroprotective. Because specific antibodies against major pathogenic factors are usually induced after bacterial infections, Turunen et al.'s result did not agree with many other reports indicating that PD is a potential risk factor of atherosclerosis. This can be indicating that pathogenesis of PD is different from mere infection of periodontopathic bacteria. Because IgM antibody is a highly conserved subclass among wide variety of species, it may have other functions than mere protection from bacterial infection, for example, regulation of other immune responses including other components in innate and adaptive immune systems.

Finally and most importantly, it is possible that results from Turunen et al.'s paper are indicating that specific immunological tolerance against gingipain on *P. gingivalis* can be broken down when an OPTM adduct that shows molecular mimicry to gingipain increases and starts inducing cross-reacting antibodies. The induced gingipain-specific antibodies can be exudated into gingival crevicular fluid and can initiate aberrant immune responses leading to chronic inflammation.

As briefly mentioned earlier, two bacterial epitopes are known to show molecular mimicry with OPTM adducts. They are phosphocholine (PC) and cardiolipin. Many bacteria express epitopes containing PC, including approximately 30 % of the strains colonizing the oral cavity such as *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, and *Streptococcus sanguis* [62, 67, 68]. Bacterial strains can be classified as PC-bearing or devoid of PC according to their ability to incorporate choline from the culture medium [69].

The significance of Turunen et al.'s paper is that they discovered a novel cross-reacting epitope on the major periodontophatic bacteria *P. gingivalis*. Interestingly, some *P. gingivalis* strain is devoid of PC [69], but bears this new cross-reacting antigen. This feature may make *P. gingivalis* a unique candidate causative agent among many oral commensal bacteria. This paper is providing a strong rationale to the involvement of protein oxidative modification in the pathogenesis of PD. Further investigations are expected if other putative periodontopathic bacteria have their own antigens which have molecular mimicry with OPTM adducts.

2.4 Conclusions

Association of protein oxidative modification in the pathogenesis of PD has been proposed many years. A new hypothesis of the potential mechanism explaining the association was discussed: it is the breakdown of immunological tolerance against the causative bacteria, which are commensal bacteria. This hypothesis can be applicable to both IBD and PD because both diseases can be caused by the breakdown of immunological tolerance to commensal bacteria. The breakdown of immunological tolerance to self-antigens caused by the accumulation of untreated OPTM adducts have been proposed in autoimmune diseases or chronic inflammatory diseases and the proposed underlying mechanism is molecular mimicries between self-antigens and OPTM adducts.

To our knowledge, this review article is the first one in which the above concept was introduced to IBD and PD research and discussed.

References

1. Stadtman ER, Levine RL (2000) Protein oxidation. Ann N Y Acad Sci 899:191–208
2. Stadtman ER (1992) Protein oxidation and aging. Science 257(5074):1220–1224

3. Chapple IL, Matthews JB (2007) The role of reactive oxygen and antioxidant species in periodontaltissuedestruction.Periodontol200043:160–232.doi:10.1111/j.1600-0757.2006.00178.x
4. Dean RT, Fu S, Stocker R, Davies MJ (1997) Biochemistry and pathology of radical-mediated protein oxidation. Biochem J 324(Pt 1):1–18
5. Shacter E (2000) Quantification and significance of protein oxidation in biological samples. Drug Metab Rev 32(3–4):307–326. doi:10.1081/DMR-100102336
6. Halliwell B (1991) Reactive oxygen species in living systems: source, biochemistry, and role in human disease. Am J Med 91(3C):14S–22S
7. Hess DT, Matsumoto A, Kim SO, Marshall HE, Stamler JS (2005) Protein S-nitrosylation: purview and parameters. Nat Rev Mol Cell Biol 6(2):150–166. doi:10.1038/nrm1569
8. Huggins TG, Wells-Knecht MC, Detorie NA, Baynes JW, Thorpe SR (1993) Formation of o-tyrosine and dityrosine in proteins during radiolytic and metal-catalyzed oxidation. J Biol Chem 268(17):12341–12347
9. Neuzil J, Gebicki JM, Stocker R (1993) Radical-induced chain oxidation of proteins and its inhibition by chain-breaking antioxidants. Biochem J 293(Pt 3):601–606
10. Bachur NR, Gordon SL, Gee MV, Kon H (1979) NADPH cytochrome P-450 reductase activation of quinone anticancer agents to free radicals. Proc Natl Acad Sci U S A 76(2):954–957
11. Chevion M (1988) A site-specific mechanism for free radical induced biological damage: the essential role of redox-active transition metals. Free Radic Biol Med 5(1):27–37
12. Levine RL, Oliver CN, Fulks RM, Stadtman ER (1981) Turnover of bacterial glutamine synthetase: oxidative inactivation precedes proteolysis. Proc Natl Acad Sci U S A 78(4): 2120–2124
13. Stadtman ER (1990) Metal ion-catalyzed oxidation of proteins: biochemical mechanism and biological consequences. Free Radic Biol Med 9(4):315–325
14. Fucci L, Oliver CN, Coon MJ, Stadtman ER (1983) Inactivation of key metabolic enzymes by mixed-function oxidation reactions: possible implication in protein turnover and ageing. Proc Natl Acad Sci U S A 80(6):1521–1525
15. Canakci CF, Cicek Y, Canakci V (2005) Reactive oxygen species and human inflammatory periodontal diseases. Biochemistry (Moscow) 70(6):619–628. doi:10.1007/s10541-005-0161-9
16. Demple B, Harrison L (1994) Repair of oxidative damage to DNA: enzymology and biology. Annu Rev Biochem 63:915–948. doi:10.1146/annurev.bi.63.070194.004411
17. Halliwell B, Gutteridge JM, Cross CE (1992) Free radicals, antioxidants, and human disease: where are we now? J Lab Clin Med 119(6):598–620
18. Cadenas E (1989) Biochemistry of oxygen toxicity. Annu Rev Biochem 58:79–110. doi:10.1146/annurev.bi.58.070189.000455
19. Nohl H, Hegner D (1978) Do mitochondria produce oxygen radicals in vivo? Eur J Biochem/FEBS 82(2):563–567
20. Shigenaga MK, Hagen TM, Ames BN (1994) Oxidative damage and mitochondrial decay in aging. Proc Natl Acad Sci U S A 91(23):10771–10778
21. Weighardt H, Feterowski C, Veit M, Rump M, Wagner H, Holzmann B (2000) Increased resistance against acute polymicrobial sepsis in mice challenged with immunostimulatory CpG oligodeoxynucleotides is related to an enhanced innate effector cell response. J Immunol 165(8):4537–4543
22. Segal AW, Abo A (1993) The biochemical basis of the NADPH oxidase of phagocytes. Trends Biochem Sci 18(2):43–47
23. Davies MJ, Truscott RJ (2001) Photo-oxidation of proteins and its role in cataractogenesis. J Photochem Photobiol B Biol 63(1–3):114–125
24. Hawkins CL, Davies MJ (2001) Generation and propagation of radical reactions on proteins. Biochim Biophys Acta 1504(2–3):196–219
25. Jeong Y, Chaupin DF, Matsushita K, Yamakuchi M, Cameron SJ, Morrell CN, Lowenstein CJ (2009) Aldosterone activates endothelial exocytosis. Proc Natl Acad Sci U S A 106(10):3782–3787. doi:10.1073/pnas.0804037106
26. Matsushita K, Morrell CN, Cambien B, Yang SX, Yamakuchi M, Bao C, Hara MR, Quick RA, Cao W, O'Rourke B, Lowenstein JM, Pevsner J, Wagner DD, Lowenstein CJ (2003) Nitric

oxide regulates exocytosis by S-nitrosylation of N-ethylmaleimide-sensitive factor. Cell 115(2):139–150
27. Matsushita K, Morrell CN, Lowenstein CJ (2004) Sphingosine 1-phosphate activates Weibel–Palade body exocytosis. Proc Natl Acad Sci U S A 101(31):11483–11487. doi:10.1073/pnas.0400185101
28. Matsushita K, Morrell CN, Mason RJ, Yamakuchi M, Khanday FA, Irani K, Lowenstein CJ (2005) Hydrogen peroxide regulation of endothelial exocytosis by inhibition of *N*-ethylmaleimide sensitive factor. J Cell Biol 170(1):73–79. doi:10.1083/jcb.200502031
29. Matsushita K, Yamakuchi M, Morrell CN, Ozaki M, O'Rourke B, Irani K, Lowenstein CJ (2005) Vascular endothelial growth factor regulation of Weibel–Palade-body exocytosis. Blood 105(1):207–214. doi:10.1182/blood-2004-04-1519
30. Into T, Kanno Y, Dohkan J, Nakashima M, Inomata M, Shibata K, Lowenstein CJ, Matsushita K (2007) Pathogen recognition by Toll-like receptor 2 activates Weibel–Palade body exocytosis in human aortic endothelial cells. J Biol Chem 282(11):8134–8141. doi:10.1074/jbc.M609962200
31. Into T, Inomata M, Nakashima M, Shibata K, Hacker H, Matsushita K (2008) Regulation of MyD88-dependent signaling events by S nitrosylation retards Toll-like receptor signal transduction and initiation of acute-phase immune responses. Mol Cell Biol 28(4):1338–1347. doi:10.1128/MCB.01412-07
32. Chang MK, Binder CJ, Miller YI, Subbanagounder G, Silverman GJ, Berliner JA, Witztum JL (2004) Apoptotic cells with oxidation-specific epitopes are immunogenic and proinflammatory. J Exp Med 200(11):1359–1370. doi:10.1084/jem.20031763
33. Toyoda K, Nagae R, Akagawa M, Ishino K, Shibata T, Ito S, Shibata N, Yamamoto T, Kobayashi M, Takasaki Y, Matsuda T, Uchida K (2007) Protein-bound 4-hydroxy-2-nonenal: an endogenous triggering antigen of anti-DNA response. J Biol Chem 282(35):25769–25778. doi:10.1074/jbc.M703039200
34. International Workshop for a Classification of Periodontal Diseases and Conditions. Papers. Oak Brook, Illinois, October 30–November 2, 1999 (1999). Ann Periodontol (the American Academy of Periodontology) 4(1):i, 1–112. doi:10.1902/annals.1999.4.1.i
35. Ara T, Kurata K, Hirai K, Uchihashi T, Uematsu T, Imamura Y, Furusawa K, Kurihara S, Wang PL (2009) Human gingival fibroblasts are critical in sustaining inflammation in periodontal disease. J Periodontal Res 44(1):21–27. doi:10.1111/j.1600-0765.2007.01041.x
36. Brandtzaeg P, Kraus FW (1965) Autoimmunity and periodontal disease. Odontol Tidskr 73:281–393
37. Gemmell E, Yamazaki K, Seymour GJ (2002) Destructive periodontitis lesions are determined by the nature of the lymphocytic response. Crit Rev Oral Biol Med 13(1):17–34
38. Indriolo A, Greco S, Ravelli P, Fagiuoli S (2011) What can we learn about biofilm/host interactions from the study of inflammatory bowel disease. J Clin Periodontol 38(Suppl 11):36–43. doi:10.1111/j.1600-051X.2010.01680.x
39. Lamster IB, Novak MJ (1992) Host mediators in gingival crevicular fluid: implications for the pathogenesis of periodontal disease. Crit Rev Oral Biol Med 3(1–2):31–60
40. Teng YT (2006) Protective and destructive immunity in the periodontium: part 1—innate and humoral immunity and the periodontium. J Dent Res 85(3):198–208
41. Zaric S, Shelburne C, Darveau R, Quinn DJ, Weldon S, Taggart CC, Coulter WA (2010) Impaired immune tolerance to *Porphyromonas gingivalis* lipopolysaccharide promotes neutrophil migration and decreased apoptosis. Infect Immun 78(10):4151–4156. doi:10.1128/IAI.00600-10
42. Pabst O, Mowat AM (2012) Oral tolerance to food protein. Mucosal Immunol 5(3):232–239. doi:10.1038/mi.2012.4
43. Kawai T, Paster BJ, Komatsuzawa H, Ernst CW, Goncalves RB, Sasaki H, Ouhara K, Stashenko PP, Sugai M, Taubman MA (2007) Cross-reactive adaptive immune response to oral commensal bacteria results in an induction of receptor activator of nuclear factor-kappaB ligand (RANKL)-dependent periodontal bone resorption in a mouse model. Oral Microbiol Immunol 22(3):208–215

44. Bostanci N, Belibasakis GN (2012) Porphyromonas gingivalis: an invasive and evasive opportunistic oral pathogen. FEMS Microbiol Lett 333(1):1–9. doi:10.1111/j.1574-6968.2012.02579.x
45. Zhu H, Li YR (2012) Oxidative stress and redox signaling mechanisms of inflammatory bowel disease: updated experimental and clinical evidence. Exp Biol Med (Maywood) 237(5):474–480. doi:10.1258/ebm.2011.011358
46. Duchmann R, Kaiser I, Hermann E, Mayet W, Ewe K, Meyer zum Buschenfelde KH (1995) Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). Clin Exp Immunol 102(3):448–455
47. Hampe J, Franke A, Rosenstiel P, Till A, Teuber M, Huse K, Albrecht M, Mayr G, De La Vega FM, Briggs J, Gunther S, Prescott NJ, Onnie CM, Hasler R, Sipos B, Folsch UR, Lengauer T, Platzer M, Mathew CG, Krawczak M, Schreiber S (2007) A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. Nat Genet 39(2):207–211. doi:10.1038/ng1954
48. Rioux JD, Xavier RJ, Taylor KD, Silverberg MS, Goyette P, Huett A, Green T, Kuballa P, Barmada MM, Datta LW, Shugart YY, Griffiths AM, Targan SR, Ippoliti AF, Bernard EJ, Mei L, Nicolae DL, Regueiro M, Schumm LP, Steinhart AH, Rotter JI, Duerr RH, Cho JH, Daly MJ, Brant SR (2007) Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. Nat Genet 39(5):596–604. doi:10.1038/ng2032
49. Brandtzaeg P (2001) Inflammatory bowel disease: clinics and pathology. Do inflammatory bowel disease and periodontal disease have similar immunopathogeneses? Acta Odontol Scand 59(4):235–243
50. Afar B, Engel D, Clark EA (1992) Activated lymphocyte subsets in adult periodontitis. J Periodontal Res 27(2):126–133
51. Berglundh T, Liljenberg B, Lindhe J (2002) Some cytokine profiles of T-helper cells in lesions of advanced periodontitis. J Clin Periodontol 29(8):705–709
52. Berglundh T, Donati M (2005) Aspects of adaptive host response in periodontitis. J Clin Periodontol 32(Suppl 6):87–107. doi:10.1111/j.1600-051X.2005.00820.x
53. Celenligil H, Kansu E, Ruacan S, Eratalay K, Caglayan G (1993) In situ characterization of gingival mononuclear cells in rapidly progressive periodontitis. J Periodontol 64(2):120–127
54. Charon J, Toto PD, Gargiulo AW (1981) Activated macrophages in human periodontitis. J Periodontol 52(6):328–335
55. Hillmann G, Krause S, Ozdemir A, Dogan S, Geurtsen W (2001) Immunohistological and morphometric analysis of inflammatory cells in rapidly progressive periodontitis and adult periodontitis. Clin Oral Investig 5(4):227–235
56. Joachim F, Barber P, Newman HN, Osborn J (1990) The plasma cell at the advancing front of the lesion in chronic periodontitis. J Periodontal Res 25(1):49–59
57. Lappin DF, Koulouri O, Radvar M, Hodge P, Kinane DF (1999) Relative proportions of mononuclear cell types in periodontal lesions analyzed by immunohistochemistry. J Clin Periodontol 26(3):183–189
58. Socransky SS, Haffajee AD, Goodson JM, Lindhe J (1984) New concepts of destructive periodontal disease. J Clin Periodontol 11(1):21–32
59. Malhotra JD, Kaufman RJ (2007) Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? Antioxid Redox Signal 9(12):2277–2293. doi:10.1089/ars.2007.1782
60. Wuttge DM, Bruzelius M, Stemme S (1999) T-cell recognition of lipid peroxidation products breaks tolerance to self proteins. Immunology 98(2):273–279
61. Naito Y, Takagi T, Yoshikawa T (2007) Molecular fingerprints of neutrophil-dependent oxidative stress in inflammatory bowel disease. J Gastroenterol 42(10):787–798. doi:10.1007/s00535-007-2096-y
62. Turunen SP, Kummu O, Harila K, Veneskoski M, Soliymani R, Baumann M, Pussinen PJ, Horkko S (2012) Recognition of *Porphyromonas gingivalis* gingipain epitopes by natural IgM binding to malondialdehyde modified low-density lipoprotein. PLoS One 7(4):e34910. doi:10.1371/journal.pone.0034910

63. Genco CA, Odusanya BM, Potempa J, Mikolajczyk-Pawlinska J, Travis J (1998) A peptide domain on gingipain R which confers immunity against *Porphyromonas gingivalis* infection in mice. Infect Immun 66(9):4108–4114
64. Gibson FC 3rd, Genco CA (2001) Prevention of *Porphyromonas gingivalis*-induced oral bone loss following immunization with gingipain R1. Infect Immun 69(12):7959–7963. doi:10.1128/IAI.69.12.7959-7963.2001
65. Yasaki-Inagaki Y, Inagaki S, Yamada S, Okuda K, Ishihara K (2006) Production of protective antibodies against *Porphyromonas gingivalis* strains by immunization with recombinant gingipain domains. FEMS Immunol Med Microbiol 47(2):287–295. doi:10.1111/j.1574-695X.2006.00091.x
66. O'Brien-Simpson NM, Paolini RA, Reynolds EC (2000) RgpA-Kgp peptide-based immunogens provide protection against *Porphyromonas gingivalis* challenge in a murine lesion model. Infect Immun 68(7):4055–4063
67. Gmur R, Thurnheer T, Guggenheim B (1999) Dominant cross-reactive antibodies generated during the response to a variety of oral bacterial species detect phosphorylcholine. J Dent Res 78(1):77–85
68. Schenkein HA, Berry CR, Purkall D, Burmeister JA, Brooks CN, Tew JG (2001) Phosphorylcholine-dependent cross-reactivity between dental plaque bacteria and oxidized low-density lipoproteins. Infect Immun 69(11):6612–6617. doi:10.1128/IAI.69.11.6612-6617.2001
69. Schenkein HA, Gunsolley JC, Best AM, Harrison MT, Hahn CL, Wu J, Tew JG (1999) Antiphosphorylcholine antibody levels are elevated in humans with periodontal diseases. Infect Immun 67(9):4814–4818

Chapter 3
Association Between Oxidative Stress and Periodontal Diseases in Animal Model Studies

Manabu Morita, Daisuke Ekuni, and Takaaki Tomofuji

3.1 Introduction

Periodontal diseases include gingivitis and periodontitis. Periodontitis is a chronic inflammatory disease characterized by gingival bleeding, periodontal pocket formation, connective tissue destruction, and alveolar bone resorption leading to tooth loss [1, 2]. Oral bacterial pathogens are responsible for the initiation and progression of periodontitis [3, 4]. The pathogenesis of periodontal diseases involves host response to subgingival periodontal bacteria [e.g., *Porphyromonas gingivalis* (*P. gingivalis*), *Fusobacterium nucleatum* (*F. nucleatum*)] [5], which is identified as exaggerated inflammation that fails to remove the causative organisms and results in chronic non-resolving inflammation and tissue damage [6].

The generation of reactive oxygen species (ROS), namely superoxide, hydroxyl and nitric oxide radicals, hydrogen peroxide and hypochlorous acid, as well as nitric oxide (NO), represents an important pathogenic mechanism for diseases associated with phagocytic infiltration and bone resorption [7, 8] as the host defense mechanism against the invading pathogen [9]. The production of ROS is an essential protective mechanism against diseases [10, 11]. However, overproduced ROS oxidizes DNA, lipids, and proteins that contribute to tissue damage [12, 13]. Increasing evidence has indicated that periodontitis induces excessive ROS production in periodontal tissue [14–18]. Clinical studies have shown that periodontitis is correlated with increased lipid peroxidation in gingival crevicular fluid and saliva [14–16]. Randomized clinical trials have also shown that periodontal treatment reduces oxidative stress [17, 18]. In animal studies, periodontitis has been reported to induce

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oxidative DNA damage [19], lipid damage [20], and protein damage [20] in connective tissue and increase hydrogen peroxide in polymorphonuclear leukocytes and epithelium [21, 22].

Animal model studies have contributed to the generation of new knowledge in biological sciences. Research on the host response using animals is critically important for analysis of periodontitis and development of improved treatment protocols [23]. Furthermore, cause and effect relationships can be established by applying inhibitors or activators or through the use of genetically modified animals. Due to important ethical considerations, it is often difficult to perform such gain or loss of function studies in humans, particularly to obtain target tissue [24]. Various methods have been used to study the pathogenesis of periodontitis and to assess therapeutic modalities against the disease. The relationship between oxidative stress and periodontitis has also been investigated in animal models. In this chapter, we summarize this relationship and potential of animal models.

3.2 Animal Models Used in Periodontology

Animal models have been extensively used in periodontal research. The model has resulted in significant advances in our understanding of etiology, pathogenesis, prevention, and treatment [25].

An optimal animal model of periodontal diseases needs to be standardized, reproducible, and share some characteristics with humans, such as periodontal anatomy, etiology, pathophysiology, disease course, and clinical outcome [26]. Other desired attributes include availability and simplicity of handling. The most commonly used models are dogs and nonhuman primates (monkeys), although other animals (rats, mice, hamsters, rabbits, miniature pigs, ferrets, and sheep) have also been used [25]. The advantages and disadvantages of different animal models in periodontal research are summarized in Table 3.1.

Table 3.1 Advantages and disadvantages of different animal models in periodontal research[a]

Model	Research on oxidative stress	Established disease model	Anatomy	Spontaneous periodontal disease	Oral microflora	Genetic association study
Nonhuman primates	D	A	A	A	A	D
Dogs	D	A	A	A	A	D
Rats/mice	A	A	D	D	D	A
Hamsters	D	D	D	D	D	D
Rabbits	D	D	D	D	D	D
Miniature pigs	D	A	A	A	A	D
Ferrets	D	D	D	A	A	D
Sheep	D	D	A	A	A	D

A advantage, *D* disadvantage

[a]Table is modified from [26]

3.2.1 Nonhuman Primates

Nonhuman primates have been used in periodontology in several studies because of their anatomical, immunological, and microbiological similarity to the human oral cavity and periodontium [26]. The organization of collagen fibers in gingival and periodontal connective tissue is similar to that of humans [25]. Clinically, healthy monkey gingiva is histologically indistinguishable from human gingiva. Nonhuman primates have naturally occurring dental plaque, calculus, oral microbial pathogens (e.g., *P. gingivalis*), and periodontal diseases. Rhesus monkeys (*Macaca mulatta*), cynomolgus monkeys (*Macaca fascicularis*), and baboons (*Papio anubis*) are susceptible to naturally occurring periodontal diseases [27]. These animal models are considered the closest to humans in terms of periodontal diseases [25, 28]. Many studies have been carried out related to periodontal healing, filling with biomaterials, guided tissue regeneration, enamel matrix derivatives, and implant surgery [29, 30].

However, these models have some limitations. The naturally occurring periodontitis appears later in life, the lesions are asymmetrical, and the teeth and pocket depths are much smaller than in humans [25, 26]. The inflammatory infiltrate associated with periodontal diseases is microscopically similar to humans in some species such as the cynomolgus monkeys (*M. fascicularis*), but the squirrel monkeys (*Saimiri sciureus*) and marmosets have limited numbers of lymphocytes and plasma cells, making them inappropriate models for studying pathogenesis of periodontitis [27, 31–34]. Furthermore, to accelerate periodontitis, plaque accumulating devices, such as orthodontic elastic ligatures or sutures, should be placed apical to the interproximal region around selected molars [35]. Research on nonhuman primates is hindered by high costs, ethical considerations, difficulty in handling, aggressiveness, high susceptibility to infections and systemic illness, and the possibility of infectious agent transmission from and to these animals [25, 26].

3.2.2 Dogs

Dogs provide an appropriate model to study naturally occurring gingivitis and periodontitis [36]. Therefore dogs, particularly beagles, are used in dental research for the study of periodontal disease progression, guided tissue regeneration, tissue wound healing, and dental implants [23, 37]. In dogs, the subgingival plaque involves predominantly anaerobic gram (–) negative cocci and rods, *P. gingivalis* and *F. nucleatum*, similar to human bacteria [38, 39]. Histological traits of the normal and diseased periodontium are similar in humans and dogs [40]. Moreover, there is a high prevalence of periodontal diseases in the canine population [41, 42]. The severity of the disease increases with age and frequently results in tooth loss [23]. Susceptibility or resistance to periodontal diseases in different breeds is mainly dictated by genetic variations [43] rather than the diet [44]. In periodontal research,

in parallel with naturally occurring periodontal diseases, it is possible to induce experimental periodontal defects by placing silk bindings around the teeth for a period of 4–6 months or to use surgically created lesions [45].

Although the gingivitis and periodontitis lesions in dogs closely resemble those in humans, there are still differences [25]. Inflammatory lesions in dogs begin in the most coronal portion of the connective tissue at the gingival margin, rather than lateral and apical to the base of the gingival sulcus as in humans. With increasing severity of gingivitis, the entire thickness of the marginal gingiva is involved and not just the tissue lateral to the gingival pocket wall [46]. The subgingival microflora in beagle dogs with relatively healthy gingiva is different from humans with a high percentage of gram-negative bacteria. In periodontal diseases, there is an increase in catalase-positive *Prevotella melaninogenica*, which is not seen in humans, and a decrease in gram-negative facultative rods isolated from ligated sites [47]. Limitations of the use of a canine model includes great inter-animal variability, high expense, limited number of bony defects available, and faster bone formation [48]. Furthermore, animal care regulations, including daily companionship, exercise, space, and maintenance, make the use of dogs less desirable in periodontal studies [23].

3.2.3 Rats and Mice

Rats and mice have been used because of their small size, low cost, easy availability, ease of handling and housing, and the detailed knowledge of their genetic makeup. Additionally, they present some anatomical and histological similarities with the human periodontium and periodontal diseases [49].

Most histologic features of the epithelium and connective tissue in the rat are similar to those of humans except for the sulcular epithelium which is keratinized [50]. One of the most successful approaches to study oral disease in rats appears to be the utilization of the gnotobiotic or germ-free rat [25]. Gnotobiotic rats have been used to demonstrate the ability of various filamentous bacteria to form plaque and induce periodontal diseases in the absence of other bacteria [51]. There is evidence from the literature demonstrating horizontal bone loss in rats infected with *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans* (*A. actinomycetemcomitans*) [52–56] or *P. gingivalis* [54–57]. Periodontitis has been induced in rats by placing a bacterial plaque-retentive silk or cotton ligature in the gingival sulcus around the molar teeth [58]. In addition, alveolar bone loss has been induced by the injection of *P. gingivalis* [59] or topical application of bacterial pathogens [3, 21, 22, 60]. These animals can also be used in periodontal tissue regeneration and bone healing studies [45].

However, significant differences in oral cavity size, dental anatomy, oral microflora, inflammatory processes, and periodontal disease lesions have been observed in rats [25, 49]. The gingival response is only an acute immune infiltrate. Lesions induced by gram-negative bacteria showed minimal inflammation [25]. The connective tissue infiltrate contained primarily neutrophils, few lymphocytes,

and no plasma cells [25]. Thus, the destructive process in response to gram-negative bacteria can occur in the absence of a cell-mediated immune response [61], which is not similar to humans. Another difference between rat and human periodontal diseases is that instead of the lesion extending along the root surface as in man, the most apical extent of the lesion is located along the central part of the interdental tissues [25]. Bone loss could occur without apical migration of the junctional epithelium [62].

The use of gnotobiotic or germ-free mice also allows for the study of the individual effects of a particular bacterium without the interference of other microorganisms. The Baker mouse model of periodontitis has been used to measure alveolar bone resorption caused by oral bacterial inoculums as an outcome for the clinical presentation of periodontitis in humans [63]. To assess the virulence of periodontal pathogens, specific pathogen-free female mice were orally infected with strains of *A. actinomycetemcomitans* and/or *P. gingivalis* [64–66]. *P. gingivalis* partially initiated experimental periodontitis by modifying the endogenous subgingival biofilm to acquire enhanced virulence [67]. This model, however, may not reproduce all aspects of human periodontitis initiation and progression. The Chemically Induced Mouse Model is also used for inducing periodontal inflammation and alveolar bone loss by using trinitrobenzene sulfonic acid or dextran sulfate sodium [68, 69]. Implementation of the inflammatory bone resorption model will enable determination of ROS contribution to inflammatory disease lesions in the oral cavity. Furthermore, the use of knockout mouse models facilitates the exploration of new concepts regarding the pathogenesis of periodontal diseases [70, 71]. However, these mouse models have the same limitations as rat models.

3.2.4 Hamsters

Hamsters have been used mainly in bacteriological studies [72]. They have been used to demonstrate the transmissibility of periodontal diseases with plaque bacteria [73] and develop experimental periodontitis using ligatures around the molar teeth [72, 74, 75]. Periodontal diseases in hamsters are similar to those in rats, and therefore present the same limitations [23, 25].

3.2.5 Rabbits

Rabbits have been used in periodontal tissue regeneration studies [45, 72]. Characterization of the oral microorganisms in rabbits shows numerous pathogenic bacteria, such as *F. nucleatum*, *Prevotella heparinolytica*, *Micromonas micros*, and *Arcanobacterium haemolyticum*, which are somehow consistent with the flora related to periodontal diseases in humans [76]. However, rabbits do not exhibit the spontaneous form of periodontal diseases and there is no standardized model with respect to relevant aspects of periodontal disease pathogenesis [77].

3.2.6 Miniature Pigs

Miniature pigs are described as useful animal models in periodontal diseases [78, 79]. The animals are very similar to humans in oral and maxillofacial anatomy and inflammatory response. Miniature pigs develop spontaneous periodontal diseases with high prevalence at a young age. These animals have been used to test the regenerative capacity of periodontal tissues [78], the effects of dental lasers on periodontal healing, and dental implant surgery [80].

3.2.7 Ferrets

The domestic ferrets naturally develop calculus and their clinical characteristics of periodontal diseases are similar to humans [25, 81, 82]. The ferret has both a deciduous and a permanent dentition [25]. The tissues respond by characteristic inflammatory reactions, which are identical in all respects to those found in human gingivitis [81]. Calculus and plaque deposition and impingement on the gingival crest lead to loss of keratin and splitting of the junctional epithelium with pocket formation [25]. The animals appear to be good alternatives to dogs and primates in the ligature-induced periodontitis model [82, 83]. Ferrets are a suitable model to study calculus, because calculus formation in ferrets does not depend on the diet unlike rodents and can be scored in live ferrets [23, 81]. However, they easily escape from standard cages and thus need special maintenance [23]. Further studies are needed to confirm the use of this animal as an appropriate model for periodontology [23].

3.2.8 Domestic Sheep

Sheep naturally develop periodontitis [84]. The periodontium, oral microflora in periodontal diseases, and bone metabolism in sheep are similar to those of humans [49]. The model is suitable for training surgical methods and for guided tissue regeneration research [85, 86]. However, there are disadvantages, such as size, cost, and handling demands, as well as the challenging diagnosis of periodontal diseases as a result of poor access to posterior teeth [49, 84].

3.3 Oxidative Stress and Periodontitis in Animal Model Studies

In the periodontal tissue, host cells such as polymorphonuclear leukocytes produce ROS as part of the host defense against bacterial pathogens [87]. However, when ROS overwhelm the cellular antioxidant defense, damage to DNA, proteins, and

lipids in host tissue also occurs (oxidative stress) [88]. Here, we summarize the relationship between oxidative stress and periodontitis in animal model studies and show that periodontal oxidative stress increases with periodontal inflammation.

3.3.1 Rats

In a rat ligature-induced periodontitis (a traditional and established model), the animals showed apical migration of junctional epithelium, inflammatory cell infiltration in the connective tissue and bone resorption [20]. These histopathological changes suggested that oxidative damage occurred in the periodontium. Higher levels of 8-hydroxydeoxyguanosine (8-OHdG) (a marker of oxidative DNA damage), hexanoyl-lysine, malonaldehyde or thiobarbituric acid-reactive substances (TBARS) (a marker of lipid peroxidation), and nitrotyrosine (a marker of oxidative protein damage) were observed in periodontitis compared to the control [20, 89–98]. The levels of other markers related to oxidative stress, such as NOx, myeloperoxidase, and inducible nitric oxide synthase (iNOS), were also higher than those in the control [93, 94, 98–100]. Decreased reduced/oxidized glutathione ratio in gingiva was induced by periodontitis [90, 91, 93]. Furthermore, in a rat model, ligature-induced periodontitis showed a higher serum level of lipid peroxidation compared to the controls [92, 101, 102]. The serum level for hexanoyl-lysine was elevated in a time-dependent manner by ligature-induced periodontitis in rats. It is suggested that the progression of periodontitis induces oxidative stress not only in periodontium but also in circulating blood.

In another rat model, oxidative stress was also observed in the periodontium during the initiation of periodontitis. Periodontitis was induced after an 8-week daily-topical application of 25 μg/μl *Escherichia coli* lipopolysaccharide (LPS), suspension in pyrogen-free water, and 2.25 U/μl proteases from *Streptomyces griseus* daily by micropipette into the palatal gingival sulcus of both maxillary first molars within 1 h [3]. The levels of ROS and 8-hydroxydeoxyguanosine were higher in periodontitis than those of the control [21, 22]. An additional study has also been conducted that focused on the roles of epithelium in periodontitis [22]. After laser-capture microscopy for the isolation of epithelium, microarray analysis revealed that of the genes induced in response to disease onset (9,031 of 19,730) only 42 showed a greater than fourfold increase in expression [22]. Within the top 10 of this group, three genes, monoamine oxidase B (Maob, 5.72-fold) and flavin-containing monooxygenase 1 (Fmo1, 6.70-fold) and 2 (Fmo2, 7.26-fold) were involved in reactive oxygen signaling [22]. Conversely, no antioxidant genes were found to be up-regulated, but instead showed consistently decreased expression [22]. Taken together, these results indicate that LPS induced overall epithelial oxidative stress.

In rats, periodontitis was also induced by a single injection of LPS from *E. coli* LPS (10 μg/μl saline) into the mandibular gingiva [103]. The periodontitis group showed a significant increase in periodontal interleukin (IL)-1β, lipid peroxidation, 8-OHdG, apical migration of junctional epithelium, alveolar bone resorption, and number of polymorphonuclear leukocytes [103].

3.3.2 Mice

There are some infection and knockout (KO) models. Toll-like receptor (TLR) 2-deficient mice developed more severe periodontitis after *Aggregatibacter actinomycetemcomitans* (previously *Actinobacillus actinomycetemcomitans*) infection, characterized by significantly higher bone loss and inflammatory cell migration to periodontal tissues [104]. Phagocytosis and NO production were diminished in macrophages and neutrophils from these TLR2(−/−) mice [104].

Mice infected recurrently with live *F. nucleatum* synthesize a significant amount of NO between 12 and 24 h after *F. nucleatum* injection [105]. In these mice, the increase of the total cell numbers caused by an increase in neutrophils, a significant NO production only after injection of live *F. nucleatum* at 24 h and identification of iNOS positive macrophages were confirmed [106].

Oral administration of *P. gingivalis* caused alveolar bone loss in the maxilla of wild-type mice, but failed to induce such a change in iNOS KO mice [107]. Furthermore, repeated ingestion of *P. gingivalis* resulted in generalized production of NO in organs and NOx in plasma in mouse model [108, 109].

Alveolar bone dehiscence has been observed in KK-A(y) mice, which are metabolic syndrome model mice with type 2 diabetes [110]. The expression of endothelial nitric oxide synthase (eNOS) was decreased in gingival keratinocytes from KK-A(y) mice compared with gingival keratinocytes from control mice.

3.3.3 Dogs

There are few studies that show direct evidence for induction of oxidative stress by periodontal diseases. The total antioxidant capacities in gingival crevicular fluid have been related to the degree of severity of periodontal disease in dogs [36]. This is likely the result of release of ROS by activated phagocytes and fibroblasts in the inflamed periodontal tissues. However, further studies are required to determine the relationship between oxidative stress and periodontal diseases in the dog models.

3.4 Effects of Antioxidants in Animal Model Studies

Antioxidants include vitamins (carotenoids, vitamins C and E), minerals (selenium and zinc), or others, such as flavonoids, and their effects on periodontal diseases are one of the notable topics in periodontal research. Antioxidant enzymes (e.g., catalase, SOD, glutathione, etc.) and specific inhibitors of ROS also have effects on periodontal diseases. This chapter gives a brief overview of antioxidants in only animal models, because other chapters describe the details.

3.4.1 Vitamins

Vitamins are essential to maintain normal metabolic processes and homeostasis within the body. Carotenoids, vitamins C and E have been suggested to limit oxidative damage, thereby lowering the risk of certain chronic diseases, such as periodontal diseases.

The antioxidant effects of systemic administration of vitamin C on ligature-induced periodontitis in rats have been reported [111]. Vitamin C intake induced an improvement in the gingival 8-OHdG level (decreased) and in the reduced:oxidized glutathione ratio (increased) as well as reduction of inflammation [111]. Systemic administration of vitamin C could be clinically beneficial in improving periodontitis-induced oxidative stress.

In male albino rats, a deficiency of vitamin E did not cause increased destruction of the periodontium in the presence of periodontitis [112]. Moreover, no beneficial effects from the therapeutic use of vitamin E to combat periodontitis were found.

3.4.2 Flavonoids

Polyphenolic flavenoids are absorbed following dietary intake of primarily vegetables, red wine, and tea [87, 114], and their uses have been reviewed [113]. There are over 4,000 kinds of known flavonoids [87], including catechin and polyphenol.

Cocoa has become a material of interest as a therapeutic natural product due to its flavonoid content [115]. The consumption of a cocoa-enriched diet decreased the 8-OHdG level and increased the reduced form glutathione (GSH)/oxidized form glutathione (GSSG) in rat periodontitis [90]. Alveolar bone loss and polymorphonuclear leukocyte infiltration after ligature placement were also inhibited by cocoa intake.

Green tea catechins have been shown to possess potent antioxidant activity several times higher than that of vitamin C and vitamin E [116]. Topical application of a green tea catechin-containing dentifrice reduced inflammatory cell infiltration in the rat periodontal lesions to a greater degree than the control dentifrice [20]. The gingiva to which green tea catechin-containing dentifrice was applied also showed a lower level of expression of hexanoyl-lysine, nitrotyrosine, and tumor necrosis factor (TNF)-α compared to the control.

Proanthocyanidin, a novel flavanoid extracted from grape seeds, has been shown to provide a significant therapeutic effect on endotoxin (*E. coli*) induced experimental periodontitis in rats [117].

Baicalin (7-glucuronic acid, 5,6-dihydroxy-flavone) is a flavonoid compound purified from the medicinal plant, *Scutellaria baicalensis* Georgi, and reported to possess anti-inflammatory and antioxidant activities [118]. Baicalin protects against tissue damage in ligature-induced periodontitis in rats, which might be mediated by its inhibitory effect on the expression of cyclooxygenase-2 and inducible nitric oxide synthase [119].

3.4.3 Other Antioxidants

Molecular hydrogen, which selectively reduces cytotoxic ROS and oxidative stress, is considered to be a novel antioxidant [120]. Drinking water containing a therapeutic dose of hydrogen (hydrogen-rich water; HW) represents an alternative mode of delivery for molecular hydrogen. In a rat ligature model, hydrogen-rich water intake inhibited an increase in serum ROS level and lowered expression of 8-OHdG and nitrotyrosine in the periodontal tissue, which prevented polymorphonuclear leukocyte infiltration and osteoclast differentiation following periodontitis progression [121].

Thymoquinone (2-isopropyl-5-methyl-1, 4-benzoquinone), the main constituent of volatile oil from Nigella sativa seeds, has a range of pharmacologic properties; the antioxidant effect of thymoquinone is considered to be one of its most significant properties [122]. Oral administration of thymoquinone diminishes alveolar bone resorption in a rat periodontitis model.

Verbascoside has previously been characterized as an effective scavenger of active free radicals and an inhibitor of lipid peroxidation [123]. In ligature-induced periodontitis, oral administration of verbascoside significantly decreased the parameters of inflammation such as, myeloperoxidase activity, NF-κB expression, iNOS expression, the nitration of tyrosine residues, and the degree of gingivomucosal tissue injury [94].

Mangiferin (C2-β-D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone), generally called C-glucosyl xanthone, is widely distributed in higher plants and has antioxidant capacity [124]. When treated with mangiferin, the alveolar bone loss of rats with experimental periodontitis was remarkably reduced [125].

Calcium gluconate, in particular, has been used to treat injuries stemming from direct contact with hydrofluoric acid [126]. Daily oral treatment with calcium gluconate effectively inhibits ligature placement-induced periodontitis and related alveolar bone loss via antioxidant effects [127].

Oral aminoguanidine (an iNOS inhibitor) treatment significantly inhibited ligature-induced bone loss in rats [128]. Mercaptoethylguanidine and guanidinoethyldisulfide, which are iNOS inhibitors and reactive nitrogen scavenging compounds, significantly reduce gingival bleeding responses and protect against associated extravasetion and bone destruction [100, 129].

Aldose reductase is an NADPH-dependent oxidoreductase that catalyzes the reduction of a wide variety of aldehydes and ketones to their corresponding alcohols [130]. Aldose reductase inhibitors, tolrestat, imirestat, and quercetin, significantly reduced LPS-induced periodontitis in animals with and without diabetes to the level where they were not different from PBS-injected sites in normal diet controls [131].

N-acetylcysteine (NAC) is a thiolic antioxidant produced by the body, which serves as a precursor of glutathione synthesis [132]. NAC prevented alveolar bone loss in the rat ligature model, in a dose-dependent manner, when administered systemically [133].

Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl) exerted beneficial effects in animal models of shock, ischemia–reperfusion injury, inflammation,

hypertension, diabetes, and endothelial cell dysfunction [97]. Intraperitonial injection of Tempol significantly decreased all the parameters of inflammation in rat ligature-induced periodontitis [97].

M40403 [manganese(II)dichloro[(4R,9R,14R,19R)-3,10,13,20,26 pentaazatetracyclo[20.3.1.0.(4,9)0(14,19)]hexacosa-1(26),-22(23),24-triene]] is a low-molecular-weight, synthetic, manganese-containing superoxide dismutase (SOD) mimetic that removes superoxide anions without interfering with other reactive species known to be involved in inflammatory responses (e.g., NO and peroxynitrite). An intraperitoneal injection of M40403 significantly decreased inflammation markers in a rat model of periodontitis [134].

In experimental periodontitis, which was induced by elastic ligatures around teeth of beagle dogs, scaling and root planing with subgingival application of liposome-encapsulated SOD suppressed periodontal inflammation [135].

3.5 Limitations

Although animal models have provided a wide range of important data, it is sometimes difficult to determine whether the findings are applicable to humans [23]. There is no single model that represents all aspects of human periodontal disease. In addition, variability in host responses to bacterial infection among individuals contributes significantly to the expression of periodontal diseases [136]. A practical and highly reproducible model that truly mimics the natural pathogenesis of human periodontal diseases has yet to be developed [23].

3.6 Future Directions

Mechanistic questions cannot be typically addressed in human studies due to important ethical considerations. Thus, animal studies are critical for establishing cause and effect relationships and for initial tests of principle for the development of advanced therapeutics. The most important issue is whether a given model is suitable for studying a specific hypothesis [137].

Recently, a new mouse model for oxidative stress was developed [138]. In the model, an oxidative stress indicator in living cells, named OKD48 (Keap1-dependent Oxidative stress Detector, No-48), is dually regulated by induction at the transcriptional level, and by protein stabilization at the post-translational level in Keap1-Nrf2 pathway. The OKD48 transgenic mice expressing the indicator significantly exhibited signals upon oxidative stress. The results indicate the usefulness of the system as an indicator of oxidative stress. Using transgenic mice may be useful for exploring dynamics of oxidative stress by periodontal diseases and effective therapeutics.

3.7 Conclusion

Animal models have contributed new knowledge to periodontology. Rodents, rabbits, pigs, dogs, and nonhuman primates have been used to model human periodontitis, each with advantages and disadvantages. A number of studies in animal models of periodontitis, especially rodent models, support the notion that ROS have a critical role in periodontitis. Antioxidants attenuate periodontal inflammation and oxidative damage in animal models. These findings elucidate both the mechanism of periodontal disease progression and the role of oxidative stress on the disease.

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References

1. Page RC, Engel LD, Narayanan AS, Clagett JA (1978) Chronic inflammatory gingival and periodontal disease. JAMA 240:545–550
2. Pihlstrom BL, Michalowicz BS, Johnson NW (2005) Periodontal diseases. Lancet 366:1809–1820
3. Ekuni D, Tomofuji T, Yamanaka R, Tachibana K, Yamamoto T, Watanabe T (2005) Initial apical migration of junctional epithelium in rats following application of lipopolysaccharide and proteases. J Periodontol 76:43–48
4. Madianos PN, Bobetsis YA, Kinane DF (2005) Generation of inflammatory stimuli: how bacteria set up inflammatory responses in the gingiva. J Clin Periodontol 32:57–71
5. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr (1988) Microbial complexes in subgingival plaque. J Clin Periodontol 25:134–144
6. Van Dyke TE (2007) Cellular and molecular susceptibility determinants for periodontitis. Periodontol 2000 45:10–13
7. Yoon SO, Park SJ, Yoon SY, Yun CH, Chung AS (2002) Sustained production of H(2)O(2) activates pro-matrix metalloproteinase-2 through receptor tyrosine kinases/phosphatidylinositol 3-kinase/NF-kappa B pathway. J Biol Chem 277:30271–30282
8. Zaragoza C, Lopez-Rivera E, Garcia-Rama C, Saura M, Martinez-Ruiz A, Lizarbe TR, Martin-de-Lara F, Lamas S (2006) Cbfa-1 mediates nitric oxide regulation of MMP-13 in osteoblasts. J Cell Sci 119:1896–1902
9. Chapple IL (1997) Reactive oxygen species and antioxidants in inflammatory diseases. J Clin Periodontol 24:287–296
10. Akalın FA, Baltacıoğlu E, Alver A, Karabulut E (2007) Lipid peroxidation levels and total oxidant status in serum, saliva and gingival crevicular fluid in patients with chronic periodontitis. J Clin Periodontol 34:558–565
11. Hyslop PA, Hinshaw DB, Scraufstatter IU, Cochrane CG, Kunz S, Vosbeck K (1995) Hydrogen peroxide as a potent bacteriostatic antibiotic: implications for host defense. Free Radic Biol Med 19:31–37
12. Fialkow L, Wang Y, Downey GP (2007) Reactive oxygen and nitrogen species as signaling molecules regulating neutrophil function. Free Radic Biol Med 42:153–164
13. Valko M, Leibffritz D, Moncol J, Cronin MT, Mazur M, Telser J (2007) Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 39:44–84
14. Halliwell B (1994) Free radicals, antioxidants and human disease: curiosity, cause or consequence. Lancet 344:721–724

15. Chapple IL, Brock GR, Milward MR, Ling N, Matthews JB (2007) Compromised GCF total antioxidant capacity in periodontitis: cause or effect? J Clin Periodontol 34:103–110
16. Tsai CC, Chen HS, Chen SL, Ho YP, Ho KY, Wu YM, Hung CC (2005) Lipid peroxidation: a possible role in the induction and progression of chronic periodontitis. J Periodontal Res 40:378–384
17. Abou Sulaiman AE, Shehadeh RM (2010) Assessment of total antioxidant capacity and the use of vitamin C in the treatment of non-smokers with chronic periodontitis. J Periodontol 81:1547–1554
18. Barnes VM, Teles R, Trivedi HM, Devizio W, Xu T, Lee DP, Mitchell MW, Wulff JE, Milburn MV, Guo L (2010) Assessment of the effects of dentifrice on periodontal disease biomarkers in gingival crevicular fluid. J Periodontol 81:1273–1279
19. Tomofuji T, Azuma T, Kusano H, Sanbe T, Ekuni D, Tamaki N, Yamamoto T, Watanabe T (2006) Oxidative damage of periodontal tissue in the rat periodontitis model: effects of a high-cholesterol diet. FEBS Lett 580:3601–3604
20. Maruyama T, Tomofuji T, Endo Y, Irie K, Azuma T, Ekuni D, Tamaki N, Yamamoto T, Morita M (2011) Supplementation of green tea catechins in dentifrices suppresses gingival oxidative stress and periodontal inflammation. Arch Oral Biol 56:48–53
21. Ekuni D, Tomofuji T, Tamaki N, Sanbe T, Azuma T, Yamanaka R, Yamamoto T, Watanabe T (2008) Mechanical stimulation of gingiva reduces plasma 8-OHdG level in rat periodontitis. Arch Oral Biol 53:324–329
22. Ekuni D, Firth JD, Nayer T, Tomofuji T, Sanbe T, Irie K, Yamamoto T, Oka T, Liu Z, Vielkind J, Putnins EE (2009) Lipopolysaccharide-induced epithelial monoamine oxidase mediates alveolar bone loss in a rat chronic wound model. Am J Pathol 175:1398–1409
23. Oz HS, Puleo DA (2011) Animal models for periodontal disease. J Biomed Biotechnol 99:102–110
24. Graves DT, Kang J, Andriankaja O, Wada K, Rossa C Jr (2012) Animal models to study host-bacteria interactions involved in periodontitis. Front Oral Biol 15:117–132
25. Weinberg MA, Bral M (1999) Laboratory animal models in periodontology. J Clin Periodontol 26:335–340
26. Albuquerque C, Morinha F, Requicha J, Martins T, Dias I, Guedes-Pinto H, Bastos E, Viegas C (2012) Canine periodontitis: the dog as an important model for periodontal studies. Vet J 191:299–305
27. Schou S, Holmstrup P, Kornman KS (1993) Non-human primates used in studies of periodontal disease pathogenesis: a review of the literature. J Periodontol 64:497–508
28. Miller DR, Aufdemorte TB, Fox WC, Waldrop TC, Mealey BL, Brunsvold MA (1995) Periodontitis in the baboon: a potential model for human disease. J Periodontal Res 30: 404–409
29. Caton J, Mota L, Gandini L, Laskaris B (1994) Non-human primate models for testing the efficacy and safety of periodontal regeneration procedures. J Periodontol 65:1143–1150
30. Fritz ME, Braswell LD, Koth D, Jeffcoat M, Reddy M, Cotsonis G (1997) Experimental peri-implantitis in consecutively placed, loaded root-form and plate-form implants in adult *Macaca mulatta* monkeys. J Periodontol 68:1131–1135
31. Brecx MC, Nalbandian J, Ooya K, Kornman KS, Robertson PB (1985) Morphological studies on periodontal disease in the cynomolgus monkey. II. Light microscopic observations on ligature induced periodontitis. J Periodontal Res 20:165–175
32. Kornman KS, Holt SC, Robertson PB (1981) The microbiology of ligature-induced periodontitis in the cynomolgus monkey. J Periodontal Res 16:363–371
33. Listgarten M, Ellegaard B (1973) Experimental gingivitis in the monkey. J Periodontal Res 8:199–214
34. Page R, Schroeder H (1982) Periodontitis in man and other animals. A comparative review. Basel, Karger, pp 57–202, 222–239, 272
35. Madden TE, Caton JG (1994) Animal models for periodontal disease. Methods Enzymol 235:106–119

36. Pavlica Z, Petelin M, Nemec A, Erzen D, Skaleric U (2004) Measurement of total antioxidant capacity in gingival crevicular fluid and serum in dogs with periodontal disease. Am J Vet Res 65:1584–1588
37. Wikesjö UM, Kean CJ, Zimmerman GJ (1994) Periodontal repair in dogs: supraalveolar defect models for evaluation of safety and efficacy of periodontal reconstructive therapy. J Periodontol 65:1151–1157
38. Egelberg J (1965) Local effect of diet on plaque formation and development of gingivitis in dogs. I. Effect of hard and soft diets. Odontol Revy 16:31–41
39. Hamp SE, Lindhe J, Löe H (1973) Experimental periodontitis in the beagle dog. J Periodontal Res 10:13–14
40. Hamp SE, Lindberg R (1977) Histopathology of spontaneous periodontitis in dogs. J Periodontal Res 12:46–54
41. Sorensen WP, Löe H, Ramfjord SP (1980) Periodontal disease in the beagle dog. A cross sectional clinical study. J Periodontal Res 15:380–389
42. Lund EM, Armstrong PJ, Kirk CA, Kolar LM, Klausner JS (1999) Health status and population characteristics of dogs and cats examined at private veterinary practices in the United States. J Am Vet Med Assoc 214:1336–1341
43. Kwon DH, Bennett W, Herberg S, Bastone P, Pippig S, Rodriguez NA, Susin C, Wikesjö UM (2010) Evaluation of an injectable rhGDF-5/PLGA construct for minimally invasive periodontal regenerative procedures: a histological study in the dog. J Clin Periodontol 37:390–397
44. Page RC, Schroeder HE (1981) Spontaneous chronic periodontitis in adult dogs: a clinical and histopathological survey. J Periodontol 52:60–73
45. Struillou X, Boutigny H, Soueidan A, Layrolle P (2010) Experimental animal models in periodontology: a review. Open Dent J 4:37–47
46. Matsson L, Attström R (1979) Histologic characteristics of experimental gingivitis in the juvenile and adult beagle dog. J Clin Periodontol 6:334–350
47. Kornman KS, Siegrist B, Soskolne WA, Nuki K (1981) The predominant cultivable subgingival flora and beagle dogs following ligature placement and metronidazole therapy. J Periodontal Res 16:251–258
48. Giannobile WV, Finkelman RD, Lynch SE (1994) Comparison of canine and non-human primate animal models for periodontal regenerative therapy. Result following a single administration of PDGF/IGF-I. J Periodontol 65:1158–1168
49. Genco CA, Van Dyke T, Amar S (1998) Animal models for *Porphyromonas gingivalis-mediated* periodontal disease. Trends Microbiol 6:444–449
50. Listgarten MA (1975) Similarity of epithelial relationships in the gingiva of rat and man. J Periodontol 46:677–680
51. Socransky SS, Hubersak C, Propas D (1970) Induction of periodontal destruction in gnotobiotic rats by a human oral strain of *Actinomyces naeslundii*. Arch Oral Biol 15:993–995
52. Taubman MA, Stoufi ED, Seymour GJ, Smith DJ, Ebersole JL (1988) Immunoregulatory aspects of periodontal disease. Adv Dent Res 2:328–333
53. Yamashita K, Eastcott JW, Taubman MA, Smith DJ, Cox DS (1991) Effect of adoptive transfer of cloned *Actinobacillus actinomycetemcomitans*-specific T helper cells on periodontal disease. Infect Immun 59:1529–1534
54. Taubman MA, Yoshie H, Wetherell JR Jr, Ebersole JL, Smith DJ (1983) Immune response and periodontal bone loss in germfree rats immunized and infected with *Actinobacillus actinomycetemcomitans*. J Periodontal Res 18:393–401
55. Roeterink CH, van Steenbergen TJ, de Jong WF, de Graaff J (1984) Histopathological effects in the palate of the rat induced by injection with different black-pigmented Bacteroides strains. J Periodontal Res 19:292–302
56. Evans RT, Klausen B, Ramamurthy NS, Golub LM, Sfintescu C, Genco RJ (1992) Periodontopathic potential of two strains of *Porphyromonas gingival* is in gnotobiotic rats. Arch Oral Biol 37:813–819

57. Klausen B, Sfintescu C, Evans RT (1991) Asymmetry in periodontal bone loss of gnotobiotic Sprague–Dawley rats. Arch Oral Biol 36:685–687
58. Breivik T, Opstad PK, Gjermo P, Thrane PS (2000) Effects of hypothalamic–pituitary–adrenal axis reactivity on periodontal tissue destruction in rats. Eur J Oral Sci 108:115–122
59. Nakajima K, Hamada N, Takahashi Y, Sasaguri K, Tsukinoki K, Umemoto T, Sato S (2006) Restraint stress enhances alveolar bone loss in an experimental rat model. J Periodontal Res 41:527–534
60. Ekuni D, Yamamoto T, Yamanaka R, Tachibana K, Watanabe T (2003) Proteases augment the effects of lipopolysaccharide in rat gingiva. J Periodontal Res 38:591–596
61. Irving JT, Socransky SS, Heeley JD (1974) Histological changes in experimental periodontal disease in gnotobiotic rats and conventional hamsters. J Periodontal Res 9:73–80
62. Heijl L, Wennstrom J, Lindhe J, Socransky SS (1980) Periodontal disease in gnotobiotic rats. J Periodontal Res 15:405–419
63. Baker PJ, Evans RT, Roopenian DC (1994) Oral infection with *Porphyromonas gingivalis* and induced alveolar bone loss in immunocompetent and severe combined immunodeficient mice. Arch Oral Biol 39:1035–1040
64. Pierce DL, Nishiyama S, Liang S, Wang M, Triantafilou M, Triantafilou K, Yoshimura F, Demuth DR, Hajishengallis G (2009) Host adhesive activities and virulence of novel fimbrial proteins of *Porphyromonas gingivalis*. Infect Immun 77:3294–3301
65. Novak EA, Shao H, Daep CA, Demuth DR (2010) Autoinducer-2 and QseC control biofilm formation and in vivo virulence of *Aggregatibacter actinomycetemcomitans*. Infect Immun 78:2919–2926
66. Polak D, Wilensky A, Shapira L, Halabi A, Goldstein D, Weiss EI, Houri-Haddad Y (2009) Mouse model of experimental periodontitis induced by *Porphyromonas gingivalis Fusobacterium nucleatum* infection: bone loss and host response. J Clin Periodontol 36:406–410
67. Kinane DF, Hajishengallis G (2009) Polymicrobial infections, biofilms, and beyond. J Clin Periodontol 36:404–405
68. Oz HS, Ebersole JL (2010) A novel murine model for chronic inflammatory alveolar bone loss. J Periodontal Res 45:94–99
69. Oz HS, Chen T, Ebersole JL (2010) A model for chronic mucosal inflammation in IBD and periodontitis. Dig Dis Sci 55:2194–2202
70. Sasaki H, Okamatsu Y, Kawai T, Kent R, Taubman M, Stashenko P (2004) The interleukin-10 knockout mouse is highly susceptible to *Porphyromonas gingivalis*-induced alveolar bone loss. J Periodontal Res 39:432–441
71. Fine DH (2009) Of mice and men: animal models of human periodontal disease. J Clin Periodontol 36:913–914
72. Hojo K, Tamura A, Mizoguchi C, Kato D, Ohshima T, Maeda N (2008) Predominant bacteria recovered from a periodontitis site in a hamster model raised by silk-ligature with *Porphyromonas gingivalis* infection. Biosci Biotechnol Biochem 72:1348–1351
73. Jordan HV, Keyes PH (1964) Aerobic, grampositive, filamentous bacteria as etiologic agents of experimental periodontal disease in Hamsters. Arch Oral Biol 9:401–414
74. Baron R, Saffar JL (1978) A quantitative study of bone remodeling during experimental periodontal disease in the golden hamster. J Periodontal Res 13:309–315
75. Miller WA, Ripley JF (1975) Early periodontal disease in the Syrian hamster. J Periodontol 46:368–374
76. Tyrrell KL, Citron DM, Jenkins JR, Goldstein EJ (2002) Periodontal bacteria in rabbit mandibular and maxillary abscesses. J Clin Microbiol 40:1044–1047
77. Hasturk H, Kantarci A, Goguet-Surmenian E, Blackwood A, Andry C, Serhan CN, Van Dyke TE (2007) Resolvin E1 regulates inflammation at the cellular and tissue level and restores tissue homeostasis in vivo. J Immunol 179:7021–7029
78. Lang H, Schüler N, Nolden R (1998) Attachment formation following replantation of cultured cells into periodontal defects – a study in minipigs. J Dent Res 77:393–405

79. Wang S, Liu Y, Fang D, Shi S (2007) The miniature pig: a useful large animal model for dental and orofacial research. Oral Dis 13:530–537
80. Singh G, O'Neal RB, Brennan WA, Strong SL, Horner JA, Van Dyke TE (1993) Surgical treatment of induced peri-implantitis in the micro pig: clinical and histological analysis. J Periodontol 64:984–989
81. King JD, Gimson AP (1947) Experimental investigations of periodontal disease in the ferret and related lesions in man. Br Dent J 83:126
82. Fischer RG, Klinge B (1994) Clinical and histological evaluation of ligature-induced periodontitis in the domestic ferret. J Clin Periodontol 21:230–239
83. Harper DS, Mann PH, Regnier S (1990) Measurement of dietary and dentifrice effects upon calculus accumulation rates in the domestic ferret. J Dent Res 69:447–450
84. Duncan WJ, Persson GR, Sims TJ, Braham P, Pack AR, Page RC (2003) Ovine periodontitis as a potential model for periodontal studies. Cross-sectional analysis of clinical, microbiological, and serum immunological parameters. J Clin Periodontol 30:63–72
85. Danesh-Meyer MJ, Pack AR, McMillan MD (1997) A comparison of 2 polytetrafluoroethylene membranes in guided tissue regeneration in sheep. J Periodontal Res 32:20–30
86. Al-Qareer AH, Afsah MR, Müller HP (2004) A sheep cadaver model for demonstration and training periodontal surgical methods. Eur J Dent Educ 8:78–83
87. Chapple IL, Matthews JB (2007) The role of reactive oxygen and antioxidant species in periodontal tissue destruction. Periodontol 2000 43:160–232
88. Circu ML, Aw TY (2010) Reactive oxygen species, cellular redox systems, and apoptosis. Free Radic Biol Med 48:749–762
89. Tomofuji T, Ekuni D, Irie K, Azuma T, Tamaki N, Maruyama T, Yamamoto T, Watanabe T, Morita M (2011) Relationships between periodontal inflammation, lipid peroxide and oxidative damage of multiple organs in rats. Biomed Res 32:343–349
90. Tomofuji T, Ekuni D, Irie K, Azuma T, Endo Y, Tamaki N, Sanbe T, Murakami J, Yamamoto T, Morita M (2009) Preventive effects of a cocoa-enriched diet on gingival oxidative stress in experimental periodontitis. J Periodontol 80:1799–1808
91. Tomofuji T, Yamamoto T, Tamaki N, Ekuni D, Azuma T, Sanbe T, Irie K, Kasuyama K, Umakoshi M, Murakami J, Kokeguchi S, Morita M (2009) Effects of obesity on gingival oxidative stress in a rat model. J Periodontol 80:1324–1329
92. Ekuni D, Tomofuji T, Sanbe T, Irie K, Azuma T, Maruyama T, Tamaki N, Murakami J, Kokeguchi S, Yamamoto T (2009) Periodontitis-induced lipid peroxidation in rat descending aorta is involved in the initiation of atherosclerosis. J Periodontal Res 44:434–442
93. de Menezes AM, de Souza GF, Gomes AS, de Carvalho Leitão RF, Ribeiro Rde A, de Oliveira MG, de Castro Brito GA (2012) S-nitrosoglutathione decreases inflammation and bone resorption in experimental periodontitis in rats. J Periodontol 83:514–521
94. Paola RD, Oteri G, Mazzon E, Crisafulli C, Galuppo M, Toso RD, Pressi G, Cordasco G, Cuzzocrea S (2011) Effects of verbascoside, biotechnologically purified by *Syringa vulgaris* plant cell cultures, in a rodent model of periodontitis. J Pharm Pharmacol 63:707–717
95. Holanda Pinto SA, Pinto LM, Cunha GM, Chaves MH, Santos FA, Rao VS (2008) Anti-inflammatory effect of alpha, beta-Amyrin, a pentacyclic triterpene from *Protium heptaphyllum* in rat model of acute periodontitis. Inflammopharmacology 16:48–52
96. Di Paola R, Mazzon E, Maiere D, Zito D, Britti D, De Majo M, Genovese T, Cuzzocrea S (2006) Rosiglitazone reduces the evolution of experimental periodontitis in the rat. J Dent Res 85:156–161
97. Di Paola R, Mazzon E, Zito D, Maiere D, Britti D, Genovese T, Cuzzocrea S (2005) Effects of Tempol, a membrane-permeable radical scavenger, in a rodent model periodontitis. J Clin Periodontol 32:1062–1068
98. Di Paola R, Marzocco S, Mazzon E, Dattola F, Rotondo F, Britti D, De Majo M, Genovese T, Cuzzocrea S (2004) Effect of aminoguanidine in ligature-induced periodontitis in rats. J Dent Res 83:343–348

99. Nishikawa T, Naruse K, Kobayashi Y, Miyajima S, Mizutani M, Kikuchi T, Soboku K, Nakamura N, Sokabe A, Tosaki T, Hata M, Ohno N, Noguchi T, Matsubara T (2012) Involvement of nitrosative stress in experimental periodontitis in diabetic rats. J Clin Periodontol 39:342–349
100. Lohinai Z, Benedek P, Fehér E, Györfi A, Rosivall L, Fazekas A, Salzman AL, Szabó C (1998) Protective effects of mercaptoethylguanidine, a selective inhibitor of inducible nitric oxide synthase, in ligature-induced periodontitis in the rat. Br J Pharmacol 123:353–360
101. Sobaniec H, Sobaniec-Lotowska ME (2000) Morphological examinations of hard tissues of periodontium and evaluation of selected processes of lipid peroxidation in blood serum of rats in the course of experimental periodontitis. Med Sci Monit 6:875–881
102. Tomofuji T, Sanbe T, Ekuni D, Azuma T, Irie K, Maruyama T, Tamaki N, Yamamoto T (2008) Oxidative damage of rat liver induced by ligature-induced periodontitis and chronic ethanol consumption. Arch Oral Biol 53:1113–1118
103. Mousavi-Jazi M, Aslroosta H, Moayer AR, Baeeri M, Abdollahi M (2010) Effects of Angipars on oxidative inflammatory indices in a murine model of periodontitis. Daru 18:260–264
104. Gelani V, Fernandes AP, Gasparoto TH, Garlet TP, Cestari TM, Lima HR, Ramos ES, de Souza Malaspina TS, Santos CF, Garlet GP, da Silva JS, Campanelli AP (2009) The role of toll-like receptor 2 in the recognition of *Aggregatibacter actinomycetemcomitans*. J Periodontol 80:2010–2019
105. Kato C, Mikami M, Saito K (2001) Nitric oxide production and iNOS mRNA expression in mice induced by repeated stimulation with live *Fusobacterium nucleatum*. Microbiol Immunol 45:69–78
106. Gyurko R, Boustany G, Huang PL, Kantarci A, Van Dyke TE, Genco CA, Gibson FC 3rd (2003) Mice lacking inducible nitric oxide synthase demonstrate impaired killing of *Porphyromonas gingivalis*. Infect Immun 71:4917–4924
107. Gyurko R, Shoji H, Battaglino RA, Boustany G, Gibson FC 3rd, Genco CA, Stashenko P, Van Dyke TE (2005) Inducible nitric oxide synthase mediates bone development and *P. gingivalis-induced* alveolar bone loss. Bone 36:472–479
108. Nemec A, Pavlica Z, Crossley DA, Sentjurc M, Jerin A, Erzen D, Vrecl M, Majdic G, Zdovc I, Petelin M, Skaleric U (2009) Chronic ingestion of *Porphyromonas gingivalis* induces systemic nitric oxide response in mice. Oral Microbiol Immunol 24:204–210
109. Nemec A, Pavlica Z, Petelin M, Crossley DA, Sentjurc M, Jerin A, Erzen D, Zdovc I, Hitti T, Skaleric U (2010) Systemic use of selective iNOS inhibitor 1400W or non-selective NOS inhibitor l-NAME differently affects systemic nitric oxide formation after oral *Porphyromonas gingivalis* inoculation in mice. Arch Oral Biol 55:509–514
110. Ohnishi T, Bandow K, Kakimoto K, Machigashira M, Matsuyama T, Matsuguchi T (2009) Oxidative stress causes alveolar bone loss in metabolic syndrome model mice with type 2 diabetes. J Periodontal Res 44:43–51
111. Tomofuji T, Ekuni D, Sanbe T, Irie K, Azuma T, Maruyama T, Tamaki N, Murakami J, Kokeguchi S, Yamamoto T (2009) Effects of vitamin C intake on gingival oxidative stress in rat periodontitis. Free Radic Biol Med 46:163–168
112. Parrish JH Jr, DeMarco TJ, Bissada NF (1977) Vitamin E and periodontitis in the rat. Oral Surg Oral Med Oral Pathol 44:210–218
113. Battino M, Bullon P, Wilson M, Newman H (1999) Oxidative injury and inflammatory periodontal diseases: the challenge of anti-oxidants to free radicals and reactive oxygen species. Crit Rev Oral Biol Med 10:458–476
114. Rice-Evans C (1999) Implications of the mechanisms of action of tea polyphenols as antioxidants in vitro for chemoprevention in humans. Proc Soc Exp Biol Med 220:262–266
115. Ramiro-Puig E, Castell M (2009) Cocoa: antioxidant and immunomodulator. Br J Nutr 101:931–940
116. Rice-Evans CA, Miller NJ, Bolwell PG, Bramley PM, Pridham JB (1995) The relative antioxidant activities of plant-derived polyphenolic flavonoids. Free Radic Res 22:375–383

117. Govindaraj J, Emmadi P, Deepalakshmi, Rajaram V, Prakash G, Puvanakrishnan R (2010) Protective effect of proanthocyanidins on endotoxin induced experimental periodontitis in rats. Indian J Exp Biol 48:133–142
118. Kubo M, Matsuda H, Tanaka M, Kimura Y, Okuda H, Higashino M, Tani T, Namba K, Arichi S (1984) Studies on *Scutellariae radix*. VII. Anti-arthritic and anti-inflammatory actions of methanolic extract and flavonoid components from *Scutellariae radix*. Chem Pharm Bull (Tokyo) 32:2724–2729
119. Cai X, Li C, Du G, Cao Z (2008) Protective effects of baicalin on ligature-induced periodontitis in rats. J Periodontal Res 43:14–21
120. Ohsawa I, Ishikawa M, Takahashi K, Watanabe M, Nishimaki K, Yamagata K, Katsura K, Katayama Y, Asoh S, Ohta S (2007) Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. Nat Med 13:688–694
121. Kasuyama K, Tomofuji T, Ekuni D, Tamaki N, Azuma T, Irie K, Endo Y, Morita M (2011) Hydrogen-rich water attenuates experimental periodontitis in a rat model. J Clin Periodontol 38:1085–1090
122. Ozdemir H, Kara MI, Erciyas K, Ozer H, Ay S (2012) Preventive effects of thymoquinone in a rat periodontitis model: a morphometric and histopathological study. J Periodontal Res 47:74–80
123. Feng R, Lu Y, Bowman LL, Qian Y, Castranova V, Ding M (2005) Inhibition of activator protein-1, NF-kappaB, and MAPKs and induction of phase 2 detoxifying enzyme activity by chlorogenic acid. J Biol Chem 280:27888–27895
124. Dar A, Faizi S, Naqvi S, Roome T, Zikr-ur-Rehman S, Ali M, Firdous S, Moin ST (2005) Analgesic and antioxidant activity of mangiferin and its derivatives: the structure activity relationship. Biol Pharm Bull 28:596–600
125. Duang XY, Wang Q, Zhou XD, Huang DM (2011) Mangiferin: a possible strategy for periodontal disease to therapy. Med Hypotheses 76:486–488
126. Bracken WM, Cuppage F, McLaury RL, Kirwin C, Klaassen CD (1985) Comparative effectiveness of topical treatments for hydrofluoric acid burns. J Occup Med 27:733–739
127. Ku SK, Cho HR, Sung YS, Kang SJ, Lee YJ (2011) Effects of calcium gluconate on experimental periodontitis and alveolar bone loss in rats. Basic Clin Pharmacol Toxicol 108:241–250
128. Herrera BS, Martins-Porto R, Maia-Dantas A, Campi P, Spolidorio LC, Costa SK, Van Dyke TE, Gyurko R, Muscara MN (2011) iNOS-derived nitric oxide stimulates osteoclast activity and alveolar bone loss in ligature-induced periodontitis in rats. J Periodontol 82:1608–1615
129. Paquette DW, Rosenberg A, Lohinai Z, Southan GJ, Williams RC, Offenbacher S, Szabó C (2006) Inhibition of experimental gingivitis in beagle dogs with topical mercaptoalkylguanidines. J Periodontol 77:385–391
130. Brownlee M (2001) Biochemistry and molecular cell biology of diabetic complications. Nature 414:813–820
131. Kador PF, O'Meara JD, Blessing K, Marx DB, Reinhardt RA (2011) Efficacy of structurally diverse aldose reductase inhibitors on experimental periodontitis in rats. J Periodontol 82:926–933
132. Gokcimen A, Cim A, Tola HT, Bayram D, Kocak A, Ozgüner F, Ayata A (2007) Protective effect of N-acetylcysteine, caffeic acid and vitamin E on doxorubicin hepatotoxicity. Hum Exp Toxicol 26:519–525
133. Toker H, Ozdemir H, Eren K, Ozer H, Sahin G (2009) N-acetylcysteine, a thiol antioxidant, decreases alveolar bone loss in experimental periodontitis in rats. J Periodontol 80:672–678
134. Di Paola R, Mazzon E, Rotondo F, Dattola F, Britti D, De Majo M, Genovese T, Cuzzocrea S (2005) Reduced development of experimental periodontitis by treatment with M40403, a superoxide dismutase mimetic. Eur J Pharmacol 516:151–157
135. Petelin M, Pavlica Z, Ivanusa T, Sentjurc M, Skaleric U (2000) Local delivery of liposome-encapsulated superoxide dismutase and catalase suppress periodontal inflammation in beagles. J Clin Periodontol 27:918–925

136. Schenkein HA (2006) Host responses in maintaining periodontal health and determining periodontal disease. Periodontol 2000 40:77–93
137. Graves DT, Fine D, Teng YT, Van Dyke TE, Hajishengallis G (2008) The use of rodent models to investigate host-bacteria interactions related to periodontal diseases. J Clin Periodontol 35:89–105
138. Oikawa D, Akai R, Tokuda M, Iwawaki T (2012) A transgenic mouse model for monitoring oxidative stress. Sci Rep 2:229

Chapter 4
Expression of Reactive Oxygen Species in Junctional and Pocket Epithelium

Daisuke Ekuni, James D. Firth, and Edward E. Putnins

4.1 Introduction

The oral cavity is a unique environment [1]. Oral mucosa serves as a critical protective interface between the external and internal environments, functions as a mechanical protective barrier, and protects the host against a myriad of pathological challenges [2–4] (Fig. 4.1). These tissues are protected by two types of immunity: innate and adaptive [4]. The major components of innate immunity include mechanical and chemical constituents. The mechanical strength of oral gingival epithelium is due in large part to its keratinization [2, 3]. This mechanical barrier resists bacterial infection and is continually renewed by cellular proliferation and desquamation. In addition to this physical barrier, the oral activity is protected by proteins and antimicrobial peptides delivered by saliva, such as histatins and defensins [5]. However, epithelial cells actively respond by secreting cytokines and chemokines to orchestrate a local cell response and attract immune regulatory cells such as neutrophils. In response to bacterial virulence factors, keratinocytes also produce antimicrobial peptides including β-defensins, cathelicidin, calprotectin, RNase 7, and CCL20/MIP-3a [6]. Many human β-defensins have been described [7] and some play a role in the defense of the oral environment [8, 9].

Teeth in a moist oral mucosal environment are the only place on the body in which hard tissue penetrates through the epithelial surface. This is in contrast to

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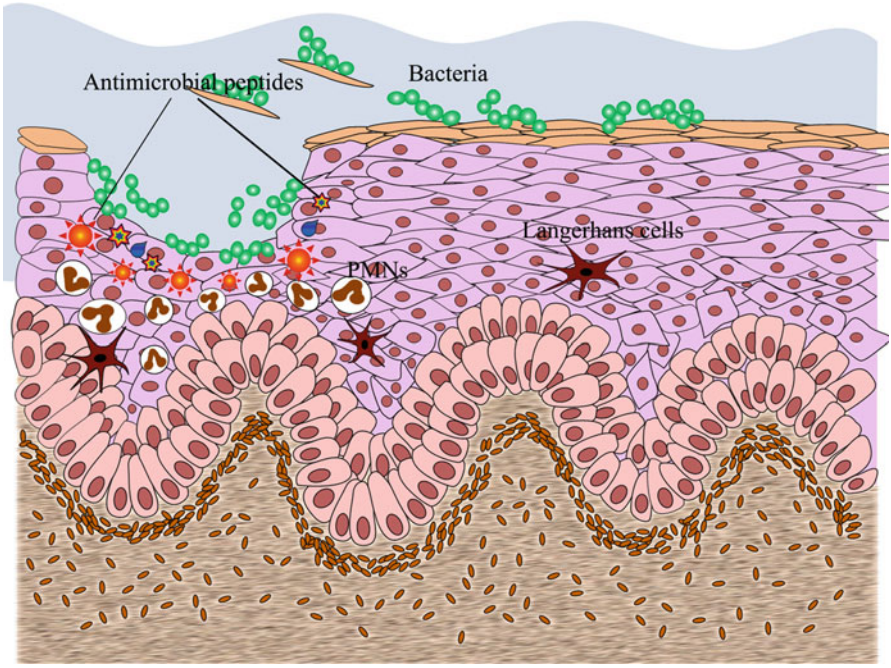

Fig. 4.1 Structural organization of oral epithelium. The oral epithelium is keratinized, serves as a protective barrier, and resists functional stresses. A shedding keratinized surface layer serves as the first defensive barrier; however, several classes of antimicrobial peptides are expressed by keratinocytes as well. *PMNs* polymorphonuclear leukocytes

other appendages, such as scales of reptiles, feathers, hair, fingernails, claws, hoofs, and antlers [10]. This means that the epithelium adjacent to teeth must be unique and specialized in order to maintain physiological homeostasis. Junctional epithelium faces the teeth; it is not keratinized but forms an attachment and relative seal (Fig. 4.2). Oral sulcular epithelium is a transition tissue between the previous two. The junctional epithelial attachment complex is structurally unique and its continual exposure to tooth-associated bacterial biofilm (i.e., dental plaque) leads to periodontal tissue vulnerability. It is at a significant risk for bacterially induced chronic inflammation.

4.2 Junctional Epithelium

The junctional epithelium is in contact with the tooth surface and forms a nonkeratinized epithelial structure that attaches the gingival soft tissue to enamel or, in the case of progressive disease, root cementum [11, 12] (Fig. 4.2). Junctional epithelial cell renewal occurs by the active proliferation of basal epithelial cells both on

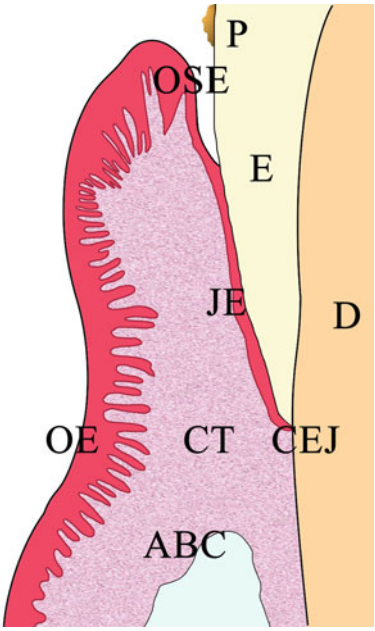

Fig. 4.2 Structural organization of the human periodontium. *ABC* alveolar bone crest, *CEJ* cemento-enamel junction, *CT* gingival connective tissue, *D* dentin, *E* enamel, *JE* non-keratinized junctional epithelium, *OE* keratinized oral epithelium, *OSE* oral sulcular epithelium, *P* dental plaque

the connective tissue side and against the hard tissue. Junctional epithelial cells facing the tooth surface form and maintain an epithelial attachment and seal against the tooth surface [10] (Fig. 4.3).

The nutrient-rich, wet and warm oral cavity provides the critical components required for micro-organism survival and proliferation. Collectively, micro-organisms establish complex ecological biofilms (i.e., dental plaque) that adhere to a glycoprotein layer (i.e., acquired pellicle) which is deposited on non-shedding surfaces. Junctional epithelial cells adjacent to the tooth surface play an important role in maintaining tissue homeostasis and defense against micro-organisms and their virulence factors [10, 13]. Junctional epithelial cells, which are juxtaposed next to the biofilm, are constantly being stimulated by bacterial virulence factors. To successfully respond to this complex environment, structural and functional adaptations have evolved in the junctional epithelium. One of the most significant protective mechanisms in other epithelia is the presence of a keratinized surface; however, junctional epithelium is not keratinized. In compensation, junctional epithelium is made up of a special structural framework and provides an antimicrobial barrier in collaboration with other non-epithelial cells. For example, the presence of relatively loose intercellular junctions due to few desmosomes, adherens junctions, and gap junctions allows for tissue exudation and inflammatory cell migration towards the gingival sulcus [11].

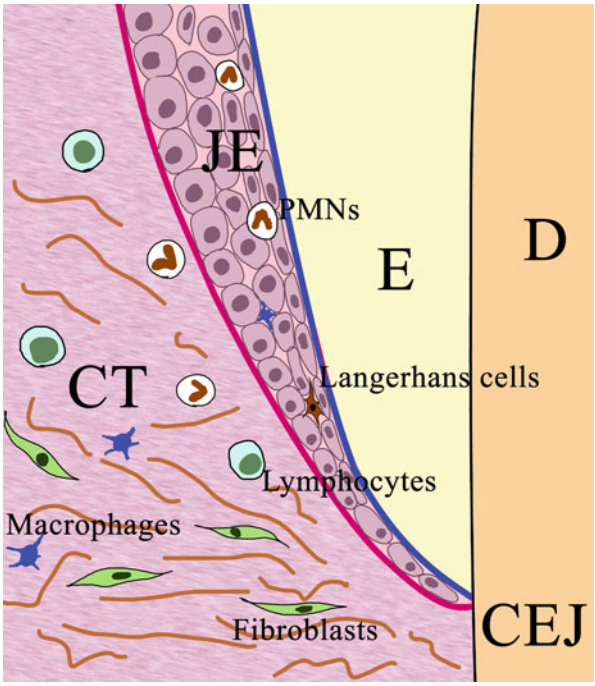

Fig. 4.3 Structural organization of epithelial attachment. Clinically healthy junctional epithelium (JE) tapers off in the apical direction and terminates at the cemento-enamel junction (CEJ). Junctional epithelial cells closest to the enamel (E) are attached to the tooth via an internal basal lamina (*blue line*) and epithelial cells closest to the connective tissue are attached via the external basal lamina (*pink line*). *CT* gingival connective tissue, *D* dentin, *PMNs* polymorphonuclear leukocytes

4.2.1 Junctional Epithelium Structure

The junctional epithelium is a stratified, squamous non-keratinizing epithelium that faces both the gingival connective tissue (i.e., the lamina propria of the gingiva) and tooth surface (Fig. 4.3). It tapers in an apical direction and consists of 15–30 cell layers coronally and only one to three cell layers at its apical point [11]. Junctional epithelial cells express cytokeratins 5, 13, 14, and 19, and occasionally weakly express cytokeratins 8, 16, and 18 [13]. Junctional epithelium is made up of only two strata, a basal (stratum basale) layer facing the gingival connective tissue and a suprabasal layer (stratum suprabasale) [11]. The basal cells and the adjacent one to two suprabasal cell layers are cuboidal to slightly spindle-form in shape. All of the remaining cells of the suprabasal layer are mostly flat and oriented parallel to the tooth surface [11]. Lysosomal bodies containing antimicrobial enzymes are found in large numbers within junctional epithelial cells [14].

In contrast to other epithelia, junctional epithelial cells have only a few desmosomes and occasionally gap junctions [10, 13, 15–20]. The gingival crevicular fluid-filled intercellular spaces vary in width, but generally are wider when compared to oral gingival or sulcular epithelium [16]. This reduced cell–cell contact accounts for its remarkable permeability to fluids and immune cells [11]. Most commonly within these interstitial spaces are polymorphonuclear leukocytes (PMNs), with the

highest proportion in the central region of the junctional epithelium and near the tooth surface (Fig. 4.3) [10]. The fluid-filled widened intercellular spaces allow significant PMN transmigration from the tissues to the oral cavity. Healthy human periodontium is associated with approximately 30,000 PMNs migrating into the oral cavity per minute [21]. The migration of PMNs through the junctional epithelium into the oral cavity is a significant host defense mechanism [10]. Thus, the presence of inflammatory cells within the junctional epithelium has been described as a normal state of homeostasis and is an essential element in this area of chronic bacterial challenge [10]. Lymphocytes, macrophages, antigen-presenting cells, and Langerhans cells and other dendritic cells also reside in the junctional epithelium [22, 23]. In conjunction with the cellular and fluid components, the basal cell layer is innervated with sensory nerve fibers [24–27].

4.2.2 Junctional Epithelium: Cell Adhesion Molecules

Since teeth represent unique transmucosal structures that perforate mucosal epithelium, a unique epithelial attachment to extracellular adhesion molecules has evolved to maintain a barrier. A basement lamina (i.e., external basal lamina) [13] is formed between the basal cells of the junctional epithelium and gingival connective tissue and a basal lamina (i.e., internal basal lamina) forms between the tooth-facing junctional epithelial cells (Fig. 4.3). The internal basal lamina (together with hemidesmosomes at the tooth–junctional epithelium interface [13]) forms an epithelial attachment and is structurally different from the external basal lamina [10]. These "directly attached to the tooth" cells have been described as DAT cells [28]. Junctional epithelial cell proliferation in primates is higher [29–31]. Cell mitosis occurs in the basal and DAT cells [32]; however, exfoliation of cells occurs at the coronal margin of the junctional epithelium where these cells desquamate [11]. Since DAT cells are connected to the basal lamina via hemidesmosomes, a remodeling of the epithelial attachment must occur to accommodate cellular desquamation [11].

Histologically, the external basal lamina contains the very same extracellular adhesion molecules present in a typical basement membrane. Lamina lucida against the basal keratinocytes and lamina densa toward the connective tissue stroma are present; however, the internal basal lamina differs significantly in terms of its protein composition [12]. Absent from the internal basal lamina are basement membrane proteins, such as laminin 111, laminin 511, type IV and VII collagens, and perlecan [33]. In contrast, cell adhesion proteins identified in the internal basal lamina include laminin 332 (laminin 5), which is also present in the external basal lamina [33–35]. Classically, normal basement membranes are produced by both basal keratinocytes and fibroblasts through paracrine-soluble mediator cross-talk [36]. Without fibroblasts, keratinocytes continue to express laminin 332, but fail to deposit laminin 111 and type IV collagen [36]. Several growth factors and cytokines induce laminin 332 expression, including transforming growth factor (TGF) β1, tumor necrosis factor (TNF)-α, keratinocyte growth factor (KGF), epidermal growth

factor (EGF), and interferon-γ [37, 38]. Junctional epithelial cells constitutively express many of these factors and may induce the expression of laminin 332 in the internal basal lamina [39, 40].

The maintenance of the periodontal tissue architecture requires the expression of cell matrix and cell–cell adhesion molecules on the membrane of epithelial cells. Specifically, integrins and cadherins are cell adhesion molecules expressed by junctional epithelial cells [11, 12, 41]. Integrins function as cell-surface receptors mediating extracellular matrix interactions [11, 42, 43]. All integrins are products of two separate genes encoding individual α and β subunits. The expression of α2β1, α3β1, α5β1, α6β4, and αvβ6 integrins has been demonstrated in junctional epithelial cells [12, 34, 44–47]. In general, basal keratinocytes, including junctional epithelial cells, interact with the C-terminal LG domains of the α3 chain of laminin 332 via α3β1 and α6β4 integrins [12, 48]. In hemidesmosomes, integrin α6β4 is a crucial component where it binds to processed laminin 332 [12, 49]. To allow for coronal migration of junctional epithelial cells that are attached to the tooth via hemidesmosomes they must be disassembled to allow for cell movement [12]. This process is not fully understood but is believed to start with β4 integrin cytoplasmic domain phosphorylation with subsequent disassociation between β4 integrin and plectin [49, 50]. The cadherins are responsible for adherens junctions between cells [41, 51]. E-cadherin, an epithelium-specific cell adhesion molecule, may play a crucial role in maintaining the structural integrity of junctional epithelium [52, 53]. Transmembrane carcinoembryonic Ag-related cell adhesion molecule 1 is expressed more strongly on the cell surface of junctional epithelium as compared to oral sulcular epithelium [54]. Thus, the dynamic regulation of junctional epithelial cell cohesion may be mediated by this molecule [54]. Carcino-embryonic Ag-related cell adhesion molecules also contribute to PMN guidance through the junctional epithelium, participate in the regulation of cell proliferation, stimulation, and co-regulation of activated T-cells, and may serve as a receptor for certain bacteria [54–59]. Additional cell adhesion molecules expressed in junctional epithelial cells include intercellular adhesion molecule-1 or CD54 and lymphocyte function-associated antigen-3 [60–64]. The expression gradient of intercellular adhesion molecule-1 in junctional epithelium is believed to be an important PMN guiding mechanism towards the sulcus [63, 64].

Claudins play an important role in regulating the cell–cell epithelial barrier at apically located tight junctions and septate junctions [65]. Claudin-1 is present in rat junctional epithelium and possibly contributes to barrier function in these tissues. Of interest, claudin 1 protein expression was reduced by lipopolysaccharide in a rat periodontal disease model and in cell culture [52, 66].

4.2.3 Junctional Epithelium: Cytokines and Growth Factors

Junctional epithelial cells, PMNs, and macrophages express a myriad of cytokines and chemokines. Elevated interleukin-8 (IL-8) expression in the coronal cells of the junctional epithelium may direct PMNs toward the bacterial challenge present in the

adherent biofilm [64, 67]. In the coronal aspect of the junctional epithelium, IL-1α, IL-1β, and TNF-α are strongly expressed [68]. After lipopolysaccharide stimulation, most cells of the junctional epithelium are strongly labeled for these cytokines [68, 69]. Thus, these cytokines play a role in the defense against bacteria that are present in the adjacent biofilm [11]. Collectively, junctional epithelium also represents a key mediator of host–parasite interactions in addition to its attachment role.

Growth factors and corresponding receptor expression have also been examined in the junctional epithelium. KGF-1 [fibroblast growth factor (FGF) 7] and KGF-2 (FGF-10) have both been described as paracrine mediators of epithelial cell growth [70, 71]. Stromal cells express KGF, but only epithelial cells express the KGF-specific receptor, which is a splice variant of FGFR2 (FGFR2-IIIb) (KGFR) and binds KGF-1, KGF-2, and acidic FGF [72–74]. For normal epidermal homeostasis, KGF-1 is required and is up-regulated during wound healing, chronic inflammatory bowel diseases, psoriasis, and periodontitis [74–82]. KGF-1 induces cell proliferation, migration, and matrix metalloproteinase (MMP) secretion in epithelial cells [83–87]. Orally, KGF-1 is expressed by fibroblasts isolated from buccal mucosa, gingiva, periodontal ligament, and in inflamed periapical stroma [39, 75, 88–94]. KGF-1 protein and gene expression in gingival fibroblasts is induced by pro-inflammatory cytokines IL-1α, IL-1β, IL-6, and TNF-α [75, 89, 90]. Conversely, KGF-2 was weakly expressed in gingival fibroblasts and not induced by pro-inflammatory cytokines [75]. In addition, lipopolysaccharide (LPS) purified from *Escherichia coli* (*E. coli*) and *Porphyromonas gingivalis* (*P. gingivalis*) induced KGF-1 protein and gene expression through a Toll-like receptor signaling pathway [94]. In human gingival biopsies from periodontal healthy patients, KGF-1 protein expression was localized to select junctional epithelial cells with more intense staining in the region of the internal and external basal lamina [39]. However, pocket epithelium in biopsies collected from patients with advanced periodontitis showed general intense staining in all cell layers [39]. Within these same health and disease groups, there was positive KGFR peri-cellular staining in most cells of the junctional epithelium; however, KGFR protein expression was significantly higher in pocket epithelium [39]. In a rat periodontitis model, KGFR was dramatically up-regulated with the induction of disease [95].

EGF is a potent mitogen involved in epithelial growth, differentiation, wound healing, and signals through the EGF receptor (EGFR) [11]. EGFR is either absent or poorly expressed in healthy human junctional epithelium, but intense labeling in proliferating cells was shown in inflamed tissues from patients with chronic periodontitis [96]. In normal rat junctional epithelium, immunohistochemical localization for EGF and EGFR was observed in the cytoplasm [95, 97].

Tissue plasminogen activator [98] and its associated inhibitor (type-2) are expressed in junctional epithelium [99, 100]. Tissue plasminogen activator is a serine protease responsible for the activation of plasminogen to plasmin. Plasmin degrades a variety of extracellular matrix proteins and activates MMPs. Matrilysin (MMP-7), a proteolytic enzyme found in many mature epithelial cells, is expressed in human suprabasal junctional epithelium [101].

The innate defense role of junctional epithelium is demonstrated by the secretion of antimicrobial peptides and proteins [4, 102]. These antimicrobial molecules include α- and β-defensins, the cathelicidin family members (LL-37), and calprotectin. Human β-defensin 1 and β-defensin 2 are poorly expressed or undetectable in the junctional epithelium; however, α-defensins and LL-37 are significantly expressed [4, 102]. Further expression by PMNs of α-defensins and LL-37 provides additional protection to the junctional epithelium and the host [11].

4.2.4 Junctional Epithelium Transition to Pocket Epithelium

The transition of junctional epithelium to pocket epithelium is a critical step in the initiation of periodontal disease. However, the detailed roles that junctional epithelium plays in disease pathogenesis and its ultimate conversion to pocket epithelium at the time of pocket formation are poorly understood [13, 103].

The loss of cellular continuity in the coronal aspect of the junctional epithelium is an early aspect of pocket formation [10, 104]. This initiation may occur via detachment of the DAT cells from the tooth surface or formation of an intraepithelial split. Degenerative changes in the second or third cell layer of the DAT cells in the coronal aspect of the junctional epithelium facing the bacterial biofilm have been described in humans, rats, and dogs [105–107]. Several hypotheses have been put forth to explain this finding. Inflammation-induced high levels of PMN emigration and an increased rate of gingival crevicular fluid passing through the intercellular junctional epithelial spaces may contribute to this separation [108–112]. In addition, increased numbers of mononuclear leukocytes, i.e., T- and B-lymphocytes and monocytes/macrophages, together with PMNs, are also considered to be factors that mediate the focal disintegration of junctional epithelium.

The intercellular junctions in junctional epithelium are relatively open, allowing cells and antimicrobial molecules to transit from the connective tissue and basal junctional epithelial cells. This works to clear and counteract bacteria and their virulence factors [11]. However, bacteria and their virulence factors can penetrate past the junctional epithelium in early disease onset [11]. This was demonstrated by immunohistochemistry in rat periodontal disease tissues in which LPS was identified in the subjacent gingival connective tissue [113]. This supports a hypothesis that initiation of pocket formation is a response to subgingival penetration of bacteria or their virulence factors [114]. In addition, direct effects of bacteria on epithelial cells are possible. *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans* and *P. gingivalis,* which have been demonstrated to be associated with aggressive and chronic periodontitis, can adhere and invade epithelial cells [115–126]. In sites of periodontal inflammation, there is a reduction in proliferation and an increase in apoptosis [127]. In a tissue culture model, bacterial internalization followed by epithelial cell apoptosis have been demonstrated [128, 129]. Gingipains, a cysteine proteinase bacterial virulence factor, can effectively disrupt epithelial cell-to-cell junctional complexes and disturb ICAM-1-mediated adhesion of PMNs to epithelial

cells [130–135]. This disruption of epithelial integrity in concert with focal sites of epithelial cell apoptosis may be critical early initiation factors in pocket formation and ultimately allow bacterial invasion and penetration of virulence factors into the epithelium and subepithelial connective tissue as disease progresses.

The overall enhanced proliferation of junctional epithelium is an important factor in the formation of Rete Ridges and the initiation of periodontal pocket formation [136]. LPS purified from *E. coli* and *P. gingivalis* induced proliferation of epithelial cells [94, 107]. The positive regulation of proliferation is critical to disease onset. KGFR is expressed in healthy junctional epithelium; however, gene (25 times) and protein (11 times) levels were dramatically up-regulated in periodontitis [95]. This up-regulation may possibly be explained by direct stimulation from LPS or secondarily due to pro-inflammatory cytokine secretion (IL-1β and TNF-α) by epithelial cells [95]. A dramatic increase in KGFR expression enables local epithelial cells to bind elevated KGF-1 ligand expressed by fibroblasts in chronically inflamed periodontal tissues [39, 75, 94]. In the same rat LPS model, the overall number of PCNA-positive basal epithelial cells increased [137]. It is possible that up-regulation of KGF-1 and KGFR protein in diseased tissues positively regulates epithelial cell proliferation associated with periodontal pocket formation [39]. Conversely, apoptosis of connective tissue and periodontal ligament fibroblasts was also demonstrated [137]. Thus, LPS promoted proliferation of epithelial cells but not fibroblasts. This imbalance in the proliferative activity between junctional epithelium and fibroblasts subjacent to the junctional epithelium may begin to explain the histological finding seen at the initial stage of disease-associated apical migration of the junctional epithelium [137].

4.3 Reactive Oxygen and Nitrogen Species

4.3.1 Pathology of Oxidative Stress

Reactive oxygen and nitrogen species (ROS/RNS), namely superoxide, hydroxyl and nitric oxide radicals, hydrogen peroxide, and hypochlorous acid, are by-products of normal cellular degradative mechanisms and can function at low concentrations as intra- and intercellular signaling molecules or in acute high concentrations as bacteriocidal agents [138–142]. Under certain pathological conditions, ROS/RNS levels may become chronically elevated and induce conditions of oxidative stress, which is an imbalance between the production and elimination of highly reactive molecular oxygen species. For instance, hydrogen peroxide may mediate important normal metabolic and signaling functions in the brain [143]. Hydrogen peroxide is lipid-soluble and diffuses across biological membranes and has been shown to be a diffusible paracrine mediator for signal cross-talk between epithelial and stromal tissue [144]. However, at higher concentrations, hydrogen peroxide is toxic [145].

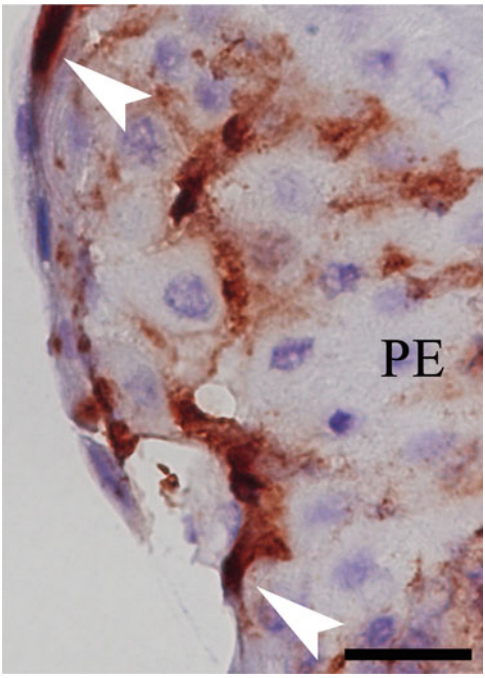

Fig. 4.4 Detection of hydrogen peroxide using colorimetric staining. Pocket epithelium from LPS-induced periodontal disease in rats stain brown for hydrogen peroxide localization (*white arrowheads*). Scale bar: 50 μm. *PE* pocket epithelium

Recently it has been shown that natural gut epithelial infections in *Drosophila* is associated with rapid ROS synthesis but flies that lack the normal ROS cycling capacity have increased mortality rates [146]. Epithelial cells from gastric mucosa when exposed to various *Helicobacter pylori* (*H. pylori*) strains demonstrated a dose-dependent increase in ROS generation. ROS levels were greater in epithelial cells isolated from *H. pylori*-infected gastric mucosal human biopsy specimens than in cells from uninfected individuals [147]. Strains of *H. pylori* bearing the cag pathogenicity island (PAI) are associated with greater peptic ulceration [148], induced higher levels of ROS, and activated the apoptosis markers caspase 3 and 8 more than isogenic cag PAI-deficient mutants did [147]. PAI may act by elevating mitochondrial ROS formation [149].

4.3.2 ROS Production in Epithelium

Oxidative stress and decreased total antioxidant status are generally associated with periodontitis and locally induce tissue damage by oxidizing DNA, lipids, and proteins. The resulting increased lipid peroxidation can be assayed in gingival crevicular fluid and saliva [150–152]. A rat model of periodontitis has proven valuable in demonstrating ROS production in the junctional epithelium during the initiation of periodontitis [69, 153] (Fig. 4.4). In this model, experimental periodontitis was induced after an 8-week daily topical application of lipopolysaccharide into the

palatal gingival sulcus of maxillary molars [69]. Histological changes associated with disease-induced chronic inflammation such as junctional epithelial downgrowth and loss of alveolar bone confirmed disease onset in this rat model [69]. Elevated local and plasma oxidative stress as measured by 8-hydroxydeoxyguanosine levels was demonstrated [153]. Hydrogen peroxide was shown to be elevated in the disease state by 3,3′-diaminobenzidine staining of histological sections. Similarly, superoxide as measured by 3,3′-diaminobenzidine/manganese chloride staining was also found to be strongly elevated in rat LPS-treated pocket epithelium [69].

These results were supported in cell culture experiments using a porcine periodontal epithelial cell line (PLE) that has previously been established as a model of junctional epithelium [154]. Lipopolysaccharide-treated PLE cultures showed significantly increased hydrogen peroxide and superoxide positive staining. Interestingly, the PLE cell line has previously been shown to express a chymase in a superoxide-dependent manner [155]. In the case of LPS treatment, the distribution of staining for both ROS species seemed to be consistent with previous reports of hydrogen peroxide extracellular diffusibility [144]. The use of cell culture modeling has the advantage of discerning ROS production from a single cell type, whereas in histological sections, hydrogen peroxide production from PMNs present in the junctional epithelial compartment cannot be ruled out. Therefore, to ascertain whether LPS-induced ROS could be of epithelial origin, PLE cultures were first assayed via flow cytometry for purity of the population by staining for the epithelium-specific marker cytokeratin 13 or the PMN-specific marker integrin-β2 (CD 11) [69]. Exclusive staining of PLEs by cytokeratin 13 confirmed epithelial cell purity and demonstrated epithelial cell generation of ROS after LPS treatment [69].

4.3.3 Gene Array Analysis of Epithelial Oxidative Stress

A major development in the analysis of the possible role of junctional epithelium and the associated role of ROS in disease onset have been made by laser-capture microscopy. This permits precise excision and isolation of the healthy and diseased experimental animal epithelial tissues (Fig. 4.5). Processed samples can then be subject to gene expression analysis on select tissue compartments [69]. There are several challenges inherent to utilizing this approach because of the small area of tissue that is involved. A typical healthy rat junctional epithelial section would contain about 100 nuclei in an area of 33,000 μm^2 and require the collection of about 60 sections totaling 2,000,000 μm^2 in order to synthesize adequate cRNA for analysis. Periodontal disease tissues in rats characteristically present with pocket epithelium-associated hyperplasia and thus yield an equivalent amount of precursor RNA from about 25 sections per animal. Rat cRNA can then be subject to expression analysis using an Affymetrix GeneChip Rat Genome 230 2.0 Array or equivalent [69]. After the generation of raw expression data, post-normalization was required by principal component and d-chip software analysis [156] to confirm significant broad differences between healthy and diseased samples [69].

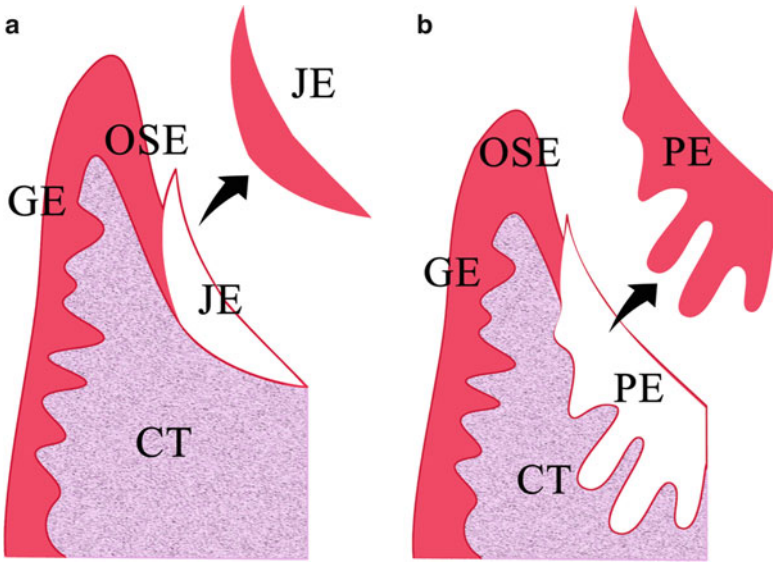

Fig. 4.5 Microdissection of epithelia. A laser-capture microdissection technique allows isolation of junctional epithelium from periodontal health (**a**) and pocket epithelium from LPS-induced periodontitis (**b**) tissue sections *CT* gingival connective tissue, *JE* non-keratinized junctional epithelium, *GE* gingival epithelium, *OSE* oral sulcular epithelium, *PE* pocket epithelium

Principal component analysis revealed that healthy and diseased samples significantly segregated into discrete groups, indicating that LPS-induced disease correlated with broad-scale changes in gene expression. Of the 33,000 sequences assayed by the chip, 19,730 transcripts were changed. Of these, about half were increased ($n = 9{,}031$) and half decreased ($n = 10{,}699$). Microarray analysis revealed that 42 showed a greater than fourfold increase in the expression of the genes induced in response to disease onset [69]. The top 10 of this group are monoamine oxidase (MAO) B (MAO-B) (5.72-fold), LRP16 protein (5.91-fold), paraoxonase 3 (6.25-fold), EGF-like protein 6 (6.28-fold), flavin-containing monooxygenase 1 (6.70-fold), 4-aminobutyrate aminotransferase (7.01-fold), flavin-containing monooxygenase 2 (7.26-fold), palate/lung/nasal carcinoma protein precursor (8.27-fold), transcription factor AP-2β (13.03-fold), and dihydropyrimidinase-like 3 (25.69-fold). Of these ten genes, three genes, MAO-B and flavin-containing monooxygenase 1 and 2, are involved in reactive oxygen signaling [69, 157, 158]. Conversely, no antioxidant genes are found to be up-regulated, but instead show consistently decreased expression [69]. Taken together, these results suggest that LPS induced overall epithelial oxidative stress.

In a more recent study using the same LPS rat periodontitis model, junctional epithelium and underlying stromal tissue were separately collected from healthy and diseased animals by laser-capture microdissection and subject to gene expression microarray analysis. This study demonstrated that separate global gene expression patterns exist between epithelia and adjacent stroma in both healthy and diseased tissues. The expression array data was further subject to GenMapp/

Mappfinder [159, 160] analysis of the global trends in gene expression. This analysis approach measured the overarching changes in gene ontology structure and function expression patterns [161] and was used to discern multiple genes of related function that could participate in a potential stromal-epithelial signaling axes. The key finding was that fibroblast ligand amphiregulin was the focus of concerted epithelial EGFR signaling [113]. Of particular significance, amphiregulin has previously been shown to be activated/released by hydrogen peroxide [162]. The LPS rat model of periodontitis has shown the resulting loss of junctional epithelial barrier with the penetration of LPS [113]. Taken together, this body of evidence suggests that hydrogen peroxide may be a key modulator of an early signaling cascade that results in the changes to junctional epithelium which is seen in early disease onset.

4.3.4 Monoamine Oxidases

MAOs belong to the protein family of flavin-containing amine oxidoreductases called flavoproteins and function to catalyze the inactivation of biogenic monoamines by oxidative deamination [163]. The mitochondrial enzymes MAO-A and MAO-B are bound to mitochondrial outer membranes in most cell types and catalyze the oxidative deamination of monoamine neurotransmitters such as dopamine, adrenaline/noradrenaline, and serotonin [164]. Based on their inhibitory profile, MAOs have been studied as drug targets for the management of neurodegenerative and neurological diseases [165]. Oxidative deamination by MAO-B generates hydrogen peroxide in brain mitochondria [166]. For example, MAOs play a vital role in inactivating catecholamine neurotransmitters that are free within the nerve terminal. MAO enzymes use oxygen to remove an amine group, resulting in the formation of the corresponding aldehyde and either ammonia (in the case of primary amines) or a substituted amine (in the case of secondary amines) and also hydrogen peroxide as a significant by-product [167]. MAO-A and MAO-B isozymes are present in most mammalian tissues bound tightly to the outer mitochondrial membrane; however, the proportions of MAO-A and MAO-B vary from tissue to tissue [168, 169]. These isoenzymes were originally distinguished via their inhibition by clorgyline and deprenyl (Selegiline), and by their substrate specificities [170]. Classically, MAO-A is inhibited by low concentrations of clorgyline, whereas MAO-B is inhibited by low concentrations of deprenyl. MAO-A and MAO-B are both inhibited by phenelzine. The differential substrate specificities exhibited by these isoforms include, for example, serotonin, melatonin, norepinephrine, and epinephrine being primarily broken down by MAO-A. Benzylamine and trace amines are broken down by MAO-B. MAO-B also acts on a broad spectrum of phenylethylamines including β-phenylethylamine. Common substrates exist for both types of MAO, such as tyramine and dopamine [167]. Heterogeneity in the behavior of MAO isoenzymes within the same species exists. MAO is known to be an imidazoline-binding enzyme in the brain and peripheral tissues; however, only ~10 % of human liver MAO-B is capable of binding imidazolines, and human platelet MAO-B has been shown to weakly bind imidazolines [171]. It is not clear if this

reflects tissue-specific differences in enzyme processing or the effects of an endogenous ligand. MAO in tissues such as the intestine, liver, lungs, and placenta protects the body by oxidizing amines from the blood or from entering into circulation. The roles of MAO-A and MAO-B in terminating neurotransmitter action and dietary amines have been extensively studied; however, less attention has been paid to the activity of MAO by-products.

4.3.5 Monoamine Oxidase Regulation of Cytokine Expression

It has been postulated that remission of rheumatoid arthritis in patients who are prescribed with MAO inhibitors may be due to inhibition of prostaglandin E2 synthesis [172]. Similarly, it has been noted that the MAO inhibitor phenelzine induced remission in patients with another chronic inflammatory disease, Crohn's disease [173]. Also, another MAOB inhibitor, pargyline, has been reported to reduce oxidant-associated inflammatory damage resulting from reperfusion ischemia in a mouse vascular surgery model [174]. MAO inhibitors may possibly inhibit these diseases by blocking cytokines. MAO-B levels are closely related to the pathogenesis of Parkinson's disease, and up-regulation of TNF-α and IL-6 mRNA is increased in the hippocampus of Parkinson's patients [175, 176]. MAO-B inhibitors are effective for the treatment of Parkinson's disease, both through their direct effect on MAO-B and in part by also activating multiple factors including anti-inflammatory cytokines [175]. In a rat chronic periodontal disease model, we find that LPS-induced TNF-α protein expression is significantly abrogated by phenelzine (MAO-A and MAO-B inhibitor) [69]. In a cell-free system, phenelzine was confirmed to also be a hydrogen peroxide scavenger, which may potentiate its antioxidant effect. These data provide additional support that MAO inhibitors impact proinflammatory cytokine expression and contribute to the inhibition of oxidative stress in periodontitis by reducing hydrogen peroxide. In a rat LPS model, the topical application of phenelzine significantly reduced proliferation and apical migration of junctional epithelium, expression of TNF-α and MAO-B, PMN infiltration, elevated circulating oxidative stress, and alveolar bone loss. TNF-α, as one of the major cytokines driving inflammation, can directly promote osteoclastogenesis via binding to TNF receptor 1 on osteoclast precursor cells or indirectly via induction of macrophage colony-stimulating factor and the receptor activator for nuclear factor κB ligand on mesenchymal cells [176]. Using PLE cultures, increased levels of hydrogen peroxide have been shown to correlate with TNF-α [69].

4.4 Conclusions

The oral cavity is the one area of the body in which hard tissues break and transverse the epithelium. Due to this unique situation, the epithelium surrounding the tooth is specialized and forms an attachment and seal around each tooth. In health,

junctional epithelium is structurally and functionally very well adapted to manage the constant pressure on the host by bacteria and their virulence factors. The conversion of junctional epithelium to pocket epithelium is one significant sign in the development of periodontitis, but the regulatory mechanism associated with this conversion is still poorly understood. A variety of host molecules (cell adhesion molecules, cytokines/chemokines, growth factors and corresponding receptors, proteases, and antimicrobial peptides) may play a significant role in regulating the cellular changes associated with periodontitis. Growing evidence speaks to the importance that ROS molecules play in disease pathogenesis and their inhibition is associated with disease reduction. For example, in a rat periodontal disease model, MAO inhibitors reduced hydrogen peroxide in junctional epithelium and reduced histological parameters that are associated with disease onset.

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References

1. Dale BA, Fredericks LP (2005) Antimicrobial peptides in the oral environment: expression and function in health and disease. Curr Issues Mol Biol 7:119–133
2. Pomahac B, Svensjo T, Tao F, Brown H, Eriksson E (1998) Tissue engineering of skin. Crit Rev Oral Biol Med 9:333–344
3. Presland RB, Dale BA (2000) Epithelial structural protein of the skin and oral cavity: function in health and disease. Crit Rev Oral Biol Med 11:383–408
4. Abiko Y, Saitoh M, Nishimura M, Yamazaki M, Sawamura D, Kaku T (2007) Role of beta-defensins in oral epithelial health and disease. Med Mol Morphol 40:179–184
5. Amerongen AV, Veerman EC (2002) Saliva – the defender of the oral cavity. Oral Dis 8:12–22
6. Niyonsaba F, Ogawa H (2005) Protective roles of the skin against infection: implication of naturally occurring human antimicrobial against beta-defensins, cathelicidin LL-37 and lysozome. J Dermatol Sci 40:157–168
7. Pazgier M, Hoover DM, Yang D, Lu W, Lubkowski J (2006) Human beta-defensins. Cell Mol Life Sci 63:1294–1313
8. Maisetta G, Botani G, Esin S, Raco G, Bottai D, Favilli F, Florio W, Campa M (2005) Susceptibility of *Streptococcus mutants* and *Actinobacillus actinomycetemcomitans* to bactericidal activity of human beta-defensin 3 in biological fluids. Antimicrob Agents Chemother 49:1245–1248
9. Ouhara K, Komatsuzawa H, Yamada S, Shiba H, Fujiwara T, Ohara M, Sayama K, Hashimoto K, Kurihara H, Sugai M (2005) Susceptibilities of periodontopathogenic and cariogenic bacteria to antibacterial peptides, β-defensins and LL37, produced by human epithelial cells. J Antimicrob Chemother 55:888–896
10. Schroeder HE, Listgarten MA (1997) The gingival tissues: the architecture of periodontal protection. Periodontol 2000 13:91–120
11. Bosshardt DD, Lang NP (2005) The junctional epithelium: from health to disease. J Dent Res 84:9–20
12. Larjava H, Koivisto L, Häkkinen L, Heino J (2011) Epithelial integrins with special reference to oral epithelia. J Dent Res 90:1367–1376
13. Schroeder HE (1996) The junctional epithelium: origin, structure, and significance. A review. Acta Med Dent Helv 1:155–167

14. Lange D, Schroeder HE (1971) Cytochemistry and ultrastructure of gingival sulcus cells. Helv Odontol Acta 15:65–86
15. Schroeder HE (ed) (1981) Differentiation of the human oral stratified epithelia. S Karger Pub, Basel
16. Schroeder HE, Münzel-Pedrazzoli S (1970) Morphometric analysis comparing junctional and oral epithelium of normal human gingiva. Helv Odontol Acta 14:53–66
17. Yamasaki A, Nikai H, Niitani K, Ijuhin N (1979) Ultrastructure of the junctional epithelium of germfree rat gingiva. J Periodontol 50:641–648
18. Saito I, Watanabe O, Kawahara H, Igarashi Y, Yamamura T, Shimono M (1981) Intercellular junctions and the permeability barrier in the junctional epithelium. A study with freeze-fracture and thin sectioning. J Periodontal Res 16:467–480
19. Sasaki T, Nakagawa T, Tominaga H, Kawahara T, Higashi S (1981) Electron microscopy of the junctional epithelium of kitten gingiva. Bull Tokyo Dent Coll 22:139–149
20. Hashimoto S, Yamamura T, Shimono M (1986) Morphometric analysis of the intercellular space and desmosomes of rat junctional epithelium. J Periodontal Res 21:510–520
21. Schiött CR, Löe H (1970) The origin and variation in number of leukocytes in the human saliva. J Periodontal Res 5:36–41
22. Schroeder HE (1973) Transmigration and infiltration of leucocytes in human junctional epithelium. Helv Odontol Acta 17:6–18
23. Juhl M, Stoltze K, Reibel J (1988) Distribution of Langerhans cells in clinically healthy human gingival epithelium with special emphasis on junctional epithelium. Scand J Dent Res 96:199–208
24. Byers MR, Holland GR (1977) Trigeminal nerve endings in gingiva, junctional epithelium and periodontal ligament of rat molars as demonstrated by autoradiography. Anat Rec 188:509–523
25. Byers MR, Mecifi KB, Kimberly CL (1987) Numerous nerves with calcitonin gene-related peptide-like immunoreactivity innervate junctional epithelium of rats. Brain Res 419: 311–314
26. Kondo T, Ayasaka N, Nagata E, Tanaka T (1992) A light and electron microscopic anterograde WGA-HRP tracing study on the sensory innervation of junctional and sulcular epithelium in the rat molar. J Dent Res 71:60–65
27. Maeda T, Sodeyama T, Hara K, Takano Y (1994) Evidence for the existence of intraepithelial nerve endings in the junctional epithelium of rat molars: an immunohistochemical study using protein gene product 9.5 (PGP 9.5) antibody. J Periodontal Res 29:377–385
28. Overman DO, Salonen JI (1994) Characterization of the human junctional epithelial cells directly attached to the tooth (DAT cells) in periodontal disease. J Dent Res 73:1818–1823
29. Skougaard M (1965) Turnover of the gingival epithelium in marmosets. Acta Odontol Scand 23:623–643
30. Skougaard MR (1970) Cell renewal, with special reference to the gingival epithelium. Adv Oral Biol 4:261–288
31. Demetriou NA, Ramfjord SP (1972) Premitotic labeling and inflammation in the gingiva of Rhesus monkeys. J Periodontol 43:606–613
32. Salonen JI (1994) Proliferative potential of the attached cells of human junctional epithelium. J Periodontal Res 29:41–45
33. Hormia M, Sahlberg C, Thesleff I, Airenne T (1998) The epithelium-tooth interface – a basal lamina rich in laminin-5 and lacking other known laminin isoforms. J Dent Res 77: 1479–1485
34. Hormia M, Owaribe K, Virtanen I (2001) The dento-epithelial junction: cell adhesion by type I hemidesmosomes in the absence of a true basal lamina. J Periodontol 72:788–797
35. Oksonen J, Sorokin LM, Virtanen, Hormia M (2001) The junctional epithelium around murine teeth differs from gingival epithelium in its basement membrane composition. J Dent Res 80:2093–2097
36. Smola H, Stark HJ, Thiekötter G, Mirancea N, Krieg T, Fusenig NE (1998) Dynamics of basement membrane formation by keratinocyte-fibroblast interactions in organotypic skin culture. Exp Cell Res 239:399–410

37. Kainulainen T, Häkkinen L, Hamidi S, Larjava K, Kallioinen M, Peltonen J, Salo T, Larjava H, Oikarinen A (1998) Laminin-5 expression is independent of the injury and the microenvironment during reepithelialization of wounds. J Histochem Cytochem 46:353–360
38. Amano S, Akutsu N, Ogura Y, Nishiyama T (2004) Increase of laminin 5 synthesis in human keratinocytes by acute wound fluid, inflammatory cytokines and growth factors, and lysophospholipids. Br J Dermatol 151:961–970
39. Li M, Firth JD, Putnins EE (2005) Keratinocyte growth factor-1 expression in healthy and diseased human periodontal tissues. J Periodontal Res 40:118–128
40. Ghannad F, Nica D, Fulle MI, Grenier D, Putnins EE, Johnston S, Eslami A, Koivisto L, Jiang G, McKee MD, Häkkinen L, Larjava H (2008) Absence of αvβ6 integrin is linked to initiation and progression of periodontal disease. Am J Pathol 172:1271–1286
41. Juliano RL (2002) Signal transduction by cell adhesion receptors and the cytoskeleton: functions of integrins, cadherins, selectins, and immunoglobulin-superfamily members. Annu Rev Pharmacol Toxicol 42:283–323
42. Gräber HG, Conrads G, Wilharm J, Lampert F (1999) Role of interactions between integrins and extracellular matrix components in healthy epithelial tissue and establishment of a long junctional epithelium during periodontal wound healing: a review. J Periodontol 70: 1511–1522
43. Danen EH, Sonnenberg A (2003) Integrins in regulation of tissue development and function. J Pathol 201:632–641
44. Hormia M, Virtanen I, Quaranta V (1992) Immunolocalization of integrin α6β4 in mouse junctional epithelium suggests an anchoring function to both the internal and the external basal lamina. J Dent Res 71:1503–1508
45. Del Castillo LF, Schlegel Gómez R, Pelka M, Hornstein OP, Johannessen AC, von den Driesch P (1996) Immunohistochemical localization of very late activation integrins in healthy and diseased human gingiva. J Periodontal Res 31:36–42
46. Thorup AK, Dabelsteen E, Schou S, Gil SG, Carter WG, Reibel J (1997) Differential expression of integrins and laminin-5 in normal oral epithelia. APMIS 105:519–530
47. Gurses N, Thorup AK, Reibel J, Carter WG, Holmstrup P (1999) Expression of VLA-integrins and their related basement membrane ligands in gingiva from patients of various periodontitis categories. J Clin Periodontol 26:217–224
48. Aumailley M, El Khal A, Knöss N, Tunggal L (2003) Laminin 5 processing and its integration into the ECM. Matrix Biol 22:49–54
49. Litjens SH, de Pereda JM, Sonnenberg A (2006) Current insights into the formation and breakdown of hemidesmosomes. Trends Cell Biol 16:376–383
50. Wilhelmsen K, Litjens SH, Kuikman I, Margadant C, van Rheenen J, Sonnenberg A (2007) Serine phosphorylation of the integrin β4 subunit is necessary for epidermal growth factor receptor induced hemidesmosome disruption. Mol Biol Cell 18:3512–3522
51. Fujita T, Hayashida K, Shiba H, Kishimoto A, Matsuda S, Takeda K, Kawaguchi H, Kurihara H (2010) The expressions of claudin-1 and E-cadherin in junctional epithelium. J Periodontal Res 45:579–582
52. Ivanov DB, Philippova MP, Tkachuk VA (2001) Structure and functions of classical cadherins. Biochemistry (Moscow) 66:1174–1186
53. Ye P, Chapple CC, Kumar RK, Hunter N (2000) Expression patterns of E-cadherin, involucrin, and connexin gap junction proteins in the lining epithelia of inflamed gingiva. J Pathol 192:58–66
54. Heymann R, Wroblewski J, Terling C, Midtvedt T, Öbrink B (2001) The characteristic cellular organization and CEACAM1 expression in the junctional epithelium of rats and mice are genetically programmed and not influenced by the bacterial microflora. J Periodontol 72:454–460
55. Odin P, Asplund M, Busch C, Öbrink B (1988) Immunohistochemical localization of cell CAM105 in rat tissues: appearance in epithelia, platelets, and granulocytes. J Histochem Cytochem 36:729–739

56. Öbrink B (1997) CEA adhesion molecules: multifunctional proteins with signal-regulatory properties. Curr Opin Cell Biol 9:616–626
57. Hauck CR, Meyer TF, Lang F, Gulbins E (1998) CD66-mediated phagocytosis of Opa52 Neisseria gonorrhoeae requires a Src-like tyrosine kinase- and Rac1-dependent signalling pathway. EMBO J 17:443–454
58. Kammerer R, Hahn S, Singer BB, Luo JS, von Kleist S (1998) Biliary glycoprotein (CD66a), a cell adhesion molecule of the immunoglobulin superfamily, on human lymphocytes: structure, expression and involvement in T cell activation. Eur J Immunol 28:3664–3674
59. Singer BB, Scheffrahn I, Öbrink B (2000) The tumor growth-inhibiting cell adhesion molecule CEACAM1 (C-CAM) is differently expressed in proliferating and quiescent epithelial cells and regulates cell proliferation. Cancer Res 60:1236–1244
60. Crawford JM, Hopp B (1990) Junctional epithelium expresses the intercellular adhesion molecule ICAM-1. J Periodontal Res 25:254–256
61. Crawford JM (1992) Distribution of ICAM-1, LFA-3 and HLA-DR in healthy and diseased gingival tissues. J Periodontal Res 27:291–298
62. Gao Z, Mackenzie IC (1992) Patterns of phenotypic expression of human junctional, gingival and reduced enamel epithelia *in vivo* and *in vitro*. Epithelial Cell Biol 1:156–167
63. Tonetti MS (1997) Molecular factors associated with compartmentalization of gingival immune responses and transepithelial neutrophil migration. J Periodontal Res 32:104–109
64. Tonetti MS, Imboden MA, Lang NP (1998) Neutrophil migration into the gingival sulcus is associated with transepithelial gradients of interleukin-8 and ICAM-1. J Periodontol 69:1139–1147
65. Furuse M, Tsukita S (2006) Claudins in occluding junctions of humans and flies. Trends Cell Biol 16:181–188
66. Fujita T, Firth JD, Kittaka M, Ekuni D, Kurihara H, Putnins EE (2012) Loss of claudin-1 in lipopolysaccharide-treated periodontal epithelium. J Periodontal Res 47:222–227
67. Tonetti MS, Gerber L, Lang NP (1994) Vascular adhesion molecules and initial development of inflammation in clinically healthy human keratinized mucosa around teeth and osseointegrated implants. J Periodontal Res 29:386–392
68. Miyauchi M, Sato S, Kitagawa S, Hiraoka M, Kudo Y, Ogawa I, Zhao M, Takata T (2001) Cytokine expression in rat molar gingival periodontal tissues after topical application of lipopolysaccharide. Histochem Cell Biol 116:57–62
69. Ekuni D, Firth JD, Nayer T, Tomofuji T, Sanbe T, Irie K, Yamamoto T, Oka T, Liu Z, Vielkind J, Putnins EE (2009) Lipopolysaccharide-induced epithelial monoamine oxidase mediates alveolar bone loss in a rat chronic wound model. Am J Pathol 175:1398–1409
70. Werner S (1998) Keratinocyte growth factor: a unique player in epithelial repair processes. Cytokine Growth Factor Rev 9:153–165
71. Rubin JS, Bottaro DP, Chedid M, Miki T, Ron D, Cheon G, Taylor WG, Fortney E, Sakata H, Finch PW et al (1995) Keratinocyte growth factor. Cell Biol Int 19:399–411
72. Igarashi M, Finch PW, Aaronson SA (1998) Characterization of recombinant human fibroblast growth factor (FGF)-10 reveals functional similarities with keratinocyte growth factor (FGF-7). J Biol Chem 273:13230–13235
73. Miki T, Fleming TP, Bottaro DP, Rubin JS, Ron D, Aaronson SA (1991) Expression cDNA cloning of the KGF receptor by creation of a transforming autocrine loop. Science 251:72–75
74. Bottaro DP, Rubin JS, Ron D, Finch PW, Florio C, Aaronson SA (1990) Characterization of the receptor for keratinocyte growth factor. Evidence for multiple fibroblast growth factor receptors. J Biol Chem 265:12767–12770
75. Sanaie AR, Firth JD, Uitto VJ, Putnins EE (2002) Keratinocyte growth factor (KGF)-1 and -2 protein and gene expression in human gingival fibroblasts. J Periodontal Res 37:66–74
76. Bajaj-Elliott M, Breese E, Poulsom R, Fairclough PD, MacDonald TT (1997) Keratinocyte growth factor in inflammatory bowel disease. Increased mRNA transcripts in ulcerative colitis compared with Crohn's disease in biopsies and isolated mucosal myofibroblasts. Am J Pathol 151:1469–1476

77. Finch PW, Cheng AL (1999) Analysis of the cellular basis of keratinocyte growth factor overexpression in inflammatory bowel disease. Gut 45:848–855
78. Finch PW, Murphy F, Cardinale I, Krueger JG (1997) Altered expression of keratinocyte growth factor and its receptor in psoriasis. Am J Pathol 151:1619–1628
79. Finch PW, Pricolo V, Wu A, Finkelstein SD (1996) Increased expression of keratinocyte growth factor messenger RNA associated with inflammatory bowel disease. Gastroenterology 110:441–451
80. Brauchle M, Madlener M, Wagner AD, Angermeyer K, Lauer U, Hofschneider PH, Gregor M, Werner S (1996) Keratinocyte growth factor is highly overexpressed in inflammatory bowel disease. Am J Pathol 149:521–529
81. Guo L, Yu QC, Fuchs E (1993) Targeting expression of keratinocyte growth factor to keratinocytes elicits striking changes in epithelial differentiation in transgenic mice. EMBO J 12:973–986
82. Werner S, Peters KG, Longaker MT, Fuller-Pace F, Banda MJ, Williams LT (1992) Large induction of keratinocyte growth factor expression in the dermis during wound healing. Proc Natl Acad Sci U S A 89:6896–6900
83. Putnins EE, Firth JD, Lohachitranont A, Uitto VJ, Larjava H (1999) Keratinocyte growth factor (KGF) promotes keratinocyte cell attachment and migration on collagen and fibronectin. Cell Adhes Commun 7:211–221
84. Putnins EE, Firth JD, Uitto VJ (1996) Stimulation of collagenase (matrix metalloproteinase-1) synthesis in histiotypic epithelial cell culture by heparin is enhanced by keratinocyte growth factor. Matrix Biol 15:21–29
85. Uitto VJ, Airola K, Vaalamo M, Johansson N, Putnins EE, Firth JD, Salonen J, López-Otín C, Saarialho-Kere U, Kähäri VM (1998) Collagenase-3 (matrix metalloproteinase-13) expression is induced in oral mucosal epithelium during chronic inflammation. Am J Pathol 152:1489–1499
86. Rubin JS, Osada H, Finch PW, Taylor WG, Rudikoff S, Aaronson SA (1989) Purification and characterization of a newly identified growth factor specific for epithelial cells. Proc Natl Acad Sci U S A 86:802–806
87. Yamasaki M, Miyake A, Tagashira S, Itoh N (1996) Structure and expression of the rat mRNA encoding a novel member of the fibroblast growth factor family. J Biol Chem 271:15918–15921
88. Gao Z, Flaitz CM, Mackenzie IC (1996) Expression of keratinocyte growth factor in periapical lesions. J Dent Res 75:1658–1663
89. Grøn B, Stoltze K, Andersson A, Dabelsteen E (2002) Oral fibroblasts produce more HGF and KGF than skin fibroblasts in response to co-culture with keratinocytes. APMIS 110:892–898
90. Ohshima M, Sakai A, Sawamoto Y, Seki K, Ito K, Otsuka K (2002) Hepatocyte growth factor (HGF) system in gingiva: HGF activator expression by gingival epithelial cells. J Oral Sci 44:129–134
91. McKeown ST, Hyland PL, Locke M, Mackenzie IC, Irwin CR (2003) Keratinocyte growth factor and scatter factor expression by regionally defined oral fibroblasts. Eur J Oral Sci 111:42–50
92. Dabelsteen S, Wandall HH, Gron B, Dabelsteen E (1997) Keratinocyte growth factor mRNA expression in periodontal ligament fibroblasts. Eur J Oral Sci 105:593–598
93. Mackenzie IC, Gao Z (2001) Keratinocyte growth factor expression in human gingival fibroblasts and stimulation of *in vitro* gene expression by retinoic acid. J Periodontol 72:445–453
94. Putnins EE, Sanaie AR, Wu Q, Firth JD (2002) Induction of keratinocyte growth factor 1 expression by lipopolysaccharide is regulated by CD-14 and toll-like receptors 2 and 4. Infect Immun 70:6541–6548
95. Ekuni D, Firth JD, Putnins EE (2006) Regulation of epithelial cell growth factor receptor protein and gene expression using a rat periodontitis model. J Periodontal Res 41:340–349
96. Nordlund L, Hormia M, Saxén L, Thesleff I (1991) Immunohistochemical localization of epidermal growth factor receptors in human gingival epithelia. J Periodontal Res 26:333–338

97. Tajima Y, Yokose S, Kashimata M, Hiramatsu M, Minami N, Utsumi N (1992) Epidermal growth factor expression in junctional epithelium of rat gingiva. J Periodontal Res 27:299–300
98. Schmid J, Cohen RL, Chambers DA (1991) Plasminogen activator in human periodontal health and disease. Arch Oral Biol 36:245–250
99. Lindberg P, Baker MS, Kinnby B (2001) The localization of the relaxed form of plasminogen activator inhibitor type 2 in human gingival tissues. Histochem Cell Biol 116:447–452
100. Lindberg P, Kinnby B, Lecander I, Lang NP, Matsson L (2001) Increasing expression of tissue plasminogen activator and plasminogen activator inhibitor type 2 in dog gingival tissues with progressive inflammation. Arch Oral Biol 46:23–31
101. Uitto VJ, Salonen JI, Firth JD, Jousimies-Somer H, Saarialho-Kere U (2002) Matrilysin (matrix metalloproteinase-7) expression in human junctional epithelium. J Dent Res 81:241–246
102. Dale BA (2002) Periodontal epithelium: a newly recognized role in health and disease. Periodontol 2000 30:70–78
103. Pöllänen MT, Laine MA, Ihalin R, Uitto VJ (2012) Host-bacteria crosstalk at the dentogingival junction. Int J Dent 2012:821383
104. Schluger S, Youdelis RA, Page RC (eds) (1977) Periodontal disease. Lea and Febiger, Philadelphia
105. Takata T, Donath K (1988) The mechanism of pocket formation. A light microscopic study on undecalcified human material. J Periodontol 59:215–221
106. Hillmann G, Vipismakul V, Donath K (1990) Die Entstehung plaquebedingter Gingivataschen im Tiermodell. Eine histologische Studie an unentkalkten Dünnschliffen. Dtsch Zahnarztl Z 45:264–266 (German)
107. Ekuni D, Yamamoto T, Yamanaka R, Tachibana K, Watanabe T (2003) Proteases augment the effects of lipopolysaccharide in rat gingiva. J Periodontal Res 38:591–596
108. Klinkhamer JM (1968) Quantitative evaluation of gingivitis and periodontal disease. I. The orogranulocytic migratory rate. Periodontics 6:207–211
109. Klinkhamer JM, Zimmerman S (1969) The function and reliability of the orogranulocytic migratory rate as a measure of oral health. J Dent Res 48:709–715
110. Attström R (1970) Presence of leukocytes in crevices of healthy and chronically inflamed gingivae. J Periodontal Res 5:42–47
111. Attström R, Egelberg J (1970) Emigration of blood neutrophils and monocytes into the gingival crevices. J Periodontal Res 5:48–55
112. Kowashi Y, Jaccard F, Cimasoni G (1980) Sulcular polymorphonuclear leucocytes and gingival exudate during experimental gingivitis in man. J Periodontal Res 15:151–158
113. Firth JD, Ekuni D, Irie K, Tomofuji T, Morita M, Putnins EE (2013) Lipopolysaccharide induces a stromal-epithelial signalling axis in a rat model of chronic periodontitis. J Clin Periodontol 40:8–17
114. Schroeder HE, Attström R (1980) Pocket formation: an hypothesis. In: Lehner T, Cimasoni G (eds) The borderland between caries and periodontal disease II. Academic/Grune & Stratton, London, pp 99–123
115. Lamont RJ, Oda D, Persson RE, Persson GR (1992) Interaction of *Porphyromonas gingivalis* with gingival epithelial cells maintained in culture. Oral Microbiol Immunol 7:364–367
116. Lamont RJ, Chan A, Belton CM, Izutsu KT, Vasel D, Weinberg A (1995) *Porphyromonas gingivalis* invasion of gingival epithelial cells. Infect Immun 63:3878–3885
117. Sandros J, Papapanou PN, Nannmark U, Dahlén G (1994) *Porphyromonas gingivalis* invades human pocket epithelium *in vitro*. J Periodontal Res 29:62–69
118. Madianos PN, Papapanou PN, Nannmark U, Dahlén G, Sandros J (1996) *Porphyromonas gingivalis* FDC381 multiplies and persists within human oral epithelial cells *in vitro*. Infect Immun 64:660–664
119. Meyer DH, Mintz KP, Fives-Taylor PM (1997) Models of invasion of enteric and periodontal pathogens into epithelial cells: a comparative analysis. Crit Rev Oral Biol Med 8:389–409

120. Njoroge T, Genco RJ, Sojar HT, Hamada N, Genco CA (1997) A role for fimbriae in *Porphyromonas gingivalis* invasion of oral epithelial cells. Infect Immun 65:1980–1984
121. Deshpande RG, Khan M, Genco CA (1998) Invasion strategies of the oral pathogen *Porphyromonas gingivalis*: implications for cardiovascular disease. Invasion Metastasis 18:57–69
122. Huard-Delcourt A, Menard C, Du L, Pellen-Mussi P, Tricot-Doleux S, Bonnaure-Mallet M (1998) Adherence of *Porphyromonas gingivalis* to epithelial cells: analysis by flow cytometry. Eur J Oral Sci 106:938–944
123. Lamont RJ, Jenkinson HF (1998) Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. Microbiol Mol Biol Rev 62:1244–1263
124. Fives-Taylor PM, Meyer DH, Mintz KP, Brissette C (1999) Virulence factors of *Actinobacillus actinomycetemcomitans*. Periodontol 2000 20:136–167
125. Forng RY, Champagne C, Simpson W, Genco CA (2000) Environmental cues and gene expression in *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*. Oral Dis 6:351–365
126. Quirynen M, Papaioannou W, van Steenbergen TJ, Dierickx K, Cassiman JJ, van Steenberghe D (2001) Adhesion of *Porphyromonas gingivalis* strains to cultured epithelial cells from patients with a history of chronic adult periodontitis or from patients less susceptible to periodontitis. J Periodontol 72:626–633
127. Carro OM, Evans SAS, Leone CW (1997) Effect of inflammation on the proliferation of human gingival epithelial cells *in vitro*. J Periodontol 68:1070–1075
128. Vitkov L, Krautgartner WD, Hannig M (2005) Bacterial internalization in periodontitis. Oral Microbiol Immunol 20:317–321
129. Andrian E, Grenier D, Rouabhia M (2004) *In vitro* models of tissue penetration and destruction by *Porphyromonas gingivalis*. Infect Immun 72:4689–4698
130. Wang PL, Shinohara M, Murakawa N, Endo M, Sakata S, Okamura M, Ohura K (1999) Effect of cysteine protease of *Porphyromonas gingivalis* on adhesion molecules in gingival epithelial cells. Jpn J Pharmacol 80:75–79
131. Katz J, Sambandam V, Wu JH, Michalek SM, Balkovetz DF (2000) Characterization of *Porphyromonas gingivalis*-induced degradation of epithelial cell junctional complexes. Infect Immun 68:1441–1449
132. Katz J, Yang QB, Zhang P, Potempa J, Travis J, Michalek SM, Balkovetz DF (2002) Hydrolysis of epithelial junctional proteins by *Porphyromonas gingivalis* gingipains. Infect Immun 70:2512–2518
133. Chen T, Nakayama K, Belliveau L, Duncan MJ (2001) *Porphyromonas gingivalis* gingipains and adhesion to epithelial cells. Infect Immun 69:3048–3056
134. Hintermann E, Haake SK, Christen U, Sharabi A, Quaranta V (2002) Discrete proteolysis of focal contact and adherens junction components in *Porphyromonas gingivalis*-infected oral keratinocytes: a strategy for cell adhesion and migration disabling. Infect Immun 70: 5846–5856
135. Tada H, Sugawara S, Nemoto E, Imamura T, Potempa J, Travis J, Shimauchi H, Takada H (2003) Proteolysis of ICAM-1 on human oral epithelial cells by gingipains. J Dent Res 82:796–801
136. Takata T, Miyauchi M, Ogawa I, Ito H, Kobayashi J, Nikai H (1997) Reactive change in proliferative activity of the junctional epithelium after topical application of lipopolysaccharide. J Periodontol 68:531–535
137. Ekuni D, Tomofuji T, Yamanaka R, Tachibana K, Yamamoto T, Watanabe T (2005) Initial apical migration of junctional epithelium in rats following application of lipopolysaccharide and proteases. J Periodontol 76:43–48
138. Yoon SO, Park SJ, Yoon SY, Yun CH, Chung AS (2002) Sustained production of H_2O_2 activates pro-matrix metalloproteinase-2 through receptor tyrosine kinases/phosphatidylinositol 3-kinase/NF-kappa B pathway. J Biol Chem 277:30271–30282
139. Zaragoza C, Lopez-Rivera E, Garcia-Rama C, Saura M, Martinez-Ruiz A, Lizarbe TR, Martin-de-Lara F, Lamas S (2006) Cbfa-1 mediates nitric oxide regulation of MMP-13 in osteoblasts. J Cell Sci 119:1896–1902

140. Chapple IL (1997) Reactive oxygen species and antioxidants in inflammatory diseases. J Clin Periodontol 24:287–296
141. Halliwell B, Whiteman M (2004) Measuring reactive species and oxidative damage *in vivo* and in cell culture: how should you do it and what do the results mean? Br J Pharmacol 142:231–255
142. Thannickal VJ, Day RM, Klinz SG, Bastien MC, Larios JM, Fanburg BL (2000) Ras-dependent and -independent regulation of reactive oxygen species by mitogenic growth factors and TGF-β1. FASEB J 14:1741–1748
143. Klann E, Thiels E (1999) Modulation of protein kinases and protein phosphatases by reactive oxygen species: implications for hippocampal synaptic plasticity. Prog Neuropsychopharmacol Biol Psychiatry 23:359–376
144. Waghray M, Cui Z, Horowitz JC, Subramanian IM, Martinez FJ, Toews GB, Thannickal VJ (2005) Hydrogen peroxide is a diffusible paracrine signal for the induction of epithelial cell death by activated myofibroblasts. FASEB J 19:854–856
145. Halliwell B, Gutteridge JM (1992) Biologically relevant metal ion-dependent hydroxyl radical generation. An update. FEBS Lett 307:108–112
146. Ha E-M, Oh C-T, Ryu J-H, Bae Y-S, Kang S-W, Jang I-H, Brey PT, Lee WJ (2005) An antioxidant system required for host protection against gut infection in *Drosophila*. Dev Cell 8:125–132
147. Ding S-Z, Minohara Y, Fan XJ, Wang J, Reyes VE, Patel J, Dirden-Kramer B, Boldogh I, Ernst PB, Crowe SE (2007) *Helicobacter pylori* infection induces oxidative stress and programmed cell death in human gastric epithelial cells. Infect Immun 75:4030–4039
148. Blaser MJ, Perez-Perez GI, Kleanthous H, Cover TL, Peek RM, Chyou PH, Stemmermann GN, Nomura A (1995) Infection with *Helicobacter pylori* strains possessing cagA is associated with an increased risk of developing adenocarcinoma of the stomach. Cancer Res 55:2111–2115
149. Handa O, Naito Y, Yoshikawa T (2007) CagA protein of *Helicobacter pylori*: a hijacker of gastric epithelial cell signaling. Biochem Pharmacol 73:1697–1702
150. Akalin FA, Baltacioğlu E, Alver A, Karabulut E (2007) Lipid peroxidation levels and total oxidant status in serum, saliva and gingival crevicular fluid in patients with chronic periodontitis. J Clin Periodontol 34:558–565
151. Chapple IL, Brock GR, Milward MR, Ling N, Matthews JB (2007) Compromised GCF total antioxidant capacity in periodontitis: cause or effect? J Clin Periodontol 34:103–110
152. Tsai CC, Chen HS, Chen SL, Ho YP, Ho KY, Wu YM, Hung CC (2005) Lipid peroxidation: a possible role in the induction and progression of chronic periodontitis. J Periodontal Res 40:378–384
153. Ekuni D, Tomofuji T, Tamaki N, Sanbe T, Azuma T, Yamanaka R, Yamamoto T, Watanabe T (2008) Mechanical stimulation of gingiva reduces plasma 8-OHdG level in rat periodontitis. Arch Oral Biol 53:324–329
154. Pan YM, Firth JD, Salonen JI, Uitto VJ (1995) Multilayer culture of periodontal ligament epithelial cells: a model for junctional epithelium. J Periodontal Res 30:97–107
155. Firth JD, Uitto VJ, Putnins EE (2008) Mechanical induction of an epithelial cell chymase associated with wound edge migration. J Biol Chem 283:34983–34993
156. Li C (2008) Automating dChip: toward reproducible sharing of microarray data analysis. BMC Bioinformatics 9:231
157. Wesseling S, Joles JA, van Goor H, Bluyssen HA, Kemmeren P, Holstege FC, Koomans HA, Braam B (2007) Transcriptome-based identification of pro- and antioxidative gene expression in kidney cortex of nitric oxide-depleted rats. Physiol Genomics 28:158–167
158. Kim YH, Lim DS, Lee JH, Shim WJ, Ro YM, Park GH, Becker KG, Cho-Chung YS, Kim MK (2003) Gene expression profiling of oxidative stress on atrial fibrillation in humans. Exp Mol Med 35:336–349
159. Doniger SW, Salomonis N, Dahlquist KD, Vranizan K, Lawlor SC, Conklin BR (2003) MAPPFinder: using Gene Ontology and GenMAPP to create a global gene-expression profile from microarray data. Genome Biol 4:R7

160. Dahlquist KD, Salomonis N, Vranizan K, Lawlor SC, Conklin BR (2002) GenMAPP, a new tool for viewing and analyzing microarray data on biological pathways. Nat Genet 31:19–20
161. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G (2000) Gene ontology: tool for the unification of biology. The gene ontology consortium. Nat Genet 25:25–29
162. Miyazaki Y, Shinomura Y, Tsutsui S, Yasunaga Y, Zushi S, Higashiyama S, Taniguchi N, Matsuzawa Y (1996) Oxidative stress increases gene expression of heparin-binding EGF-like growth factor and amphiregulin in cultured rat gastric epithelial cells. Biochem Biophys Res Commun 226:542–546
163. Edmondson DE, Mattevi A, Binda C, Li M, Hubálek F (2004) Structure and mechanism of monoamine oxidase. Curr Med Chem 11:1983–1993
164. Weyler W, Hsu YP, Breakefield XO (1990) Biochemistry and genetics of monoamine oxidase. Pharmacol Ther 47:391–417
165. Binda C, Milczek EM, Bonivento D, Wang J, Mattevi A, Edmondson DE (2011) Lights and shadows on monoamine oxidase inhibition in neuroprotective pharmacological therapies. Curr Top Med Chem 11:2788–2796
166. Werner P, Cohen G (1993) Glutathione disulfide (GSSG) as a marker of oxidative injury to brain mitochondria. Ann N Y Acad Sci 679:364–369
167. Magyar K, Szende B (2004) (−)-Deprenyl, a selective MAO-B inhibitor, with apoptotic and anti-apoptotic properties. Neurotoxicology 25:233–242
168. Tipton KF, Boyce S, O'Sullivan J, Davey GP, Healy J (2004) Monoamine oxidases: certainties and uncertainties. Curr Med Chem 11:1965–1982
169. Shih JC, Chen K, Ridd MJ (1999) Monoamine oxidase: from genes to behavior. Annu Rev Neurosci 22:197–217
170. Johnston JP (1968) Some observations upon a new inhibitor of monoamine oxidase in brain tissue. Biochem Pharmacol 17:1285–1297
171. Raddatz R, Parini A, Lanier SM (1995) Imidazoline/guanidinium binding domains on monoamine oxidases. Relationship to subtypes of imidazoline-binding proteins and tissue-specific interaction of imidazoline ligands with monoamine oxidase B. J Biol Chem 270: 27961–27968
172. Lieb J (1983) Remission of rheumatoid arthritis and other disorders of immunity in patients taking monoamine oxidase inhibitors. Int J Immunopharmacol 5:353–357
173. Kast RE (1998) Crohn's disease remission with phenelzine treatment. Gastroenterology 115:1034–1035
174. Chaaya R, Alfarano C, Guilbeau-Frugier C, Coatrieux C, Kesteman AS, Parini A, Fares N, Gue M, Schanstra JP, Bascands JL (2011) Pargyline reduces renal damage associated with ischaemia-reperfusion and cyclosporin. Nephrol Dial Transplant 26:489–498
175. Nagatsu T, Sawada M (2006) Molecular mechanism of the relation of monoamine oxidase B and its inhibitors to Parkinson's disease: possible implications of glial cells. J Neural Transm Suppl 71:53–65
176. Lam J, Takeshita S, Barker JE, Kanagawa O, Ross FP, Teitelbaum SL (2000) TNF-α induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. J Clin Invest 106:1481–1488

Chapter 5
DNA Damage Caused by Oral Malodorous Compounds in Periodontal Cells In Vitro: Novel Carcinogenic Pathway

Bogdan Calenic and Ken Yaegaki

5.1 Introduction

Under normal conditions, cellular metabolism generates endogenous reactive oxygen species (ROS) that can be detrimental to the cell. Following toxic insults, the intracellular levels of ROS are increased, and they inevitably interact with different cellular molecules including DNA, causing DNA damage. This phenomenon has been well documented in a number of diseases such as cardiovascular diseases and neurodegenerative conditions [1]. Also numerous studies have established a connection between DNA damage caused by ROS and the initiation of the carcinogenetic process [2]. Increased ROS causes apoptosis/necrosis and mitochondrial dysfunction, which initiates the aging process. In order to maintain DNA integrity, cells have evolved a number of defense systems that will protect them from DNA damage or cell death due to irreparable DNA damage. Cell response is largely dependent on the amount of accumulated ROS and DNA damage, resulting in one of DNA repair, cell-cycle arrest, or activation of the apoptotic process that ultimately leads to cell death. Increased ROS and DNA damage are also present in a number of inflammatory diseases such as periodontitis. Moreover, increased ROS itself and/or DNA damage caused by ROS might be one of the causes of the aging process in oral tissues, since H_2S, which causes the production of ROS, is always present in the oral cavity. Recent studies show that periodontal inflammation increases circulating levels of lipid peroxide and causes DNA damage in distant organs such as brain, heart, liver, and kidney [3].

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5.2 H_2S and ROS

Aside from its role in halitosis, hydrogen sulfide plays an important part in the initiation and development of periodontitis [4–6]. H_2S has been shown to activate apoptosis in vitro in many cellular types derived from periodontal tissue: oral fibroblasts [7], oral keratinocytes [8], oral keratinocyte stem cells [9], dental-pulp stem cells [10] and osteoblasts [11], and also in vivo in osteoblasts [12]. These studies have also described the genotoxic effects of H_2S, suggesting that this compound may have pathological effects on human oral mucosa at a genomic level. As pointed out earlier, DNA damage is closely linked to an increase in ROS levels. Several reports have focused on the connection between H_2S and an increase in ROS, especially at the mitochondrial level. Cytochrome c oxidase (COX) is a key enzyme of the mitochondrial respiratory chain. The enzyme plays a central role in the energy production and storage of aerobic cells involved in ATP synthesis [13]. Studies show that H_2S can act as a strong inhibitor of COX [14, 15]. The inhibition mechanism can be explained by H_2S binding to the heme iron subunit of the COX enzyme, which in turn inhibits ATP synthesis. As well, COX inhibition by H_2S ultimately leads to increased ROS production inside the mitochondria, which causes a disruption in the electrochemical gradient of the inner mitochondrial membrane. The collapse of this gradient is followed by the release of cytochrome c into cytosol; the apoptosome is assembled and then activates caspase-9, which in turn triggers executioner caspases such as caspase-3.

Our results show that in gingival fibroblasts physiological levels of H_2S significantly inhibit both CuZn-SOD (superoxide dismutase) and Mn-SOD. SOD is an important enzyme of the antioxidant system known to catalyze the reduction of superoxide. As well, ROS production is enhanced after both 1 and 2 days of H_2S incubation [7]. In another study [8], similar results were obtained for gingival keratinocytes, showing that periodontal tissues like oral dermis and epidermis may react in the same way when faced with increased levels of VOCs. Flow-cytometric data show that in oral keratinocyte cells more than 40 and 50 % of the cells were positive for MitoSOX after 24 and 48 h of H_2S incubation, respectively. The increase in ROS was followed by activation of the mitochondrial apoptotic pathway.

5.3 DNA Damage Caused by H_2S

DNA strand breaks have been observed in a number of cell types derived from important periodontal tissues. We have reported an increase in the number of DNA strand breaks following an increase in ROS levels caused by H_2S in oral fibroblasts [7]. The genotoxic effects of H_2S have been further observed in oral keratinocyte cells, using single-cell gel electrophoresis [8]. A further in vitro study focusing on osteoblasts also observed DNA strand breaks following H_2S exposure [11]. Placed in an electrophoretic field, the intact DNA migrates slowly while the damaged DNA fragments move much faster, producing a comet-shaped form. Image analysis offers

quantitative data on the number of DNA strand breaks by correlating them with parameters like tail moment, DNA in tail, and tail length. The combined data show that of the three cell types, osteoblasts show the highest percentage of DNA damage, followed by oral keratinocytes and oral fibroblasts [7, 8, 11].

Another molecular event triggered by DNA damage as a result of ROS increments is the activation of p53 protein and its pathway. p53 Protein, "the guardian of the genome," is a tumor-suppressor gene functioning as a transcriptional factor, and a key regulator of cellular fate [16]. Depending on the level of cellular stress, p53 can prevent the replication of damaged DNA by activating apoptosis, cell-cycle arrest, or DNA repair [17]. Specific phosphorylation of p53 plays an important role in deciding cellular fate [18]. Thus after severe DNA damage, p53-dependent apoptosis is triggered via p53-phosphorylation of serine-46 [19]. We have recently shown that in keratinocyte stem cells derived from human skin, serine-46-phosphorylated-p53 levels were increased after H_2S incubation for 1 and 2 days [9]. Apoptosis was induced via the mitochondrial intrinsic pathway. The same study reported that Ser-46-Phospho-p53 levels represented approximately 2/3 of the total activated p53. These data suggest that following DNA damage, p53 may also be involved in other biological processes besides apoptosis.

In a further study done in oral keratinocyte stem cells, DNA damage was induced after 2 days of H_2S incubation [20]. As a result, multiple molecules along the p53 pathway were activated. qRT-PCR data showed that the level of the Chek2 gene, a DNA checkpoint protein [20], was significantly elevated when compared to controls. E2F2, E2F4, and E2F6 are factors that play important roles in regulating cell-cycle control and pro-apoptotic signals [21–25]. Thus E2F4 and E2F6 are pro-cell-cycle exit genes while over-expression of E2F2 can promote apoptosis. Cell-cycle arrest at the G1 phase following H_2S exposure has also been previously observed in normal keratinocytes [26]. Besides apoptosis and cell-cycle arrest, several genes along the DNA repair pathway have been activated as a result of H_2S incubation.

DNA repair function is of paramount importance for the normal homeostasis of oral epithelial tissue. The proteins in the GADD45 family are important checkpoints in the cellular-cycle and DNA-repair processes [27, 28]. Our study showed that levels of GADD45G, GADD45A, and GADD45B genes were significantly higher after 48 h of H_2S incubation. As a result of oxidative stress, Sirtuin proteins are activated along the sites of DNA damage and participate in DNA repair [29]. Both Sirtuin 3 and 6 genes were at significantly higher levels than in controls. p53 Activation also initiated apoptosis by activating the caspase cascade, caspase-3, -6, -7, -9, and other molecules along the intrinsic mitochondrial pathway.

5.4 p53 and Aging

The accumulation of mitochondrial ROS is caused by problems of the respiratory chain or by less active SOD. H_2S, as a mitochondrial toxic compound, is one of the most important factors in the aging process [30]. Aging is the result of dilapidated

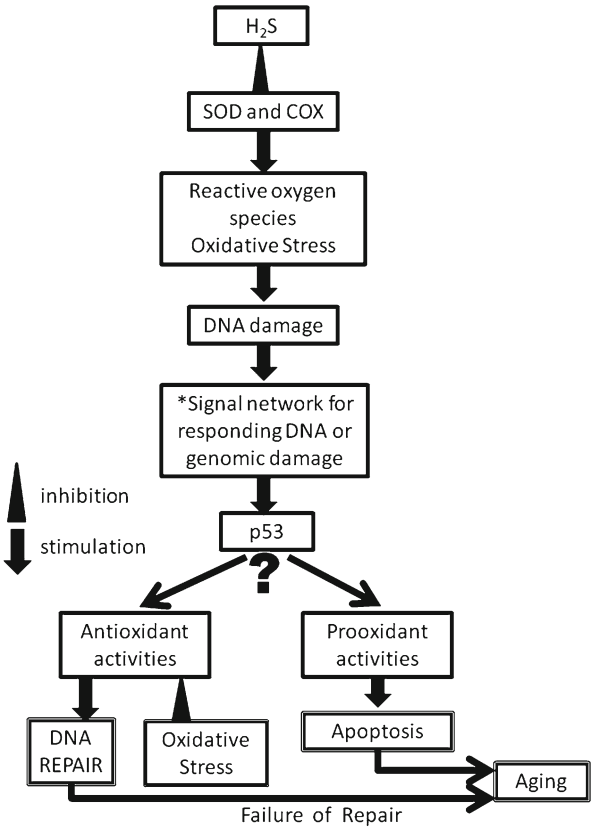

Fig. 5.1 Hydrogen sulfide causes antioxidant or pro-oxidant activity of the aging process. *Refer Chap. 7

homeostasis caused by the inadequate nature of the defense mechanisms against environmental factors and host–environment interactions [31]. Intracellular oxidative stress also injures intracellular mechanisms and may cause lifestyle-related diseases and/or age-related diseases such as diabetes or cancer [32]. DNA damage caused by ROS includes oxidized bases, basic sites (apurinic/apyrimidinic sites), double-strand DNA breaks, and single-strand DNA breaks. These errors are repaired with nucleotide excision repair (NER) or base excision repair (BER)/single-strand break repair. Hence dysfunction of BER or NER might lead to cancer or aging [33]. Constant low-level activation of p53 causes deregulated expression of p53 and/or response to DNA damage, allowing premature aging in animals [34, 35]. In other words, strong oxidative damage is not always required to initiate the aging process. However, it is agreed that normal activation of p53 by low oxidative stresses in normal conditions and environments protects cells from oxidative damage, by activating p53-induced antioxidant and repair functions to eliminate oxidative stress; i.e., normal activation of p53 defends against the aging process. p53 Prevents the accumulation of high levels of oxidative stresses that may induce p53-dependent apoptosis and senescence. On the other hand, strong and persistent oxidative stresses cause pro-oxidative activities and then lead to apoptosis, promoting the process of aging (Fig. 5.1). It has been

suggested that the balance of p53 antioxidant with pro-oxidant activities caused by oxidative stresses determines whether the suppression of the accumulation of oxidative stresses and DNA damage results in cell survival or aging. In contrast, irregularly and highly activated p53 produced by strong oxidative stresses can result in apoptosis and the host's aging [36, 37]. It has been shown that elevated p53 activity promotes aging, while a normally regulated p53 response defends against aging.

Apoptosis, which is often caused in all gingival tissues by H_2S, diminishes or inhibits the proliferation of cells including stem cells [20]. Aging in tissue depends on the replacement of damaged cells by newly produced sister cells from stromal stem cells. The replacement maintains the tissues' homeostasis. Maintenance of stem-cell viability and functions also depends on the balance between the removal of damaged cells by apoptosis and the survival and proliferation of the cells after DNA repair. Maintaining this balance is extremely important in preserving homeostasis of the host. However, in the oral cavity hydrogen sulfide or other volatile sulfur compounds easily create an unbalance by causing apoptosis, and thus accelerate the aging of oral tissues [7–11, 20]. A failure in self-renewal of stem cells happens because of aging in the stem cells themselves, as mentioned above. Therefore, the number of stem cell declines with age. The lower number of stem cells may contribute further to the aging process [31]. It has been found that overactive p53 accelerates the aging process and reduces the self-renewal of stem cells in animals [38]. We conclude that p53-mediated apoptosis permanently diminishes the number of stem cells in the tissues and becomes one of the causes of the aging process.

It has been found in the oral cavity that fibroblasts in oral submucous fibrosis accumulate senescent cells through increased ROS production and DNA double-strand breaks, producing damaged mitochondria. Interestingly, the presence of irreparable DNA double-strand breaks in normal oral fibroblasts increases dramatically the secretion of inhibitors of the metalloproteinases 1(TIMP-1) and TIMP-2. TIMP may be connected with the aging process in oral mucosal fibroblasts [39]. Moreover, the senescence phase of normal human oral keratinocytes includes accumulations of intracellular ROS. Senescing normal human oral keratinocytes also accumulate oxidative DNA damage because of increased ROS. As we have demonstrated above, DNA damage caused by H_2S could cause the aging process of oral tissues.

5.5 Conclusion

The present available scientific data show that H_2S induces DNA damage in cells derived from oral epithelia, oral dermis, and bone. The molecular pathways activated include cell-cycle arrest, DNA repair, and apoptosis (Fig. 5.2). However, more studies are needed in order to elucidate in more detail the genotoxic effects of H_2S on oral tissues.

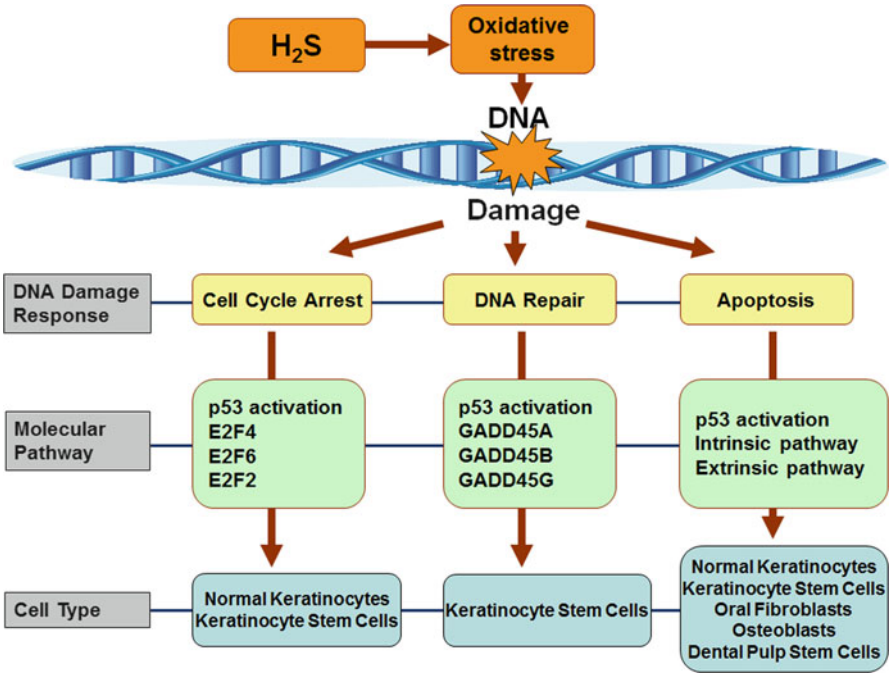

Fig. 5.2 H_2S-induced DNA damage. In response to DNA damage following H_2S exposure, cells can activate several defense mechanisms such as cell-cycle arrest, DNA repair, and the apoptotic process. Apoptosis is triggered in cells derived from oral epithelium, oral dermis, bone or dental pulp. In oral keratinocyte stem cells the p53 molecular pathway is activated, together with genes involved in the DNA repair process. Molecules playing important roles in cell-cycle arrest are activated in both normal and keratinocyte stem cells following H_2S incubation

References

1. Cooke MS, Evans MD, Dizdaroglu M, Lunec J (2003) Oxidative DNA damage: mechanisms, mutation, and disease. FASEB J 17:1195–1214
2. Jin B, Robertson KD (2013) DNA methyltransferases, DNA damage repair, and cancer. Adv Exp Med Biol 754:3–29
3. Tomofuji T, Ekuni D, Irie K, Azuma T, Tamaki N, Maruyama T, Yamamoto T, Watanabe T, Morita M (2011) Relationships between periodontal inflammation, lipid peroxide and oxidative damage of multiple organs in rats. Biomed Res 32:343–349
4. Yaegaki K, Sanada K (1992) Volatile sulfur compounds in mouth air from clinically healthy subjects and patients with periodontitis. J Periodontal Res 27:233–238
5. Yaegaki K (1995) Oral malodor and periodontal disease. In: Rosenberg M (ed) Bad breath: research perspectives. Ramot Publishing-Tel Aviv University, Tel Aviv, pp 87–108
6. Johnson P, Yaegaki K, Tonzetich J (1996) Effect of methyl mercaptan on synthesis and degradation of collagen. J Periodontal Res 31:323–329
7. Yaegaki K, Qian W, Murata T, Imai T, Sato T, Tanaka T, Kamoda T (2008) Oral malodorous compound causes apoptosis and genomic DNA damage in human gingival fibroblasts. J Periodontal Res 43:391–399

8. Calenic B, Yaegaki K, Murata T, Imai T, Aoyama I, Sato T, Ii H (2010) Oral malodorous compound triggers mitochondrial-dependent apoptosis and causes genomic DNA damage in human gingival epithelial cells. J Periodontal Res 45:31–37
9. Calenic B, Yaegaki K, Kozhuharova A, Imai T (2010) Oral malodorous compound causes oxidative stress and p53-mediated programmed cell death in keratinocyte stem cells. J Periodontol 81:1317–1323
10. Kobayashi C, Yaegaki K, Calenic B, Ishkitiev N, Imai T, Ii H, Aoyama I, Kobayashi H, Izumi Y, Haapasalo M (2011) Hydrogen sulfide causes apoptosis in human pulp stem cells. J Endod 37:479–484
11. Aoyama I, Yaegaki K, Calenic B, Ii H, Ishkitiev N, Imai T (2012) The role of p53 in an apoptotic process caused by an oral malodorous compound in periodontal tissues: a review. J Breath Res 6:017104. doi:10.1088/1752-7155/6/1/017104
12. Irie K, Ekuni D, Yamamoto T et al (2009) One shot of hydrogen sulfide application induces a transient osteoclast differentiation with RANKL expression in the rat model. Arch Oral Biol 54:723–729
13. Fontanesi F, Soto IC, Barrientos A (2008) Cytochrome c oxidase biogenesis: new levels of regulation. IUBMB Life 60:557–568
14. Dorman DC, Moulin FJ, McManus BE, Mahle KC, James RA, Struve MF (2002) Cytochrome oxidase inhibition induced by acute hydrogen sulfide inhalation: correlation with tissue sulfide concentrations in the rat brain, liver, lung, and nasal epithelium. Toxicol Sci 65:18–25
15. Beauchamp RO Jr, Bus JS, Popp JA, Boreiko CJ, Andjelkovich DA (1984) A critical review of the literature on hydrogen sulfide toxicity. Crit Rev Toxicol 13:25–97
16. Meek DW (2009) Tumor suppression by p53: a role for the DNA damage response? Nat Rev Cancer 10:714–723
17. Rufini A, Melino G (2011) Cell death pathology: the war against cancer. Biochem Biophys Res Commun 414:445–450
18. Kruse JP, Gu W (2009) Modes of p53 regulation. Cell 137:609–622
19. D'Orazi G, Cecchinelli B, Bruno T et al (2002) Homeodomain-interacting protein kinase-2 phosphorylates p53 at Ser 46 and mediates apoptosis. Nat Cell Biol 4:11–19
20. Calenic B, Yaegaki K, Ishkitiev N, Kumazawa Y, Imai T, Tanaka T (2013) p53-Pathway activity and apoptosis in hydrogen sulfide-exposed stem cells separated from human gingival epithelium. J Periodontal Res 48:322–330
21. Chehab NH, Malikzay A, Appel M, Halazonetis TD (2000) Chk2/hCds1 functions as a DNA damage checkpoint in G1 by stabilizing p53. Genes Dev 14:278–288
22. DeGregori J, Johnson DG (2006) Distinct and overlapping roles for E2F family members in transcription, proliferation and apoptosis. Curr Mol Med 6:739–748
23. Trimarchi JM, Lees JA (2002) Sibling rivalry in the E2F family. Nat Rev Mol Cell Biol 3:11–20
24. Giangrande PH, Zhu W, Schlisio S, Sun X, Mori S, Gaubatz S, Nevins JR (2004) A role for E2F6 in distinguishing G1/S- and G2/M-specific transcription. Genes Dev 18:2941–2951
25. Polager S, Ginsberg D (2009) p53 and E2f: partners in life and death. Nat Rev Cancer 9:738–748
26. Takeuchi H, Setoguchi T, Machigashira M, Kanbara K, Izumi Y (2008) Hydrogen sulfide inhibits cell proliferation and induces cell cycle arrest via an elevated p21 Cip1 level in Ca9-22 cells. J Periodontal Res 43:90–95
27. Liebermann DA, Hoffman B (2008) Gadd45 in stress signaling. J Mol Signal 3:15
28. Rosemary Siafakas A, Richardson DR (2009) Growth arrest and DNA damage 45 alpha (GADD45alpha). Int J Biochem Cell Biol 41:986–989
29. Milne JC, Denu JM (2008) The Sirtuin family: therapeutic targets to treat diseases of aging. Curr Opin Chem Biol 12:11–17
30. Nakanishi H, Wu Z (2009) Microglia-aging: roles of microglial lysosome- and mitochondria-derived reactive oxygen species in brain aging. Behav Brain Res 201:1–7

31. Jones DL, Rando TA (2011) Emerging models and paradigms for stem cell ageing. Nat Cell Biol 13:506–512
32. Ishii T, Miyazawa M, Hartman PS et al (2011) Mitochondrial superoxide anion (O(2)(−)) inducible "mev-1" animal models for aging research. BMB Rep 44:298–305
33. Hegde ML, Izumi T, Mitra S (2012) Oxidized base damage and single-strand break repair in mammalian genomes: role of disordered regions and posttranslational modifications in early enzymes. Prog Mol Biol Transl Sci 110:123–153
34. Tyner SD, Venkatachalam S, Choi J et al (2002) p53 Mutant mice that display early ageing – associated phenotypes. Nature 415:45–53
35. Maier B, Gluba W, Bernier B et al (2004) Modulation of mammalian life span by the short isoform of p53. Genes Dev 18:306–319
36. Vigneron A, Vousden KH (2010) p53, ROS and senescence in the control of aging. Aging 2:471–474
37. Liu D, Xu Y (2010) p53, oxidative stress, and aging. Antioxid Redox Signal 15:1669–1678
38. Pehar M, O'Riordan KJ, Burns-Cusato M et al (2010) Altered longevity – assurance activity of p53:p44 in the mouse causes memory loss, neurodegeneration and premature death. Aging Cell 9:174–190
39. Pitiyage GN, Lim KP, Gemenitzidis E et al (2012) Increased secretion of tissue inhibitors of metalloproteinases 1 and 2 (TIMPs −1 and −2) in fibroblasts are early indicators of oral submucous fibrosis and ageing. J Oral Pathol Med 41:454–462

Chapter 6
Apoptotic Pathways Triggered by Oral Malodorous Compounds in Periodontal Cells: Novel Periodontal Pathologic Cause

Bogdan Calenic and Ken Yaegaki

6.1 Introduction

Apoptosis, a form of programmed cell death, evolved as a key regulator of tissue homeostasis in all multicellular organisms. Deregulation of the apoptotic molecular pathways is often encountered in many systemic diseases as well as in oral infections. Periodontitis causes strong oral malodor [1], and the oral malodor compounds, volatile sulfur compounds (VSCs), are cytotoxic, especially in gingival tissues [2, 3]. Recently we found that one of the VSCs, hydrogen sulfide, causes apoptosis in human gingival cells [4–9]. Apoptosis is both a very old and a novel topic in periodontal pathogenesis. After apoptosis had been described generally, some research endeavors focused on apoptosis, but they observed only the occurrence of apoptosis; the background and mechanisms of apoptosis had not yet been investigated in periodontal pathogenesis. Hence no one has described the important role apoptosis plays in periodontal pathogenesis. However, the aging process of oral tissue is different from that in other tissues, e.g. the loss of periodontal tissues without suffering periodontitis. Such an apoptotic process might be one of the reasons for the nature of aging in oral tissues.

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6.2 Apoptosis in General

Apoptosis, or programmed cell death, is a vital biological process involved in the regulation of cell number and organ size in all living organisms. The apoptotic process is tightly regulated by a group of caspases (cysteine-dependent aspartate-specific proteases). The caspase cascade is similar to the pathway of coagulation, with downstream caspases being activated through proteolysis by upstream ones. So far 11 caspases have been discovered, with eight of them being involved in apoptosis: caspase-1, -2, -8, -9, and -10 are initiator caspases while caspase-3, -6, and -7 are executioner caspases. The activation of apical caspases is depended on different mechanisms. Thus in the death ligand extrinsic apoptotic pathway, the Fas receptor associates with Fas-associated death-domain protein (FADD) and forms the death-inducing signaling complex (DISC) that activates caspase-8. In the intrinsic mitochondrial pathway, cytochrome c released into cytosol forms the apoptosome that binds and activates initiator caspase-9.

Programmed cell death is one of the key biological processes responsible for tissue homeostasis. Therefore dysfunctions along the apoptotic pathways can lead to either premature cell death or prolonged cell survival. Thus abnormal apoptotic levels are found in a wide range of diseases such as human cancers, degenerative disorders, infections, or inflammation.

6.3 Apoptosis in Oral Tissues

Periodontal disease is the most common chronic inflammatory disease and represents a major global health problem, affecting around 40 % of the general population over 35 years old [10]. The disease is characterized by the breakdown of the tissues supporting the teeth. Thus the condition progressively affects the oral epithelial and dermal compartments, followed by bone resorption.

It is well established that apoptosis is actively involved in the initiation and development of periodontal disease [11–16]. Thus apoptosis is involved in the host immune response and inflammation: gingival tissues affected by periodontal disease exhibit increased apoptotic levels in polymorphonuclear leukocytes. Apoptotic levels are also increased in the initial stages of periodontal diseases among periodontal ligament fibroblasts [17]. Apoptotic markers are elevated in human gingival fibroblasts from periodontal tissues affected as a result of *Porphyromonas gingivalis* infection. Bacterial exposure also triggers intrinsic mitochondrial-pathway activation and DNA degradation [18]. In another study, butyric acid induced apoptosis in gingival fibroblasts isolated from periodontal tissues [19]. Tissues affected by chronic periodontal disease show increased levels of apoptotic markers such as the caspase cascade or MAP kinases [20, 21]. Pro-apoptotic genes such as p53, Bcl-2, or caspase-3 are markedly increased in aggressive severe periodontitis [22].

6.4 H_2S in General Systems and the Oral Cavity

In the human body, hydrogen sulfide is one of the endogenous gaseous transmitters, or "gasotransmiters." H_2S is an important modulator of key physiological processes in the central nervous system, cardiovascular system, and gastrointestinal tract [23–25]. Recent data show that in hepatic cells H_2S regulates LPS-mediated apoptosis [26], and we have also proved that H_2S strongly promotes hepatogenic differentiation from human dental-pulp stem cells [27].

In the oral cavity, hydrogen sulfide is the main VSC responsible for oral malodor. However, H_2S has attracted much attention not only for its esthetic factor but also for its toxicity in oral tissues. Previous research has demonstrated that VSC levels are increased in patients with periodontitis as compared to healthy subjects [1]. VSCs in general, and H_2S in particular, have been shown to play a role in the initiation and development of periodontitis. VSCs can inhibit epithelial-cell proliferation [28], basal-membrane synthesis [29], collagen production [2], and protein synthesis [3]. VSCs can raise the levels of important mediators of tissue inflammation such as PG-E_2 and collagenase. VSCs increase oral epithelial permeability and enhance LPS penetration into gingival tissues. H_2S has also been shown to activate p21 protein and to induce cell-cycle arrest in oral epithelial cells [30].

6.5 VSCs Induce Apoptosis in Periodontal Tissues

In several recent studies our group has focused on the relationship between programmed cell death, VSCs, and periodontal tissues. Physiological concentrations of H_2S have been shown to induce apoptosis in several cell types: osteoblast cells, human oral fibroblasts isolated from human gingiva, normal keratinocytes, keratinocyte stem cells, and dental-pulp stem cells. The apoptotic molecular pathways were also investigated (Table 6.1).

In normal conditions oral epithelium forms a protective barrier against oral pathogens and their toxic compounds. This protective role is heavily affected during the initiation and development of periodontal disease. Normally, the integrity of the epithelium depends on continuous cell renewal of keratinocyte cells and more specifically of keratinocyte stem cells. Elevated levels of apoptosis in these cells impair tissue homeostasis and affect the process of constant epithelial regeneration. Our group has demonstrated that H_2S induces apoptosis in several types of keratinocyte cells [7–9]: normal epithelial cells derived from human epithelium (Ca9-22 cell line), clonal human keratinocyte stem cells derived from adult skin, and human gingival keratinocyte stem cells isolated from human gingiva. In all experiments, cells were exposed for 24 or 48 h to an H_2S concentration of 0.5 μmol/l, a lower concentration than that normally found in gingival crevicular fluids taken from patients with periodontitis [5, 7]. Although accurate measurement of H_2S from gingival crevicular fluids is not simple because of its highly volatile nature, the H_2S concentration mentioned above is not a pathological concentration.

Table 6.1 Apoptotic events in different cell types following H_2S incubation

Cell type	Origin	H_2S level/time	Apoptotic process
Fibroblast	Human oral mucosa	50 ng/ml Air 24 and 48 h	Main event—early apoptosis Intrinsic apoptotic pathway—activated DNA damage
Keratinocyte stem cells	Human oral mucosa	50 ng/ml Air 24 h	Main event—early apoptosis Intrinsic apoptotic pathway—activated DNA damage
Keratinocyte-normal	Ca9-22—human oral cancer cell line	50 ng/ml Air 24 and 48 h	Main event—early apoptosis Intrinsic apoptotic pathway—activated DNA damage
Keratinocyte stem cells	Human skin cell line	50 ng/ml Air 24 and 48 h	Main event—early apoptosis Total and phosphorylated p53 activity increased BAX activity—increased Intrinsic apoptotic pathway—activated DNA damage
Dental-pulp cell	Human dental pulp	50 ng/ml Air 24 and 48 h	Main event—early apoptosis Intrinsic apoptotic pathway—activated DNA damage
Osteoblast	Mouse calvaria cell line	50 ng/ml Air 24 and 48 h	Main event—early apoptosis Intrinsic apoptotic pathway—activated Extrinsic apoptotic pathway—activated DNA damage

In the Ca9-22 cell line after both 1 and 2 days of incubation the main biological event was early apoptosis, while necrosis and late apoptosis remained under 5 %. Since H_2S strongly inhibits human-gingival superoxide dismutase, and as H_2S at higher concentrations completely inhibits cytochrome c oxidase, levels of reactive oxygen species (ROS) causing DNA strand break are increased. Thus DNA damage initiates the apoptotic process [31].

As described earlier, the two main pathways by which apoptosis is activated are the intrinsic mitochondrial pathway and the extrinsic death ligand pathway. In order to distinguish between the two mechanisms we analyzed mitochondrial changes. As shown in Fig. 6.1, flow cytometry data showed that levels of ROS were increased following H_2S exposure. This event causes a significant loss of inner mitochondrial-membrane potential. In Ca9-22 membrane potential was collapsed after both 1 and 2 days of incubation. In the next step our data show that cytochrome c was released from the mitochondria into cytosol in a time-dependent manner. Cytochrome c binds to Apaf-1 participating in the formation of the apoptosome. In turn the

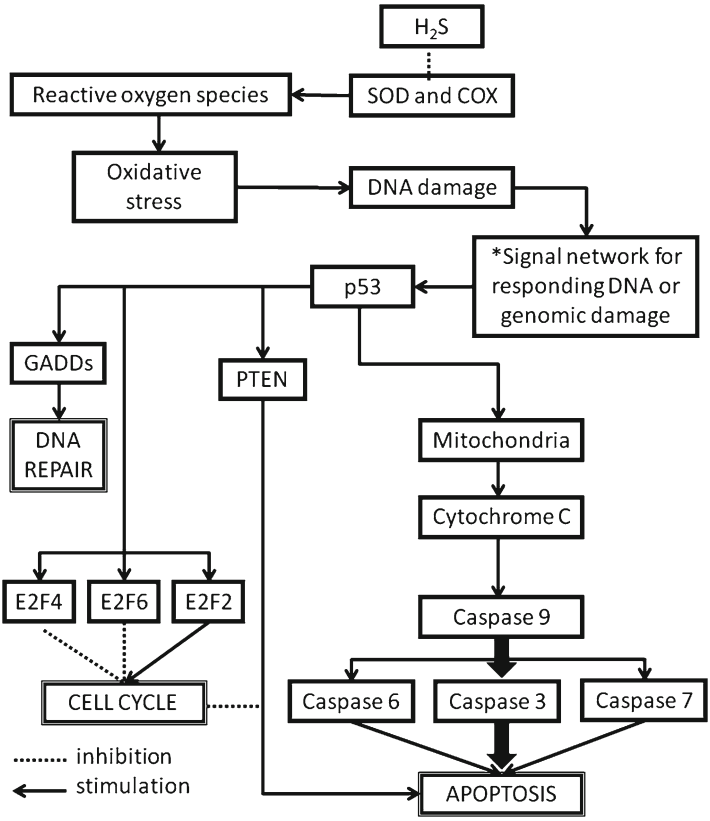

Fig. 6.1 Hydrogen sulfide causes p53-dependent apoptotic pathway involving cell-cycle arrest and DNA repair. *Refer Chap. 7

apoptosome activates initiator caspase-9. Our data show that caspase-9 levels were significantly increased following H_2S exposure. Caspase-9 is responsible for the downstream regulation of apoptosis activating the caspase cascade, including executioner caspases such as caspase-3. Incubation with VSCs increased caspase-3 levels, especially after 48 h. Furthermore, an increased number of DNA strand breaks were observed using single-gel cell electrophoresis. Thus H_2S was shown to have a genotoxic effect on Ca2-99 cells. Interestingly, caspase-8 levels remained comparable to those in negative controls, suggesting that the extrinsic death ligand pathway is not involved in the apoptotic process.

The results were further expanded in keratinocyte stem cells derived from human skin. In the first part of the experiment the obtained data were consistent with those from normal keratinocyte cells: ROS was increased, mitochondrial-membrane potential was decreased, cytochrome c release into cytosol was elevated, and caspase-9 and -3 levels were increased. Thus H_2S induced apoptosis by activating

the mitochondrial intrinsic pathway. At the same time, caspase-8 levels were low with no significant increment when compared to negative controls, showing that the extrinsic pathway is inactive. New insights came from analyzing p53 activity following H_2S exposure. It is well established that activation of p53, "the guardian of the genome," can temporarily arrest the cell cycle allowing for DNA repair or can prevent further genomic DNA damage by initiating apoptosis. p53 Activity is regulated by its phosphorylation site, i.e. serine-46 phosphorylation occurs following severe DNA damage and directs the cell's fate towards apoptosis. After H_2S incubation for either 24 or 48 h, keratinocyte stem cells had significantly higher levels of total p53 as well as increased levels of serine-46 phosphorylated-p53. Furthermore RT-PCR analysis showed that the level of BAX gene, a member of the Bcl-2 family, was elevated following H_2S incubation. Studies show that BAX is mostly activated in response to p53 activation and plays a key role in p53-mediated apoptosis. Taken together, these results lead to the conclusion that H_2S induces apoptosis through stabilization of p53 and mitochondrial-pathway activation.

One interesting finding of the study was that of the total stabilized p53, 1/3 was not phosphorylated at serine 46. This suggests that p53 might also play other roles besides induction of apoptosis. This observation was further explored in another study using human keratinocyte stem cells derived from oral mucosa. RT-PCR analysis showed that H_2S incubation induces p53 activation, which further activates genes involved not only in the apoptotic process but also in cell-cycle arrest and DNA repair. Briefly, the following genes were activated as apoptotic markers: caspase-9, -3, -6, -7, and PTEN; cell-cycle arrest markers: E2F2, E2F4, E2F6, and HDAC6; DNA-repair markers: GADD45A and GADD45B (Fig. 6.1). For a detailed discussion of the roles of p53 following VSC incubation, please refer to Chap. 7 of the present book and also Aoyama et al.'s [32] recent review.

Apoptosis was increased in cells from other periodontal tissues such as osteoblasts or gingival fibroblasts. Thus 24 or 48 h of incubation with H_2S triggered early apoptotic events in oral fibroblasts only by activating the mitochondrial apoptotic pathway [4, 5]. At the same time DNA damage was observed. Interestingly, so far osteoblasts have been the only cell type that reacted to VSCs not only by activation of the intrinsic pathway but also through the extrinsic death ligand pathway [33]. Thus after H_2S exposure, osteoblasts showed increased levels of both caspase-8 and -9 initiator caspases. In another study, H_2S also increased apoptotic levels in dental-pulp stem cells, but only by activating the mitochondrial pathway [6].

So far the previous studies have shown that the apoptotic response to H_2S is largely dependent on the cell type. As shown in Fig. 6.2, after 24 h of incubation oral-keratinocyte stem cells have the highest apoptotic levels. This, together with the DNA damage data, may suggest that apoptosis is rapidly increased in stem cells as a response to extrinsic stressors such as H_2S, preventing further accumulation of DNA damage caused by H_2S. Also cells belonging to the dermal and alveolar periodontal compartments seem to be more resistant to H_2S than cells isolated from the oral epidermis.

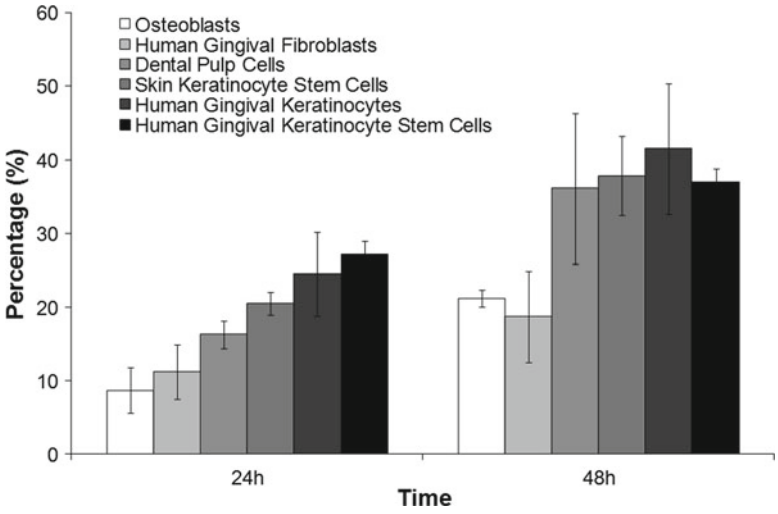

Fig. 6.2 Apoptotic levels in different cell types after 24 and 48 h of H_2S incubation

References

1. Yaegaki K, Sanada K (1992) Volatile sulfur compounds in mouth air from clinically healthy subjects and patients with periodontitis. J Periodontal Res 27:233–238
2. Johnson P, Yaegaki K, Tonzetich J (1996) Effect of methyl mercaptan on synthesis and degradation of collagen. J Periodontal Res 31:323–329
3. Johnson P, Yaegaki K, Tonzetich J (1992) Effect of volatile thiol compounds on protein metabolism by human gingival fibroblasts. J Periodontal Res 27:553–561
4. Yaegaki K, Qian W, Murata T, Imai T, Sato T, Tanaka T, Kamoda T (2008) Oral malodorous compound causes apoptosis and genomic DNA damage in human gingival fibroblasts. J Periodontal Res 43:391–399
5. Fujimura M, Calenic B, Yaegaki K, Murata T, Ii H, Imai T, Sato T, Izumi Y (2010) Oral malodorous compound activates mitochondrial pathway inducing apoptosis in human gingival fibroblasts. Clin Oral Investig 14:367–373
6. Kobayashi C, Yaegaki K, Calenic B, Ishkitiev N, Imai T, Ii H, Aoyama I, Kobayashi H, Izumi Y, Haapasalo M (2011) Hydrogen sulfide causes apoptosis in human pulp stem cells. J Endod 37:479–484
7. Calenic B, Yaegaki K, Murata T, Imai T, Aoyama I, Sato T, Ii H (2010) Oral malodorous compound triggers mitochondrial-dependent apoptosis and causes genomic DNA damage in human gingival epithelial cells. J Periodontal Res 45:31–37
8. Calenic B, Yaegaki K, Kozhuharova A, Imai T (2010) Oral malodorous compound causes oxidative stress and p53-mediated programmed cell death in keratinocyte stem cells. J Periodontol 81:1317–1323
9. Calenic B, Yaegaki K, Ishkitiev N, Kumazawa Y, Imai T, Tanaka T (2012) p53-Pathway activity and apoptosis in hydrogen sulfide-exposed stem cells separated from human gingival epithelium. J Periodontal Res. doi:10.1111/jre.12011
10. Petersen PE, Bourgeois D, Ogawa H, Estupinan-Day S, Ndiaye C (2005) The global burden of oral diseases and risks to oral health. Bull World Health Organ 83:661–669

11. Gamonal J, Bascones A, Acevedo A, Blanco E, Silva A (2001) Apoptosis in chronic adult periodontitis analyzed by in situ DNA breaks, electron microscopy, and immunohistochemistry. J Periodontol 72:517–525
12. Lucas H, Bartold PM, Dharmapatni AA, Holding CA, Haynes DR (2010) Inhibition of apoptosis in periodontitis. J Dent Res 89:29–33. doi:10.1177/0022034509350708
13. Das P, Chopra M, Sun Y, Kerns DG, Vastardis S, Sharma AC (2009) Age-dependent differential expression of apoptosis markers in the gingival tissue. Arch Oral Biol 54:329–336. doi:10.1016/j.archoralbio
14. Sawa T, Nishimura F, Ohyama H, Takahashi K, Takashiba S, Murayama Y (1999) In vitro induction of activation-induced cell death in lymphocytes from chronic periodontal lesions by exogenous Fas ligand. Infect Immun 67:1450–1454
15. Gamonal J, Sanz M, O'Connor A, Acevedo A, Suarez I, Sanz A, Martínez B, Silva A (2003) Delayed neutrophil apoptosis in chronic periodontitis patients. J Clin Periodontol 30:616–623
16. González OA, Stromberg AJ, Huggins PM, Gonzalez-Martinez J, Novak MJ, Ebersole JL (2011) Apoptotic genes are differentially expressed in aged gingival tissue. J Dent Res 90: 880–886. doi:10.1177/0022034511403744
17. Ekuni D, Tomofuji T, Yamanaka R, Tachibana K, Yamamoto T, Watanabe T (2005) Initial apical migration of junctional epithelium in rats following application of lipopolysaccharide and proteases. J Periodontol 76:43–48
18. Urnowey S, Ansai T, Bitko V, Nakayama K, Takehara T, Barik S (2006) Temporal activation of anti- and pro-apoptotic factors in human gingival fibroblasts infected with the periodontal pathogen, *Porphyromonas gingivalis*: potential role of bacterial proteases in host signaling. BMC Microbiol 6:26
19. Kurita-Ochiai T, Seto S, Suzuki N, Yamamoto M, Otsuka K, Abe K, Ochiai K (2008) Butyric acid induces apoptosis in inflamed fibroblasts. J Dent Res 87:51–55
20. Ekuni D, Tomofuji T, Yamanaka R, Tachibana K, Yamamoto T, Watanabe T (2005) Caspase activation is involved in chronic periodontitis. FEBS Lett 579:5559–5564
21. Leung WK, Wu Q, Hannam PM, McBride BC, Uitto V-J (2002) *Treponema denticola* may stimulate both epithelial proliferation and apoptosis through MAP kinase signal pathways. J Periodontal Res 37:445–455
22. Bulut S, Uslu H, Ozdemir BH, Bulut OE (2006) Expression of caspase-3, p53, and Bcl-2 in generalized aggressive periodontitis. Head Face Med 2:17
23. Zhao W, Wang R (2002) H(2)S-induced vasorelaxation and underlying cellular and molecular mechanisms. Am J Physiol Heart Circ Physiol 283:H474–H480
24. Abe K, Kimura H (1996) The possible role of hydrogen sulfide as an endogenous neuromodulator. J Neurosci 16:1066–1071
25. Fiorucci S, Distrutti E, Cirino G, Wallace JL (2006) The emerging roles of hydrogen sulfide in the gastrointestinal tract and liver. Gastroenterology 131:259–271
26. Yan J, Teng F, Chen W, Ji Y, Gu Z (2012) Cystathionine β-synthase-derived hydrogen sulfide regulates lipopolysaccharide-induced apoptosis of the BRL rat hepatic cell line in vitro. Exp Ther Med 4:832–838
27. Ishkitiev N, Calenic B, Aoyama I, Ii H, Yaegaki K, Imai T (2012) Hydrogen sulfide increases hepatic differentiation in tooth-pulp stem cells. J Breath Res 6:017103. doi:10.1088/1752-7155/6/1/017103
28. Setoguchi T, Machigashira M, Yamamoto M, Yotsumoto Y, Yoshimori M, Izumi Y, Yaegaki K (2002) The effects of methyl mercaptan on epithelial cell growth and proliferation. Int Dent J 52:241–246
29. Yaegaki K (1995) Oral malodor and periodontal disease. In: Rosenberg M (ed) Bad breath: research perspectives. Ramot Publishing-Tel Aviv University, Tel Aviv, pp 87–108
30. Takeuchi H, Setoguchi T, Machigashira M, Kanbara K, Izumi Y (2008) Hydrogen sulfide inhibits cell proliferation and induces cell cycle arrest via an elevated p21 Cip1 level in Ca9-22 cells. J Periodontal Res 43:90–95

31. Aoyama I, Yaegaki K, Calenic B, Ii H, Ishkitiev N, Imai T (2012) The role of p53 in an apoptotic process caused by an oral malodorous compound in periodontal tissues: a review. J Breath Res 6:017104. doi:10.1088/1752-7155/6/1/017104
32. Yaegaki K (2008) Oral malodorous compounds are periodontally pathogenic and carcinogenic. Jpn Dent Sci Rev 44:100–108
33. Aoyama I, Calenic B, Imai T, Ii H, Yaegaki K (2012) Oral malodorous compound causes caspase-8 and -9 mediated programmed cell death in osteoblasts. J Periodontal Res 47: 365–373. doi:10.1111/j.1600-0765.2011.01442.x

Chapter 7
The Role of p53 in Carcinogenesis and Apoptosis in Oral Tissues

Ken Yaegaki

7.1 Introduction

This chapter's objective is simply to describe and discuss the function of p53 in carcinogenesis and apoptosis, especially in the prevention of malignant formations in oral tissue. The original roles of p53 are to cause apoptosis if the DNA damage is irreparable, or to facilitate repair of the errors in DNA strands during the arrest of the cell cycle mediated by p53 [1–3]. As described in Fig. 7.1, an increase in reactive oxygen species (ROS) damages DNA, causing, e.g., DNA double-strand breaks (DSBs) or single-strand breaks, since ROS are a major cause of DNA damage. Increases in ROS are caused by environmental factors, etc.; however, the presence of certain volatile sulfur compounds is the most likely reason in the oral cavity [4–10]. Following DNA damage, a cell can take the route of either apoptosis or DNA repair, whereas cell-cycle arrest must always happen [5, 11].

In consequence, the genome guardian p53 maintains the integrity of the genome by preventing malignant formations in cells [12, 13]. In previous chapters, DNA damage and apoptosis caused by oral malodorous compounds were described. As previously described, oral malodorous compounds can produce malignancy in oral tissues when the functioning of p53 or the checkpoints for DNA damage are impaired. In the following sections, the author gives details. Admittedly, the description may reflect the author's bias in the explanation of a great deal of the information about these facts reported previously along with too much speculation.

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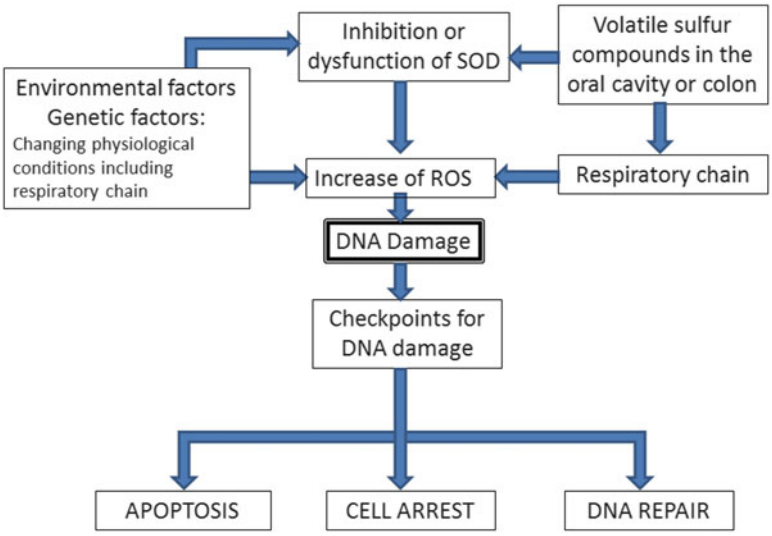

Fig. 7.1 Choices in the destiny of a cell: reactive oxygen species (ROS) damages DNA. ROS is increased by environmental and host factors, etc. Volatile sulfur compounds are the most likely cause in the oral cavity. Damage is checked by checkpoints, and the system starts either apoptosis or DNA repair

7.2 Checkpoints for the Choice Between DNA Repair and Apoptosis

To identify the process of the signal network for p53-induced apoptosis or the repair of DNA damage, we must reconfirm that all processes are carried out in a single cell, preserving the integrity of the host, not in the whole body system of the host. If DNA or genomic damage is detected, all effectors at every checkpoint of the process are stimulated or activated to find DNA damage and to repair it. Figure 7.2 shows the signal network for responding to DNA or genomic damage by selecting either apoptosis or the repair of DNA breaks after cellular DNA or genomic damage. The signal network for responding to DNA or genomic damage works together with the cell cycle (Fig. 7.3). For details of the cell cycle in oral carcinogenesis, see Todd et al.'s excellent review [14].

The primary function of p53 is to determine a cell's destiny following genomic injury as described above. In this system of checkpoints, ataxia-telangiectasia and Rad3-related (ATR), and Ataxia-telangiectasia mutated (ATM) [15] protein kinase have important roles. One of the initial signals upon the appearance of DNA DSBs is the phosphorylation of Rad3 [16]. Phosphorylation of the histone H2A variant phosphorylated histone H2A (H2AX) to generate γ-H2AX is also another indicator of DSBs [2, 3, 17], ATM is activated by phosphorylated cyclin-dependent kinase 5 (Cdk5), γ-H2AX, Mre11–Rad50–Nbs1/Xrs2 (MRN) complex, and/or DNA DSB

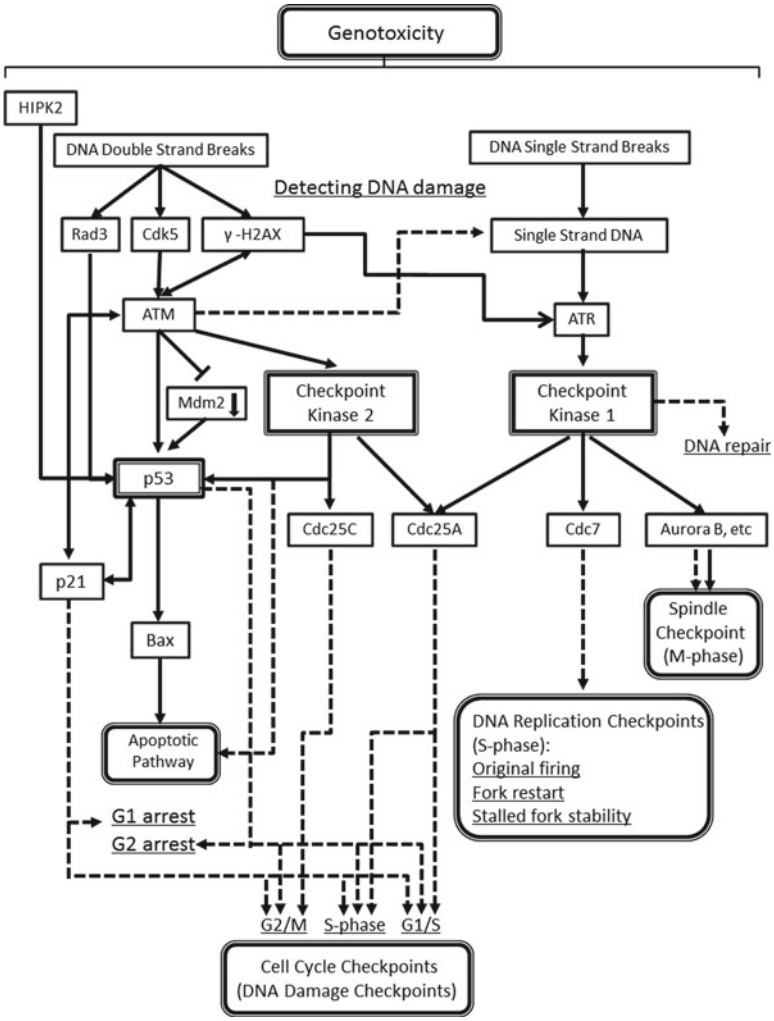

Fig. 7.2 Signal network for responding to DNA or genomic damage: DNA double-strand breaks are found by Rad5, Cdk5, and γ-H2AX, which then activate Ataxia-telangiectasia mutated (ATM) protein kinase and other elements downstream, including p53. γ-H2AX and single-strand DNA itself activate Ataxia-telangiectasia and Rad3-related (ATR). p53, ATM and ATR play a crucial role. Genotoxicity controls the network, including the checkpoints, indirectly. There are three groups of checkpoints: cell-cycle checkpoints (DNA-damage checkpoints), DNA-replication checkpoints, and the spindle checkpoint. These checkpoints detect DNA damage, then promote either DNA repair or cell death. Most of the references are described in the text. *Solid line*: direct effect. *Broken line*: indirect effect

decreasing DNA strand strain [16–19]. ATM dimer becomes a monomer in the active form after activation and then triggers both p53 and Checkpoint kinase 2 (Chk2) pathways directly. Murine double mutant 2 (Mdm2), which is a suppressor for p53, is restrained by ATM [20]. Consequently p53 is stimulated. The functions

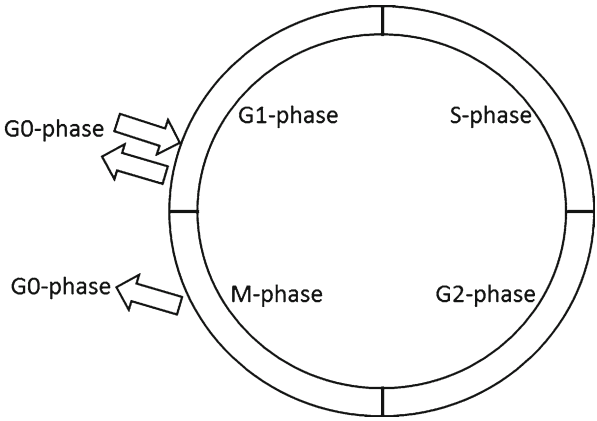

Fig. 7.3 Cell cycle. S (synthesis) phase: check DNA damage and replication; M (mitosis) phase: check DNA damage, chromosome and spindle attachment; G_0 (Gap 0) phase: resting and no proliferative cell action; G_1 (Gap 1) phase: check DNA damage, proliferation, and nucleotide; G_2 (Gap 2) phase: check DNA damage, cell size and completing DNA replication

of p53 in the cell cycle include controlling DNA-damage checkpoints: the G_1/S transition, S-phase, and the G_2/S transition [21]. At the DNA-damage checkpoint, p53 causes G_1 cell-cycle arrest following the detection of DNA injury or initiates the apoptotic pathway if the DNA damage is not repairable [22]. p53 also stimulates p21, resulting in G_1 arrest [23]. Thus if apoptosis is not chosen, the DNA damage is repaired before starting DNA replication. Dysfunction of p53 is caused by inhibition, degradation, or mutation of p53; this may cause accretion of DNA damage in each cell. Finally, accumulated DNA damage may result in starting malignant formations [22].

ATR is activated by DNA single-strand breaks, and γ-H2AX phosphate is activated by DNA DSB [17, 24], and then the downstream part of the ATR pathway, checkpoint kinase 1 (Chk1), is stimulated. Thus Chk1, Chk2, and p53 play crucial roles in the DNA-damage response signaling system for determining a cell's destiny. The ATM pathway is mainly activated by DNA DSBs, whereas the ATR pathway is stimulated mainly by single-strand breaks. The system involves several checkpoints to identify DNA damage and to repair DNA. Checkpoints are distinguished by their functions: DNA-damage, DNA-replication, and spindle checkpoints [24]. During the cell cycle there are G_1/S, S phase, and G_2/M checkpoints [2, 3]. These checkpoints are also recognized as DNA-damage checkpoints. All DNA-damage checkpoint signaling involving G_2/M, S- and G_1/S phases is indirectly stimulated by p53, Chk1 and Chk2. DNA-replication checkpoints and the spindle checkpoint are indirectly controlled by Chk1 [16, 24]. At DNA-replication checkpoints three kinds of DNA problems are screened: original firing, fork restart, and replication fork stability [24].

The number and position of cell-cycle or other checkpoints is still disputed. Only G_1, G_2, and spindle checkpoints are confirmed in many papers. The author, however, has described the number and sites of each checkpoint following basically the description of Dai and Grant [24]. A huge part of DNA-damage response signaling and the checkpoint system has not yet been unveiled. Furthermore most of the system or network is extremely complicated; so many contradictions have been found. We cannot describe them all completely.

7.3 Original Firing, Fork Restart, and Stalled-Fork Stability

The initiation of DNA replication is not simple, involving multistep processes with preservation of genomic stability. Replication starts from certain regions, replication origins, in DNA: the process is known as origin-firing. For a single replication origin there is a number of candidates to be the origin, but most of these will not be employed for replication. The ATR pathway controls other origins at the DNA-replication checkpoint (S-phase) and determines which origins fire early and efficiently. That is, the DNA-replication checkpoint excludes inefficient origins once efficient ones have fired. In this way, the checkpoint can limit the number of origins. Although origins fire at different times during S phase, all late-firing origins will be unnecessary for the completion of DNA replication as described above [25–27].

The process of DNA replication is also coupled to cell-cycle progression and to DNA repair. Replication of genomes must be an extremely coordinated process to maintain genome integrity. That is, replication of a DNA double-strand starts at replication forks formed at the DNA double-strand end as shown in Fig. 7.4. Helicase, DNA polymerases, DNA ligase, Okazaki fragments, and other proteins are required for replication-fork progression; the system is called a replisome [28]. Helicase unwinds DNA at the replication fork formed by the DNA double-strand end, and DNA polymerase produces a leading or a lagging strand. The DNA single-strand synthesis takes place only from 5′ to 3′, not from 3′ to 5′. On the leading strand, the DNA single-strand can be replicate continuously along the DNA template from 5′ to 3′ [29]. On the lagging strand, DNA synthesis must periodically restart with short RNA primers after exposing part of the unwound 3′ to 5′ template of the lagging strand. RNA is replaced with DNA later, and DNA ligase connects one fragment to another. This RNA chain is called an Okazaki fragment. Logically, in vitro it might be possible to replicate a much longer fragment of the lagging strand than the Okazaki fragment; however, the possibility of causing genomic error would be dramatically increased because of the length of time that the DNA would be exposed to the surrounding cell elements. This discontinuous synthesis of the lagging strand is the reason for the succession of Okazaki fragments.

DNA-repair and DNA-replication checkpoints respond to replication stress, allowing them to maintain genome stability. Checkpoint signals may be represented by stretches of single-strand DNA originating from DNA lesions because the forks generate single-stranded DNA [30]. In other words, single-strand involves less tension than double-strand does. The signals would be accumulated for further consideration by the DNA-duplication checkpoint or the DNA-damage-response signaling system.

Collapsed replication forks may lose their function. In fact, such a fork causes a major signal for downstream events, including fork repair and checkpoint activation [31, 32]. The replisome at the forks is enhanced by mediators working to repair the DNA [33]. The process may maintain the replication fork until the DNA damage is repaired. If replication is not easily accomplished, it may cause DNA errors and collapse in one-ended DSB [34]. When the replication fork is kept stalled for more

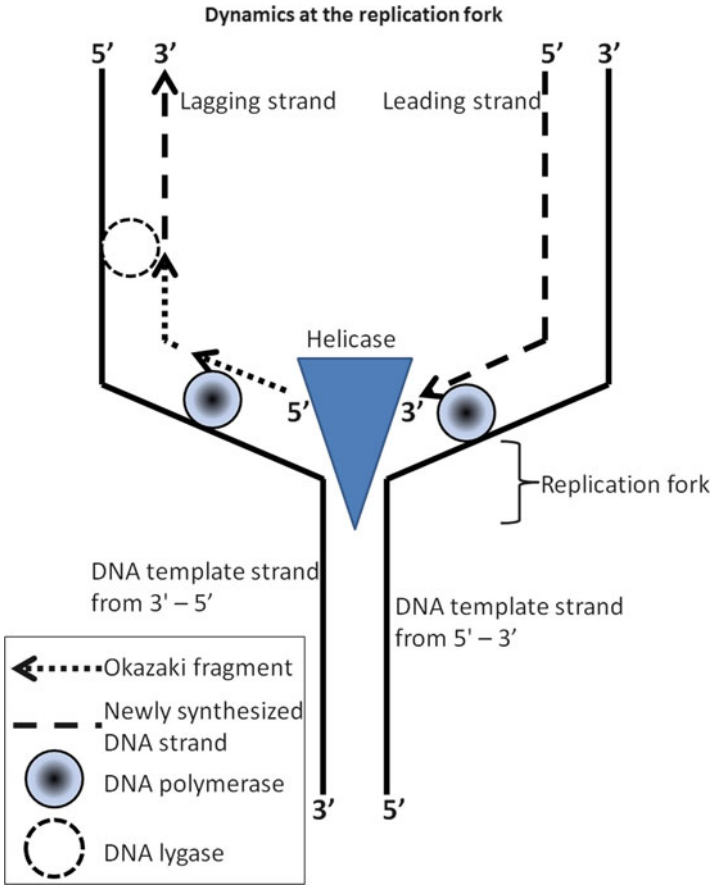

Fig. 7.4 Replication fork. Replisome consists of helicase, DNA polymerases, DNA ligase, the Okazaki fragment, and the replication fork

than 12 h, the number of fork-associated DSBs increases [35]. Therefore stalled-fork stability is important in preventing further DNA damage. When a replication fork encounters a single-strand break in a template strand, this event probably collapses the fork to a DNA double-strand end. The collapsed fork causes another origin to fire, along with homologous recombination-mediated repair, but this might not be completed [31]. When replication is not easily carried out, the DNA becomes further damaged.

On the other hand, replication forks may collapse at DNA lesions or genomic gaps on the template strand [30]. These actions would ruin the integrity of the genome, but homogeneous recombination or other damage-bypass processes cause the fork to restart [35]. These signals ensure that the response to DNA damage enables DNA repair in S phase. Hence checking the fork restart promotes the preservation of the replication fork, maintaining genomic integrity.

Therefore ATM and ATR associated with DNA-replication checkpoints prevent DSB-accumulation during DNA replication by regulating origin-firing, by recovering collapsed stalled replication forks, and by stimulating fork restart [36].

7.4 Spindle Checkpoint

The spindle checkpoint controls cell-cycle progression during mitosis: two spindles originating from opposite poles attach to the chromosomes. Failure in attachment results in unequal segregation of chromosomes, which may cause cell death or disease [37]. During this process, the spindle checkpoint works to avoid producing chromosome instability or other problems, or to detect them. The spindle microtubules bind to proteins rather than directly to the chromosome: the protein structures, known as kinetochores, settle on the centromere of each chromosome. The centromere is a tightly attached central region where the two chromatins are held together at the cross point of the two chromatins forming an X shape. As soon as the two kinetochores on a pair of sister-chromosomes attach to spindle microtubules from the two poles, they congregate at the center of the two daughter cells that are separating; this stage is known as anaphase [38]. Once the microtubules of the spindle make contact with the kinetochore, many molecules start interactions or communication, working to complete mitosis. This process regulates both the attachment and the movement along the microtubule [39]. The checkpoint is situated so as to monitor these events or signaling.

There is another reason why a checkpoint during mitosis is essential. In metaphase, chromosomes arrange themselves in a line in the middle of the cell before separating into the two daughter cells. Due to the natural randomness of the process it is critical that chromosome segregation proceeds only when all the chromosomes have collected to form the metaphase plate [38], which is required to ensure that the entire genomic information including even the smallest part is transferred into the two daughter cells. This is achieved by a surveillance mechanism, the spindle checkpoint. This checkpoint finds any lack of attachment or tension at the kinetochores. When such an event is detected, the checkpoint restrains the chromosome segregation to prevent the transference of wrong genomic information to the daughter cells from the mother cell [40, 41]. A single unattached kinetochore can delay the segregation of the chromosomes [42]: an inhibitory checkpoint signal is produced by this kinetochore to block chromosome segregation.

During the process of connecting chromosomes to spindle kinetochores from the two opposite poles to produce sister nuclei, tension is caused across the sister kinetochores on opposite sides of the segregated chromosome by the pulling forces of the spindle, to produce two sister strands of chromatin. This event produces intra-kinetochore stretch or tension [43]. Kinetochores attach multiple microtubules: it has been suggested that each microtubule-binding site is activated at a different time. Therefore, it has also been suggested that it would be prudent to study intra-kinetochore stretching [44]. May and Hardwick [37] showed that decreased tension

caused by decreased microtubule attachment initiates the checkpoint. Moreover syntelic attachment, that is losing kinetochore tension because of incidental or non-regulated attachment of both sister genomes to microtubules from the same pole, activates the checkpoint as a response to lack of tension. Moreover, chemical inhibition of the spindle microtubule attachment has been carried out to relieve tension but not to lose the attachments, following which the checkpoint was found to be activated [45]. It has also been claimed that the checkpoint signal is produced by this kinetochore, blocking chromosome segregation [42]. The kinetochore is the apparent source of the checkpoint signal in all systems. Meiosis never happens in the oral tissue, but intra-kinetochore tension signaling is also found acting as a checkpoint control in meiosis [44].

One of the targets of the spindle checkpoint is the anaphase-promoting complex/cyclosome (APC/C). In response to a sister-chromatid being incorrectly attached to the spindle, the checkpoint promotes proteins that inhibit the activity of APC/C and/or delay the start of anaphase. This confirms that this checkpoint detects improper chromosome segregation, which would be followed by the inaccurate division of the genome [37, 44, 46].

7.5 p53 in Carcinogenesis

Failure of the signal network in responding to DNA damage and in repairing DNA damage or genomic damage may lead to carcinogenesis (Fig. 7.2). p53 in particular has an extremely important role in suppressing carcinogenesis, although all the factors in the upper part of the stream of the signal network for responding to DNA or genomic damage are essential to prevent malignant tumor formation [2]. The reason is that p53 plays another important role in the DNA-repair pathway, as well as in the signal network for responding to DNA or genomic damage. p53 Induces cell-cycle arrest for p53-facilitated DNA repair, promoting nucleotide excision repair (NER) and base excision repair (BER). In BER, p53 activates both Gadd44 and p48XPE and then removes and corrects only damaged bases. Furthermore p53 may activate ER through interaction with DNA polymerase β [47–49].

Smith et al. [50, 51] investigated NER in ultraviolet-light (UV)-damaged DNA in cells involving wild-type p53 or a p53 mutant which lowers considerably the effect of p53. Cells with a malfunction of p53 showed defective repair of DNA damage caused by UV. p53 Mutation is one of the most frequent genetic changes in human cancer. p53 Mutant may obstruct caspase-3 function: caspase-3 is an excision enzyme in apoptosis, especially in p53-dependent apoptosis. It has been suggested that cells producing p53 mutants may remain as malignant cells in the oral tissues by means of inhibiting caspase-3 [23].

Human papillomavirus E6 protein inactivates p53 by causing its degradation [52]. Moreover Raf/MEK/ERK and PI3K/Akt pathways also interact with p53 directly or indirectly, e.g. Akt activates MDM2, which suppress p53 activity through these very complicated interactions [53]. Interestingly, there are p53

backup systems involving CHK1 and/or CHK2-driven E2F1 activation and p73 upregulation, moreover another backup system exists: DNA damage inhibits RNA synthesis, which causes reduction of the products of significant genes, and then eventually the death-receptor is activated [54]. However, anticancer activities in vivo of these backup systems are still obscure. The level of importance of these systems is not clear, especially compared with the p53-dependent apoptotic pathway.

There are other important anticancer systems, although p53 is not involved in them directly. Briefly, the primary response triggered by DNA-damaging agents is a sustained proliferation block, and not apoptosis. The proliferation block reflects stress-induced premature senescence in strongly p53-positive cells [23]. Since DNA methylation is one of the causes of malignant tumors, demethylation of methyl-DNA can prevent malignancies. In fact, DNA demethylation may be involved in the BER process [55]. Moreover, phosphorylated H2A(X) has been described as one of the signals from DNA DSB: the phosphorylation of H2A(X) has been demonstrated to involve an important function in preventing carcinogenesis [56]. H2A(X) has another important function. The basic unit of chromatin is the nucleosome, which is formed by DNA base pairs wrapping histone proteins [19]. There are entry and exit points of DNA from the nucleosome. The DNA entry and exit points are localized at histone proteins such as H2A/H2B dimer; H2A(X) may be near the dimer [19]. H2A(X) DNA-damage response is a cause of motif phosphorylation. Most of a motif's functions are related to DNA metabolism and communications among proteins and DNA [57]. Phosphorylation revises the nucleosome, providing the correct entry/exit points on the DNA [19].

7.6 Conclusion

A signal network for DNA or genomic damage consists of checkpoints and related mediators, including those for detecting DNA damage. p53, ATR, and ATM protein kinase have important roles. The signal network works together with the cell cycle. Checkpoints in the network are cell-cycle checkpoints (DNA-damage checkpoints), DNA-replication checkpoints, and the spindle checkpoint. When these functions are damaged or lost, the possibility of carcinogenesis is increased. Selecting either apoptosis or the repair of DNA breaks after genomic damage is the crucial role of this system.

References

1. Enoch T, Norbury C (1995) Cellular responses to DNA damage: cell-cycle checkpoints, apoptosis and the roles of p53 and ATM. Trends Biochem Sci 20:426–430
2. Meyn MS (1995) Ataxia-telangiectasia and cellular responses to DNA damage. Cancer Res 55:5991–6001
3. Meyn MS (1997) Ataxia-telangiectasia and cellular responses to DNA damage. Cancer Res 57:2313–2315

4. Aoyama I, Calenic B, Imai T et al (2012) Oral malodorous compound causes caspase-8 and -9 mediated programmed cell death in osteoblasts. J Periodontal Res 47:365–373
5. Calenic B, Yaegaki K, Ishkitiev N et al (2012) p53-Pathway activity and apoptosis in hydrogen sulfide-exposed stem cells separated from human gingival epithelium. J Periodontal Res. doi:10.1111/jre.12011
6. Calenic C, Yaegaki K, Kozhuharova A et al (2010) Oral malodorous compound causes oxidative stress and p53-mediated programmed cell death in keratinocyte stem cells. J Periodontol 81:1317–1323
7. Calenic B, Yaegaki K, Murata T et al (2010) Oral malodorous compound triggers mitochondrial-dependent apoptosis and causes genomic DNA damage in human gingival epithelial cells. J Periodontal Res 45:31–37
8. Kobayashi C, Yaegaki K, Calenic B et al (2011) Hydrogen sulfide causes apoptosis in human pulp stem cells. J Endod 37:479–484
9. Fujimura M, Calenic B, Yaegaki K et al (2010) Oral mal odorous compound activates mitochondrial pathway inducing apoptosis in human gingival fibroblasts. Clin Oral Investig 14(367–373):2010
10. Yaegaki K, Qian W, Murata T et al (2008) Oral malodorous compound causes apoptosis and genomic DNA damage in human gingival fibroblasts. J Periodontal Res 43:391–399
11. Bellini MF, Cadamuro AC, Succi M et al (2012) Alterations of the TP53 gene in gastric and esophageal carcinogenesis. J Biomed Biotechnol 2012:891961
12. Lane DP (1992) p53, guardian of the genome. Nature 358:15–16
13. Sato Y, Tsurumi T (2012) Genome guardian p53 and viral infections. Rev Med Virol. doi:10.1002/rmv.1738
14. Todd R, Hinds PW, Munger K et al (2002) Cell cycle dysregulation in oral cancer. Crit Rev Oral Biol Med 13:51–61
15. Trenz K, Smith E, Smith S et al (2006) ATM and ATR promote Mre11 dependent restart of collapsed replication forks and prevent accumulation of DNA breaks. EMBO J 25:1764–1774
16. Langerak P, Russell P (2011) Regulatory networks integrating cell cycle control with DNA damage checkpoints and double-strand break repair. Philos Trans R Soc Lond B Biol Sci 366:3562–3571
17. Landau DA, Slack FJ (2011) MicroRNAs in mutagenesis, genomic instability and DNA repair. Semin Oncol 38:743–751
18. Tian B, Yang Q, Mao Z (2009) Phosphorylation of ATM by Cdk5 mediates DNA damage signaling and regulates neuronal death. Nat Cell Biol 11:211–218
19. Kinner A, Wu W, Staudt C et al (2008) c-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin. Nucleic Acids Res 36:5678–5694
20. Lau AW, Fukushima H, Wei W (2011) The Fbw7 and beta-TRCP E3 ubiquitin ligases and their roles in tumorigenesis. Front Biosci 17:2197–2212
21. Doorbar J (2005) The papillomavirus life cycle. J Clin Virol 32S:S7–S15
22. Feller L, Wood NH, Khammissa RA et al (2010) Human papillomavirus-mediated carcinogenesis and HPV-associated oral and oropharyngealsquamous cell carcinoma. Part 1: human papillomavirus-mediated carcinogenesis. Head Face Med 6:14
23. Mirzayans R, Andrais B, Scott A et al (2012) New insights into p53 signaling and cancer cell response to DNA damage: implications for cancer therapy. J Biomed Biotechnol 2012:170325
24. Dai Y, Grant S (2010) New insights into checkpoint kinase 1 (Chk1) in the DNA damage response (DDR) signaling network: rationale for employing Chk1 inhibitors in cancer therapeutics. Clin Cancer Res 16:376–383
25. Machida YJ, Hamlin JL, Dutta A (2005) Right place, right time, and only once: replication initiation in metazoans. Cell 123:13–24
26. Boye E, Grallert B (2009) In DNA replication, the early bird catches the worm. Cell 136: 812–814
27. Katsuno Y, Suzuki A, Sugimura K et al (2009) Cyclin A-Cdk1 regulates the origin firing program in mammalian cells. Proc Natl Acad Sci U S A 106:3184–3189

28. Soultanas P (2012) Loading mechanisms of ring helicases at replication origins. Mol Microbiol 84:6–16
29. Nethanel T, Reisfeld S, Dinter-Gottlieb G et al (1988) An Okazaki piece of simian virus 40 may be synthesized by ligation of shorter precursor chains. J Virol 62:2867–2873
30. Branzei D, Foiani M (2005) The DNA damage response during DNA replication. Curr Opin Cell Biol 17:568–575
31. Petermann E, Helleday T (2010) Pathways of mammalian replication fork restart. Nat Rev Mol Cell Biol 11:683–687
32. Branzei D, Foiani M (2009) The checkpoint response to replication stress. DNA Repair 8:1038–1046
33. Shimada K, Oma Y, Schleker T et al (2008) Ino80 chromatin remodeling complex promotes recovery of stalled replication forks. Curr Biol 18:566–575
34. Allen C, Ashley AK, Hromas R et al (2011) More forks on the road to replication stress recovery. J Mol Cell Biol 3:4–12
35. Saintigny Y, Delacote F, Vares G et al (2001) Characterization of homologous recombination induced by replication inhibition in mammalian cells. EMBO J 20:3861–3870
36. Uchida KS, Takagaki K, Kumada K et al (2009) Kinetochore stretching inactivates the spindle assembly checkpoint. J Cell Biol 184:383–390
37. May KM, Hardwick KG (2006) The spindle checkpoint. J Cell Sci 119:4139–4142
38. Cleveland DW, Mao Y, Sullivan KF (2003) Centromeres and kinetochores: from epigenetics to mitotic checkpoint signaling. Cell 112:407–421
39. Maiato H, DeLuca J, Salmon ED et al (2004) The dynamic kinetochore-microtubule interface. J Cell Sci 117:5461–5477
40. Musacchio A, Hardwick KG (2002) The spindle checkpoint: structural insights into dynamic signalling. Nat Rev Mol Cell Biol 3:731–741
41. Yu H (2002) Regulation of APC-Cdc20 by the spindle checkpoint. Curr Opin Cell Biol 14:706–714
42. Rieder CL, Cole RW, Khodjakov A et al (1995) The checkpoint delaying anaphase in response to chromosome monoorientation is mediated by an inhibitory signal produced by unattached kinetochores. J Cell Biol 130:941–948
43. Maresca TJ, Salmon ED (2009) Intrakinetochore stretch is associated with changes in kinetochore phosphorylation and spindle assembly checkpoint activity. J Cell Biol 184:373–381
44. Musacchio A (2011) Spindle assembly checkpoint: the third decade. Philos Trans R Soc Lond B Biol Sci 366:3595–3604
45. Skoufias DA, Andreassen PR, Lacroix FB et al (2001) Mammalian mad2 and bub1/bubR1 recognize distinct spindle attachment and kinetochore-tension checkpoints. Proc Natl Acad Sci U S A 98:4492–4497
46. Bharadwaj R, Yu H (2004) The spindle checkpoint, aneuploidy, and cancer. Oncogene 23:2016–2027
47. Smith ML, Seo YR (2002) p53 Regulation of DNA excision repair pathways. Mutagenesis 17:149–156
48. Lu X, Nguyen TA, Appella E et al (2004) Homeostatic regulation of base excision repair by a p53-induced phosphatase: linking stress response pathways with DNA repair proteins. Cell Cycle 3:1363–1366
49. Wilson DM 3rd, Kim D, Berquist BR et al (2011) Variation in base excision repair capacity. Mutat Res 711:100–112
50. Smith ML, Chen IT, Zhan Q et al (1995) Involvement of the p53 tumor suppressor in repair of UV-type DNA damage. Oncogene 10:1053–1059
51. Smith ML, Ford JM, Hollander MC et al (2000) p53-Mediated DNA repair responses to UV radiation: studies of mouse cells lacking p53, p21, and/or gadd45 genes. Mol Cell Biol 20:3705–3714
52. Li X, Coffino P (1996) High-risk human papilloma virus E6 protein has two distinct binding sites within p53, of which only one determines degradation. J Virol 70:4509–4516

53. McCubrey JA, Steelman LS, Chappell WH et al (2007) Roles of the Raf/Mek/Erk pathway in cell growth, malignant transformation and drug resistance. Biochim Biophys Acta 1773: 1263–1284
54. Roos WP, Kaina B (2006) DNA damage-induced cell death by apoptosis. Trends Mol Med 12:440–450
55. Chen ZX, Riggs AD (2011) DNA methylation and demethylation in mammals. J Biol Chem 286:18347–18353
56. Downs JA (2007) Chromatin structure and DNA double-strand break responses in cancer progression and therapy. Oncogene 26:7765–7772
57. Baxevanis AD, Arents G, Moudrianakis EN et al (1995) A variety of DNA-binding and multimeric proteins contain the histone fold motif. Nucleic Acids Res 23:2685–2691

Chapter 8
Physiological Roles of a Periodontopathic Bacterial Membrane-Bound Quinol Peroxidase

Kiyoshi Konishi

8.1 Introduction

Upon exposure to high oxygen concentrations, most aerobic organisms (including aerobic bacteria, plants, and animals) exhibit toxic phenotypes, including increased mutation rate, growth decrease, and finally loss of viability. These effects typically do not reflect direct reaction of oxygen (O_2) itself with biological molecules [1]. Instead, the sequential addition of an electron to molecular oxygen generates superoxide anion, hydrogen peroxide, hydroxy radical, and water, in the following series: $O_2 \rightarrow O_2^-$ (−160 mV; standard redox potential at pH 7.0), $O_2^- \rightarrow H_2O_2$ (+940 mV), $H_2O_2 \rightarrow \cdot OH$ (+380 mV), $\cdot OH \rightarrow H_2O$ (+2,330 mV).

Molecular oxygen can obtain electrons from the dihydroflavin cofactors of a wide range of reduced redox enzymes. The higher the oxygen concentration, the more rapidly the reaction occurs [2, 3]. As a mixture of superoxide and hydrogen peroxide is usually generated in vivo, cells often employ superoxide dismutases and reductases as scavengers for superoxide, and catalases and peroxidases as scavengers for hydrogen peroxide. In aerobic cultures of *Escherichia coli*, H_2O_2 is formed at a steady-state rate of 10–15 μM/s/OD (optical density of bacteria at 600 nm) [4]. The rate of generation increases in proportion to oxygen concentration, consistent with enzymatic observations and accounting for the toxicity of hyperoxia. These peroxide doses are sufficient to require the existence of scavenging systems, since either superoxide ion- or H_2O_2-scavenging enzyme-deficient bacterial mutants exhibit growth deficiencies, and the phenotype becomes more severe at increasing environmental oxygen concentrations [5–8]. Peroxide levels in excess of 1 μM efficiently oxidize the loose (non-protein complexed) ferrous ion pool in H_2O_2-stressed

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E. coli cells, generating hydroxyl radicals that in turn induce DNA damage [9]. This process is referred to as the Fenton reaction:

$$H_2O_2 + Fe^{2+} \rightarrow OH^- + \cdot OH + Fe^{3+}$$

In a mechanism similar to the Fenton reaction, H_2O_2 oxidizes and destroys solvent-exposed Fe/S (iron–sulfur) clusters. The resulting inactivation of Fe/S cluster-containing enzymes, such as aconitase, blocks key pathways necessary for biosynthesis and bioenergy production [10], and H_2O_2 also inactivates non-redox enzymes that have a single ferrous iron as a substrate-activating factor [11, 12]. Thus, the enzymes vulnerable to H_2O_2 stress catalyze a diverse set of reactions, ranging from epimerization to dehydrogenation.

In vitro data demonstrate that the rate constant for the reaction of H_2O_2 with iron centers may range from 1×10^3 to 5×10^4 M^{-1} s^{-1} [13], suggesting that even submicromolar H_2O_2 can inactivate the above-mentioned enzymes within a few minutes, and explaining why cells maintain high titers of peroxidase and catalase enzyme activities. For example, the levels of these scavenger enzymes in *E. coli* are sufficient to restrict the steady-state amount of H_2O_2 to approximately 20 nM, despite the rapidity of endogenous H_2O_2 formation [14].

In addition to the autoxidation of flavoproteins, oxidants are generated by extracellular redox reaction mechanisms. On redox reaction interfaces, reduced metals and sulfur species can spontaneously encounter oxygen-containing water, and subsequent redox reaction generates the O_2^- and H_2O_2 with which bacteria have to struggle. The same reactive oxygen species (ROS) are made when ultraviolet light or short wavelength visible light illuminates extracellular chromophores, including (for example) pigments released by decaying plants. At physiological pH, O_2^- is a charged molecule and therefore cannot cross the bacterial cytoplasmic membrane, while H_2O_2 penetrates phospholipid bilayers with a permeability coefficient similar to that of water [14, 15]. When environmental H_2O_2 concentrations exceed 0.2 μM in *E. coli*, the rate of H_2O_2 influx is more than that of its endogenous formation, and the steady-state intracellular peroxide concentration begins to increase [14]. A similar effect is expected when bacteria are exposed to H_2O_2 secreted by lactic acid bacteria (LAB), which has been proposed to serve as an LAB competitive strategy [16], a mechanism for limiting or eliminating competitive flora including common pathogens such as *Haemophilus influenzae* or *Neisseria meningitidis* that share the same microenvironment. Similarly, when bacteria are captured in macrophages (cells of the animal immune system), bacteria are exposed to 5–10 μM H_2O_2, which is expected to elevate intracellular H_2O_2 levels by more than one order of magnitude [17]. Scavenging enzymes are important defenses against H_2O_2/O_2^-, and when rates of H_2O_2 generation or exposure are high, the basal levels of defenses may not be sufficient. For this reason, most organisms respond to increasing H_2O_2 levels by raising the rate of synthesis of catalases and/or peroxidases. These adaptive responses have been demonstrated in bacteria and in eukaryotes (including yeast).

The present work describes the characterization of an H_2O_2/O_2^- scavenger protein detected in a periodontopathic bacterium, *Actinobacillus actinomycetemcomitans*. However, this report first reviews other bacterial scavenger proteins

before returning to the quinol peroxidase (QPO) of this bacterium, some of which may be candidates for ROS scavenger of *A. actinomycetemcomitans*, as mentioned in the later part.

8.2 Catalase and Peroxidase

8.2.1 Properties of Catalase and Peroxidase

It was proposed in 1900 that higher organisms universally harbor an enzyme (designated catalase) that degrades hydrogen peroxide (H_2O_2) to oxygen (O_2) and water (H_2O) (8.1) [18]. At the time, few proteins were known to catalyze this reaction; later, catalase activity was identified in many bacteria as well. Subsequently, a separate class of H_2O_2-degrading enzymes (designated peroxidases) was defined as the activity is able to reduce H_2O_2 disproportionately (8.2).

$$H_2O_2 + H_2O_2 \rightarrow O_2 + 2H_2O \tag{8.1}$$

$$RH_2 + H_2O_2 \rightarrow R + 2H_2O \tag{8.2}$$

Peroxidase is able to use RH_2 as an electron donor, which can correspond to any of a range of molecules, including NADH, NADPH, glutathione, thioredoxin, or cytochrome *c*. Some enzymes with peroxidase activity can oxidize nonphysiological electron donors, such as dyes, although the actual (physiologically relevant) electron donor may be unknown. Although many organisms employ multiple, abundant peroxide scavenging activities, the basis and need for this apparent redundancy remains unresolved. For example, the model bacterium *E. coli* produces at least nine enzymes proposed as catalases or peroxidases.

8.2.2 OxyR

OxyR, a well-known transcription factor associated with H_2O_2 activation, is present in many bacteria, including *E. coli*. The protein is inactive until H_2O_2 oxidizes a target cysteine residue, thereby triggering the generation of a disulfide bond that converts the protein into an active conformation [19, 20]. Activated (H_2O_2-oxidized) OxyR binds to the promoter regions of the OxyR-regulon, which includes the genes encoding catalases and peroxidases; promoter-bound OxyR then recruits RNA polymerase and induces transcription.

An alternative H_2O_2 sensor, PerR, is used in other bacteria in place of OxyR [21, 22]. PerR generally binds iron, which is in a metallated form; in this form, PerR binds to promoters of the PerR regulon and represses their transcription. When the concentration of H_2O_2 increases, the Fenton reaction leads to the oxidation of the

bound iron and simultaneous oxidation of the corresponding PerR histidine ligand, irreversibly inactivating the PerR protein. The inactivated PerR cannot bind to the promoter regions of PerR regulon members, and the genes then are available for transcription. The PerR regulon has been studied in *Bacillus subtilis*, where the regulon includes separate genes that encode a peroxidase (Ahp) and a catalase (KatA) [23].

8.2.3 Heme Catalase

The majority of characterized bacteria harbor catalase activities, with the exceptions of enterococci, streptococci, and leuconostocs [13]. Catalases are categorized based on their cofactor, a system that distinguishes catalases into heme and non-heme (i.e., manganese) classes. Catalases catalyze the typical proponate H_2O_2 reaction as indicated above by (1), and some catalases additionally exhibit peroxidatic activities. That is, a "monofunctional catalase" has only catalase activity; an enzyme having both catalase and peroxidase activities is termed a "bifunctional catalase," a "catalase-peroxidase," or a "catalase/peroxidase." Originally, all bacterial catalases detected among aerobic and anaerobic bacteria were presumed to be monofunctional enzymes [24, 25]. The bifunctional catalases are less abundant. Crystal structures have been described for monofunctional catalases from *E. coli* [26, 27], *Proteus mirabilis* [28], *Micrococcus lysodeikticus* [29], *Helicobacter pylori* [30], and *Pseudomonas syringae* [31]. Structures have also been determined for the bifunctional enzymes from *Synechococcus* PCC 7942 [32], *Mycobacterium tuberculosis* [33], *Listeria seeligeri* [31], and *Burkholderia pseudomallei* [34]. While *E. coli* contains a catalase/peroxidase, a structure has been published only for the C-terminal domain of this enzyme [35]. The overall structures differ between monofunctional and bifunctional catalases, but the classes share the presence of a deeply buried heme that is accessed through a narrow channel [36]. This channel selects for the entry of H_2O_2 molecules, and thus provides substrate specificity by preventing catalysis of organic hydroperoxides. The apparent values of K_m and k_{cat} of monofunctional catalases are in the range of 38 mM (*Pseudomonas aeruginosa* KatB)–599 mM (*P. mirabilis*) and 54,000–83,300 s^{-1}, respectively, but the K_m and k_{cat} values of catalase activity of catalase-peroxidases are 2.4 mM (*M. tuberculosis* KatG)–4.5 mM (*B. pseudomallei* KatG) and 4,350–5,680 s^{-1}, respectively [37–40]. Consequently, catalytic efficiencies (k_{cat}/K_m) of both types of enzymes are in the range of approximately 10^5–10^6 M^{-1} s^{-1}, demonstrating that enzymes of the two classes are proficient in degrading H_2O_2.

Initially, catalases were thought to be the primary H_2O_2 scavengers, with peroxidases playing a secondary role. More recent observations suggest that peroxidases are often the primary scavengers when the dose of H_2O_2 is in the low-micromolar range [7], as is typically found in nature. The activity of catalase dominates only at higher peroxide levels, when peroxidase is saturated because of limiting rates of electron delivery and/or enzymatic inactivation due to over-oxidation. Many

bacteria have more than one catalase isozyme, and bifunctional catalases seem to be the preferred enzyme in exponential phase. These bifunctional catalases are induced by OxyR or PerR protein that detects environmental H_2O_2, but the monofunctional catalases are induced in stationary phase by a sigma factor specific for stationary phase [41–48]. Monofunctional enzymes may be more structurally stable and persist during the long periods of stasis that bacteria must periodically endure [13].

The monofunctional catalase is generally a cytoplasmic protein. Some bifunctional catalases are secreted to the periplasmic space, including *E. coli* O157: H7 KatP [49] and *Brucella abortus* KatA [50]. These bacteria also harbor cytoplasmic catalases, and such distinct localizations potentially supply an advantage to bacteria that must survive host–phagosomal environments. For phagocytosed bacteria, the existence of H_2O_2-scavenging systems in both cytoplasm and periplasm would provide a two-stage system, which may protect cytoplasmic targets against exogenous H_2O_2 more effectively than a similar amount of catalase located exclusively in the cytoplasm.

8.2.4 Manganese Catalase

The manganese catalase reacts with substrate in two steps, a process like that used by heme catalase.

$$2Mn^{2+} + H_2O_2 + 2H^+ \rightarrow 2Mn^{3+} + 2H_2O \tag{8.3}$$

$$2Mn^{3+} + H_2O_2 \rightarrow 2Mn^{2+} + 2H^+ + O^2 \tag{8.4}$$

Some bacteria harbor catalase activities that are azide- and cyanide-insensitive [51]. This type of catalase was isolated from *Pediococcus* grown in the absence of heme source [52]. Biochemical data, including atomic absorption spectroscopy data, have shown that this enzyme uses manganese atoms as cofactors, in place of heme [53, 54]. Manganese catalases have also been isolated from *Thermus thermophilus* [55], *Salmonella enterica* [56], *Lactobacillus plantarum* [53], *Thermoleophilum album* [54], and *Pyrobaculum calidiofontis* [57]. Crystal structures are available for the *L. plantarum* and *T. thermophilus* catalases [58, 59]. Two manganeses were contained in each subunit of these enzymes [60]. The dimanganese active site of the enzyme has a narrow channel, which provides substrate specificity for H_2O_2.

The kinetics of this class was studied using the enzymes of *L. plantarum*, *T. album*, *T. thermophilus*, and *P. calidifontis*. The K_m values for H_2O_2 are in the range of 15 mM (*T. album*)–350 mM (*L. plantarum*), similar to those of the heme catalases, but the k_{cat}/K_m values range between 1.7×10^5 M^{-1} s^{-1} (*P. calidifontis*) and 3.1×10^6 M^{-1} s^{-1} (*T. thermophilus*), which is an order of magnitude similar to those of the heme catalases [53, 54, 57, 61]. An inverse correlation between manganese catalase content and the H_2O_2 accumulation was reported for aerobically grown

L. plantarum, confirming that the manganese enzyme functions as a scavenger of H_2O_2 [53]. The heme catalases are more widespread than manganese catalases, with genes for manganese catalases found so far in only 100 bacteria (Peroxidase database, November, 2011) [62]. The Mn enzyme is commonly found only among cyanobacteria, but is rarely observed in other bacteria. High level of manganese catalase activity in a manganese-rich bacterium, *L. plantarum*, in which manganese is accumulated to an intracellular level of approximately 25 mM and is unable to synthesize heme, was observed only in a medium devoid of any heme source [63]. These results suggest that the manganese enzyme serves as a catalase only when more efficient heme catalases are not available.

In *S. enterica* the heme catalase (KatE) switches to the manganese enzyme in stationary phase. When iron levels decrease in the stationary phase, manganese import is induced in enteric bacteria, due to inactivation of Fur: Fe transcriptional repressor. The manganese is imported through that mechanism, and activate manganese-specific isozyme (NrdEF: manganese-dependent ribonuclease) instead of iron-dependent ribonuclease (NrdAB). The iron-dependent superoxide dismutase (SodB) is also replaced by the manganese isozyme (SodA), which is normally repressed by Fur, like manganese transporter itself [64–66].

8.3 Thiol Peroxidase (Peroxiredoxin)

8.3.1 Properties of Thiol Peroxidase (Peroxiredoxin)

Peroxidases are classified into two categories: thiol-based peroxidases, also called peroxiredoxins, and non-thiol peroxidases. The peroxiredoxins all have a conserved peroxidatic cysteine residue that can react with H_2O_2 or organic hydroperoxide, resulting in the formation of a cysteine sulfenic acid (−SOH). This sulfenic acid subsequently forms an inter- or intramolecular disulfide bond with another cysteine, and the resulting cysteine is converted back to two cysteines by reduction with another compound, such as a reduced dithiol-containing protein or NAD(P)H. Peroxiredoxins can be classified into four groups: (1) alkyl hydroperoxide reductase (AhpCF); (2) thiol peroxidases (Tpx); (3) bacterioferritin comigratory proteins (BCP); (4) glutathione peroxidases (Gpx) [13].

A eukaryotic non-heme, H_2O_2-scavenging enzyme was first reported in 1957, but was not further described until the 1970s. In 1988, a thiol-specific antioxidant (TSA) was identified in yeast as a protein that was able to inhibit glutathione synthesis from metal-catalyzed oxidation during dithionite/ferrous ion/oxygen exposure [67]. TSA function requires dithiothreitol (DTT) as an artificial electron donor.

In 1995, Cha et al. [68] purified and characterized a thiol-based peroxidatic protein of *E. coli*; this activity was designated originally as p20, and later as thiol peroxidase (Tpx). Tpx proteins contain a conserved cysteine in the N-terminal region. However, these proteins do not exhibit homology in their primary structure (amino

acid sequence). The Tpx prepared from various bacteria show conservation of Cys61 and Cys95 (using the consensus *E. coli* Tpx numbering), and these cysteines form an intramolecular disulfide bond after exposure to H_2O_2 [69]. Crystal structures have been solved for the reduced and oxidized forms [70, 71]. Site-directed mutagenesis has demonstrated that the Cys61 residue is essential for peroxidatic activity [69, 72]. The physiological reductant for Tpx is not known. However, Tpx reacts in vitro with H_2O_2, *t*-butyl hydroperoxide, cumene hydroperoxide, and linoleic acid hydroperoxide in the presence of DTT as an electron donor [73]. The k_{cat}/K_m values for a thioredoxin/thioredoxin reductase system were determined to be 7.7×10^6 and 4×10^4 M^{-1} s^{-1} for cumene hydroperoxide and H_2O_2, respectively [69], suggesting that this system could serve to scavenge organic hydroperoxides. The localization of Tpx remains unclear. Tpx was first prepared from the periplasmic fraction of *E. coli*, but later studies indicated that the *E. coli* enzyme is cytoplasmic, and Tpx of a Gram-positive bacteria also was cytoplasmic [74, 75]. *E. coli*, *Campylobacter jejuni*, *H. pylori*, or *Enterococcus faecalis* lacking Tpx did not show any phenotypes under aerobic conditions, but the *E. coli* mutant showed modest sensitivity to organic hydroperoxides in anaerobic culture [73, 75–77]. However, *tpx* mutants of *H. pylori* and *E. faecalis* were sensitive to exogenous H_2O_2 and cumene hydroperoxide [76, 77]. In conclusion, definition of the biological function of Tpx requires further analysis. Although the enzyme activity has been demonstrated in vitro, the in vivo phenotypes of *tpx* mutants are modest and detected only under conditions of stress. In *E. coli* and other bacteria growing under laboratory conditions, Ahp-containing systems are the predominant scavengers of organic peroxide and H_2O_2; other peroxidase enzymes are used only under specific circumstances (e.g., when Ahp and corresponding catalases are nonfunctional) or with particular (as-yet unidentified) substrates [13].

In 1989, a thiol-based peroxidase, AhpC, was isolated from *Salmonella typhimurium*, and was characterized as a member of the OxyR regulon, which is regulated by H_2O_2 stress [78]. The Ahp system consists of two cytoplasmic proteins, AhpC and AhpF, and is found throughout the aerobic and anaerobic microbiota [79].

8.3.2 Bacterioferritin Comigratory Protein

BCP exhibits thiol peroxidase activity in vitro, but the in vivo function of BCP remains much less obvious. BCP originally was identified in *E. coli* as a factor that co-migrates with bacterioferritin on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [80, 81]. Primary sequences of BCPs revealed homology to AhpC, and biochemical characterization confirmed the ability to react with H_2O_2. Homologs of BCP are present in a wide range of Gram-positive and Gram-negative bacteria. A cysteine residue in the N-terminal region (Cys48 in *Xanthomonas campestris* BCP) is a peroxidatic thiol, and another conserved cysteine (Cys84) may contribute to the formation of a disulfide bond. Activity is lost upon deletion of either of these cysteine residues [82, 83]. Crystal structures are available for the

BCPs of *X. campestris* [83] and *Aeropyrum pernix* (2CX4 released in 2005 by RIKEN Structural Genomics/Proteomics Initiative). While the reduction mechanism for BCP remains unknown, catalytic turnover with H_2O_2 and organic hydroperoxide has been demonstrated using both thioredoxin and glutathione/glutaredoxin (as physiological electron donors) and DTT (as an artificial electron donor) [75, 83–85]. The thioredoxin system has been proposed as the most likely in vivo electron donor. Although BCP is strongly expressed during exponential growth in *E. coli*, a mutant strain that lacks both Ahp and catalase exhibit increased H_2O_2 levels, indicating that the BCP acts in some other role. The BCP of *E. coli* reacts with linoleic acid hydroperoxide (10 μM) and *t*-butylhydroperoxide (40 μM) as well as with H_2O_2 (50 μM), suggesting that an organic hydroperoxide may be the physiological substrate [86]. However, the biological source of such an organic hydroperoxide is unknown in bacteria. Mutants of *H. pylori*, *C. jejuni*, and *Porphyromonas gingivalis* harboring deletions exhibit reduced viability under aerobic conditions as well as increased sensitivity to cumene hydroperoxide, an artificial organic hydroperoxide, but do not exhibit altered sensitivity to H_2O_2 [75, 85, 87, 88]. In both *Desulfovibrio vulgaris* and *E. coli*, *bcp* expression is increased under aerobic conditions [86, 89]. However, there is no evidence of a response to H_2O_2 or other oxidants in the other BCPs, and the physiological role of BCP remains unknown.

8.4 Scavengers of Superoxide

Spontaneous chemical dismutation (that is, the reaction of O_2^-) is not sufficient for maintaining low intracellular concentrations of O_2^-, since the rate of this reaction is very slow at physiological O_2^- concentrations and decreases sharply at lower O_2^- concentrations [13]. Gram-negative bacteria usually contain both cytoplasmic and periplasmic isozymes of superoxide dismutase (SOD) as a defense against O_2^-. *E. coli* has two cytoplasmic SOD isozymes, a manganese-cofactored version (MnSOD) and an iron-cofactored version (FeSOD). *E. coli* also secretes a periplasmic copper, zinc-cofactored superoxide dismutase (CuZnSOD, also called Sod). O_2^- cannot flow between cytoplasmic and periplasmic spaces, and thus the physiological roles of each enzyme must be considered separately [90, 91]. In eukaryotes, mitochondrial and cytoplasmic SODs employ Mn and CuZn cofactors, respectively. The role(s) of periplasmic SODs remain undefined. These isozymes may protect unidentified periplasmic target(s) from the O_2^- that leaks from respiratory chain components on the outer face of the cytoplasmic membrane [92]. Periplasmic SODs also might contribute to bacterial pathogenesis by helping to neutralize the oxidative bursts imposed by host macrophages or neutrophils [93].

The SOD of *A. actinomycetemcomitans* has, to our knowledge, been the subject of a limited number of papers. Notably, a CuZnSOD was purified from *A. actinomycetemcomitans* and was shown to interact with LtxA, a leukotoxin secreted by this bacterium [94]. This work suggested that this CuZnSOD may protect the bacterium and LtxA from reaction with O_2^- produced by host inflammatory cells during

infection [94]. In another paper, multiple MnSOD (SodA) of *Haemophilus* spp. and *A. actinomycetemcomitans* were characterized, and comprehensive sequence-based phylogenetic analysis was provided [95].

8.5 QPO Produced by Periodontopathic Bacterium

8.5.1 Properties of a Periodontopathic Bacterial QPO

A. actinomycetemcomitans is a facultatively anaerobic, carbon dioxide-requiring, Gram-negative bacterium in the gamma subdivision of the Proteobacteria [96]. The family *Pasteurellaceae* contains the well-studied genera *Haemophilus* and *Pasteurella. A. actinomycetemcomitans* is associated with a variety of human infections, including LAP, a severe disease of adolescents that is characterized by tissue and bone destruction and potential tooth loss [97].

In mammalian hosts, this bacterium must be able to survive exposure to ROS including O_2^- and H_2O_2, which are produced by host immune system [98]. H_2O_2 is a major bactericidal agent against *A. actinomycetemcomitans* in the periodontal pocket [99], so the bacteria must be resistant to host-produced ROS [100]. In addition, the primary source of endogenous ROS is assumed to be the redox enzymes of the respiratory chain in the bacterial cytoplasmic membrane [3, 100–106]. Addition of rotenone and antimycin A, respiratory chain inhibitors, increased the H_2O_2 production from the cytoplasmic membrane of *E. coli*. Inverted membrane vesicles of *E. coli* incubated with NADH generated superoxide anion and H_2O_2 in the presence of cyanide [104]. When reduced forms of NADH: quinone oxidoreductase II (NDH-2), succinate: quinone oxidoreductase (SQR), quinol: fumarate oxidoreductase, or sulfite reductase were exposed to oxygen, all generated O_2^- and H_2O_2 [3, 104, 107].

Thus, scavenging enzymes are expected to be critical for the resistance of *A. actinomycetemcomitans* to H_2O_2. The catalase of this bacterium is reported to have a critical role in bacterial resistance to hydrogen peroxide, but a catalase-deficient mutant can still grow in the presence of H_2O_2 (0.3 mM), suggesting that the other proteins, such as peroxidases, also can contribute to ROS scavenging activity [108].

Cytochrome *c* peroxidase (CCP) has been reported in yeast and several bacteria and catalyzes the reduction of H_2O_2 to water using a reduced *c*-type cytochrome as an electron donor. The yeast CCP is monoheme *b* peroxidase of 294 amino acid residues that is found in the intermembrane space of the mitochondrion, a location that is functionally analogous to the bacterial periplasm. In contrast, bacterial cytochrome *c* peroxidase (BCCP) is a di-heme *c* protein of 300–400 amino acid residues that localizes to the periplasmic space. BCCP (from *P. aeruginosa*) was first purified and characterized in 1970 [109]. BCCPs subsequently have been isolated from *Pseudomonas nautica*, *Paracoccus pantotrophus*, *Rhodobacter capsulatus*, *Nitrosomonas europaea*, and *Methylococcus capsulatus* [110–114]. The 3D structures have been determined for BCCPs from *P. aeruginosa*, *P. nautica*, *N. europaea*, *R. capsulatus*, and *P. pantotrophus* [115–119].

The BCCPs of *P. aeruginosa* and *P. pantotrophus* have been studied extensively [111, 115, 120–129], and the spectrum-data of these enzymes suggested the existence of a complicated reaction mechanism that involves changes of the redox states of the heme group and spin states of the corresponding electrons. For the *Pseudomonas* BCCPs, completely oxidized proteins are inactive, whereas the enzymes in the mixed-valence (half-reduced) state react rapidly with H_2O_2. In completely oxidized BCCPs, the high-potential electron-transferring C-terminal heme group is in a high/low-spin equilibrium and is ligated by a histidine and a methionine [130]. The second heme (in the N-terminal domain) is low potential and is coordinated by two histidines in oxidized form (IN-form) [115]. In the mixed-valence (half-reduced) state, Ca^{2+} induces a spin-state switch at the low-potential heme, converting this heme from a low- to high-spin state. In the calcium–iron-activated mixed-valence form, the distal histidine residue that ligands the N-terminal heme is released from iron, permitting H_2O_2 to enter the active site and be reduced (OUT-form) [119]. The overall reaction is indicated in Fig. 8.1a.

The *N. europaea* enzyme exhibits high sequence similarity to that of *P. aeruginosa.* However, in contrast to the *Pseudomonas* protein, the *N. europaea* BCCP reacts with H_2O_2 in the mixed-valence state or fully oxidized state [113, 117, 131]. The 3D structure of the fully oxidized *N. europaea* enzyme revealed that the CCP is already in OUT-form, in which low-potential heme is coordinated by five ligands, similar to the mixed-valence state of the *P. aeruginosa* CCP [117]. While the calcium ion dependency of the *N. europaea* enzyme has not been fully examined, the crystal structure includes a calcium ion, suggesting that Ca^{2+} may be important for the *N. europaea* protein's enzymatic activity. For most bacteria, the *c*-type cytochrome of the aerobic respiratory chain is thought to be the physiologically relevant electron donor, providing the reducing equivalent to BCCP [118, 120]. However, some bacteria, including *E. coli*, that lack cytochrome *c* in their aerobic respiratory chain contain an additional homolog of BCCP of unknown function. This homolog incorporates an N-terminal extension of 140–150 amino acid residues that contains a potential heme *c*-binding motif [121]. As part of the *E. coli* aerobic respiratory chain, NADH: quinone oxidoreductase I (NDH-1), NDH-2, SQR, and D-lactate: quinone oxidoreductase can reduce quinones, which are then reoxidized by two terminal quinol oxidases, cytochrome b_{558}-*d* and b_{562}-*o* [132]. Notably, *E. coli* lacks the quinol: cytochrome *c* oxidoreductase and cytochrome *c* oxidase activities. There are three different quinones: ubiquinone (a benzoquinone), and menaquinone and dimethyl menaquinone (naphthoquinones). During aerobic growth, ubiquinone serves as the major quinone, but this molecule is replaced by the naphthoquinones during anaerobic growth in the presence of fumarate, nitrate, or dimethyl sulfoxide, which serve as electron acceptors [132].

As we discuss here, we have identified and purified a BCCP homolog from the membrane fraction of *A. actinomycetemcomitans*. In vitro, this BCCP functions as a QPO that uses ubiquinol-1 as an electron donor, performing an overall reaction that can be summarized as follows:

$$ubiquinol\text{-}1 + H_2O_2 \rightarrow ubiquinone\text{-}1 + 2H_2O$$

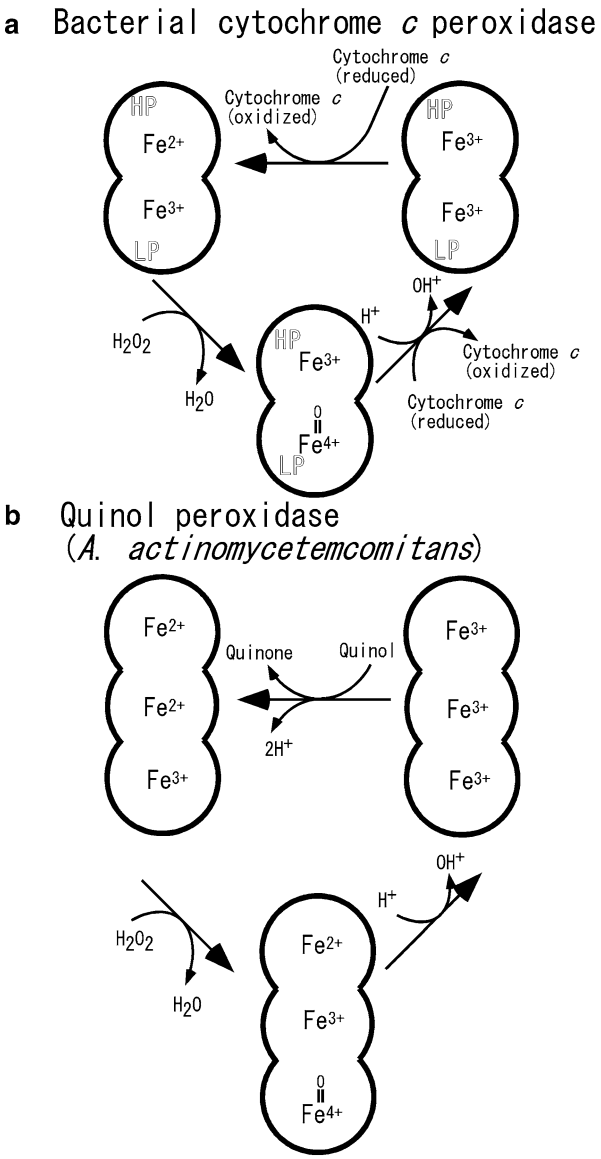

Fig. 8.1 Mechanism of H_2O_2 degradation by cytochrome *c* peroxidase (**a**) or quinol peroxidase (**b**)

On the other hand, cytochrome *c* oxidase activity was not detected in *A. actinomycetemcomitans* membrane vesicles. Genomic DNA sequence data showed that this bacterium has a cytochrome *bd*-encoding gene, but lacks genes encoding cytochromes *c*, bc_1, or aa_3. Thus, it appears that *A. actinomycetemcomitans* exclusively employs a quinol oxidase. This enzyme is expected to serve as the terminal oxidase of the respiratory chain, a role resembling that of cytochromes *bd* and *bo* in *E. coli* [132, 133]. BCCP activity was not observed in any of the cell fractions of

A. actinomycetemcomitans. Our results also suggest that QPO-dependent peroxidatic activity using substrates of the respiratory chain, including NADH and succinate, was present in the cytoplasmic membrane vesicles of *A. actinomycetemcomitans* [134], as mentioned below.

The QPO activity of the cytoplasmic membrane fraction was measured under anaerobic conditions, since the ubiquinol-1 oxidase activity of the respiratory chain otherwise interfered with the assay of ubiquinol-1 peroxidase activity. After being solubilized from the membrane fraction with detergent SM-1200, QPO was purified using a combination of Macro-prep Ceramic Hydroxyapatite, HiTrap Phenyl HP, AF-red-560M, and HiPrep Sephacryl S-200HR chromatography. The purified enzyme was reddish in color, had a specific activity of 327 μmol min^{-1} mg^{-1}, and migrated as a single 49-kDa band on SDS-PAGE [134]. Gel filtration of QPO from the Sephacryl S-200 HR column in the presence of detergent SM-1200 showed that QPO was approximately 40–50 kDa, suggesting that the QPO exists as a monomeric protein. However, this observation would be inconsistent with previous demonstrations that the active form of BCCP is a dimer [120, 135].

Based on genomic sequences, we cloned the *qpo* open reading frame (ORF) along with 120 bp of upstream sequence, including a potential ribosome-binding site (AGGACA) centered six bases upstream of the putative start codon. The 1,383-base pair ORF is delimited by an ATG start codon and a TAA stop codon. The *qpo* ORF is predicted to encode a 460-amino acid polypeptide, with a predicted molecular mass of 51,730 Da, which is slightly larger than that estimated by SDS-PAGE (about 49 kDa). The amino acids Phe4-Tyr26 were predicted by SOSUI [136] to comprise a transmembrane region.

Comparison of the predicted protein to the sequences of other BCCPs suggested that the protein consists of two domains: an N-terminal QPO-unique region that contains one heme-binding motif, and a BCCP-homologous region that has two heme-binding motifs. The deduced amino acid sequence of QPO shared 46–54 % identity with homologs from *Bacteroides fragilis* (NCBI: AAL09840.1), *E. coli* (NCBI: AAC76543.1), *S. enterica* (NCBI: AAO71193.1), *Shigella flexneri* (NCBI: AAN45044.2), and *Yersinia pestis* (NCBI: AAM84433.1). The BCCP-homologous region also shared 40–43 % identity with the BCCPs of *N. europaea* (PDB: 1IQC), *P. aeruginosa* (PDB: 1EB7), *P. pantotrophus* (PDB: 2C1V), and *R. capsulatus* (PDB: 1ZZH). The Ca^{2+}-binding residues (Asn231, Thr407, and Pro409), a tryptophan residue acting as conduit for electron transfer between two hemes (Trp246), a histidine residue interacting with the C-terminal heme propionate D group (His412), and a distal methionine ligand of the C-terminal heme (Met426) were conserved in QPO [115, 117–119, 121, 122, 134]. The three putative heme *c*-binding motifs (CXXCH) occurred at amino acids Cys56-His60, Cys203-His207, and Cys345-His349. The putative distal ligand for heme in the N-terminal region was predicted to lay at Met122 or His 131 based on conservation between QPO and its homologs. The histidine that acts as a distal ligand for N-terminal heme of the oxidized form of BCCP (His85 in *P. pantotrophus* BCCP) is not conserved in QPO. We did not find an alternative residue for the distal ligand of heme in the middle of QPO, suggesting that this heme would be coordinated by five ligands. Although the oxidized form could possibly react with hydrogen peroxide via a mechanism similar to

monoheme peroxidase and BCCP from *N. europaea*, the absorption spectrum for QPO did not change on addition of H_2O_2. This result suggests that the oxidized form of QPO does not react with H_2O_2, thus resembling the BCCPs of *P. aeruginosa* and *P. pantotrophus*.

The crystal structure of *P. aeruginosa* BCCP contains bound Ca^{2+} even without addition of calcium to the media used for purification and crystallization, suggesting that BCCP has in inherent affinity for Ca^{2+} [115]. When QPO was incubated with chelator (1 mM EGTA or EDTA), the activity of QPO was not affected. This result contrasts with *P. pantotrophus* BCCP, which is inactivated upon chelator treatment. Likewise, incubation of QPO with 1 mM $CaCl_2$ had no effect on the enzyme's activity. We conclude that QPO activity is Ca^{2+}-independent, in contrast to other BCCPs, although putative Ca^{2+}-binding residues (Asn231, Thr407, and Pro409) are retained in QPO.

8.5.2 Absorption Spectra of QPO

The absorption spectra of QPO showed that absorption maxima of the oxidized form were detected at 412 and 530 nm; absorption maxima of the dithionite-reduced form were detected at 419 nm (Soret band), 524 nm (beta band), and 553 nm (alpha band) [134].

8.5.3 Kinetic Characterization

The K_m value for ubiquinol-1 in the presence of H_2O_2 was 107 μM, which is similar to the values determined for *E. coli* cytochromes *bo* and *bd* (48 and 110 μM, respectively) [133, 134, 137]. The k_{cat} value for QPO with ubiquinol-1 as the substrate was 582 s^{-1}, which is comparable to that of BCCP from *P. pantotrophus* (1,040 s^{-1}) [120]. We propose a reaction mechanism (Fig. 8.1b) in which the first step can be modeled as a Ping–Pong Bi Bi mechanism:

$$Oxidized\text{-}QPO + \mathrm{Q1H2} \leftrightarrow reduced\text{-}QPO + \mathrm{Q1}$$
$$\left(\mathrm{Q1H2}; ubiquinol\text{-}1, \mathrm{Q1}; ubiquinone\text{-}1\right)$$

We have since confirmed this mechanism by kinetic and spectroscopic analyses (Kawarai and Konishi, unpublished observations).

8.5.4 Generation of qpo Null Mutant

To analyze the physiological role of QPO, an *A. actinomycetemcomitans qpo* null mutant (designated strain QPS003) was derived from strain IDH781 using a

homologous gene deletion method. The specific activity of the IDH781 (parent) membrane fraction was 6.33 μmol min^{-1} mg^{-1}; the activity was not detectable in the QPS003 (mutant) membrane fraction. The transformation of QPS003 cells with a plasmid containing full-length *qpo* (pVJTqpo) restored the QPO activity (6.54 μmol min^{-1} mg^{-1}) [138].

If QPO uses endogenous quinols as physiological substrates in the respiratory chain, the QPO activity is expected to be functionally connected to the respiratory chain of *A. actinomycetemcomitans*. To test this hypothesis, QPO activities in membrane fractions of IDH781, QPS003, and QPS (pVJTqpo) were examined using two respiratory chain substrates, NADH and succinate. The specific activities of IDH781 were 341 and 811 nmol min^{-1} mg^{-1} with NADH and succinate, respectively; these activities were not detected from QPS003. In addition, NADH-peroxidase and succinate-peroxidase activities of QPS003 (pVJTqpo) membrane vesicles were 299 and 326 nmol min^{-1} mg^{-1} with NADH and succinate, respectively. These data indicate that NADH- and succinate-peroxidase activities are dependent on QPO, confirming that QPO is functionally connected to the respiratory chain in *A. actinomycetemcomitans* [138].

8.5.5 Properties of qpo *Null Mutant*

Many systemic antibiotic therapies are unable to consistently suppress subgingival *A. actinomycetemcomitans* to undetectable levels. For instance, systemic therapy with metronidazole or tetracycline may markedly reduce oral *A. actinomycetemcomitans*, but not eradicate the organism [139]. Thus, novel chemotherapeutic agents for the treatment and prevention of LAP would be highly desirable. Leukotoxin (LtxA) is one of the major virulence factors of this bacterium [140] and is a member of the RTX toxin family. LtxA, which is secreted as an approximately 114-kDa protein, disrupts human leukocyte and erythrocyte cytoplasmic membranes [94, 141, 142]. Because the amino acid sequence of the C-terminal region of QPO shares about 40 % identity with di-heme BCCP, we grouped QPO and BCCP together in a single enzyme family, and we named this protein group the bacterial multiheme peroxidase family [143]. The *qpo* homologous genes in *E. coli* and *B. fragilis* are known each to encode BCCP with an N-terminal extension containing an additional heme *c*-binding motif, although the enzymatic activity and membrane localization of the proteins encoded by these genes have not been determined [144, 145]. Recently, a QPO homolog (ZmCytC) of *Zymomonas mobilis* was reported as a member of an aerobic respiratory chain; ZmCytC was postulated to bind to the bc_1 complex in addition to ubiquinone [146]. To study the physiological role of QPO from *A. actinomycetemcomitans*, a comparison was performed between the *qpo* null mutant (QPS003) and a strain (IKM001) harboring a transposon insertion mutation of the catalase (KatA)-encoding gene. KatA localizes to the cytoplasm of this bacterium, where the protein serves as an antioxidant enzyme [108]. Although QPS003 exhibited a decrease in

growth under aerobic conditions, IKM001 grew normally, as reported previously [108]. The reduction of growth seen with the *qpo* mutation might result from oxidative stress. Indeed, QPS003 cells showed increased protein damage compared to the parent strain (IDH781) and to the *katA* mutant strain (IKM001) [138], suggesting that QPO (but not KatA) plays an important role in scavenging endogenous ROS. This difference may reflect distinctions in the nature of the ROS substrates. Notably, as an uncharged ROS like a H_2O_2 can move freely across the cytoplasmic membrane; in contrast, a charged ROS like an O_2^- is relatively membrane-impermeable and so can be compartmentalized in the periplasmic space [14]. Thus, membrane-permeable H_2O_2 generated from the respiratory chain could be scavenged by antioxidant enzymes in the periplasm, in the membrane (e.g., QPO), or in the cytoplasm (e.g., KatA), while periplasmic O_2^- would effectively be inaccessible for scavenging by KatA that is localized in cytoplasm in a *qpo* mutant.

A disk diffusion assay using exogenous oxidants showed that *qpo* mutant strain QPS003 had decreased sensitivity to H_2O_2, but not to cumene hydroperoxide or *t*-butyl hydroperoxide. These results suggest that QPO has an exclusive role in protecting against exogenous oxidative stress induced by H_2O_2, and furthermore imply that QPO scavenges H_2O_2, but not organic hydroperoxides [138]. These results are consistent with the observations that purified QPO catalyzes the reduction of H_2O_2, but not that of cumene hydroperoxide or *t*-butyl hydroperoxide. The amount of lipid hydroperoxide, including linoleic acid hydroperoxide, remained undetectable in the *qpo* mutant, although the membrane localization of QPO implies that this enzyme plays a role in lipid hydroperoxide scavenging.

E. coli is known to express several enzymes that have been proposed to be peroxide scavenging enzymes. The scavenged molecules (H_2O_2 or organic hydroperoxides) then are reduced, mainly by monofunctional catalase, bifunctional catalases, peroxiredoxins, and the AhpCF system [13, 102, 147]. Genomic sequence analysis (Oralgen genome database; http://www.oralgen.lanl.gov/) shows that *A. actinomycetemcomitans* harbors at least three loci encoding predicted peroxiredoxins that might scavenge organic hydroperoxides: AA02348 (*bcp*), encoding a bacterioferritin comigratory protein; AA01514 (*grx*), encoding a peroxiredoxin 2 family protein/glutaredoxin; and AA02462 (*tpx*), encoding a thiol peroxidase [138].

We next studied LtxA production in the *qpo* mutant. SDS-PAGE analysis of secreted protein from aerobically cultured cells revealed that QPS003 exhibited a defect in production of LtxA, while IDH781 and QPS003 (pVJTqpo) secreted considerable amounts of LtxA [138]. Notably, accumulation of *ltxA* transcript did not differ among these strains [138], indicating that LtxA depletion was mediated by a posttranscriptional event. Recent results show that LtxA is degraded by ROS exposure in vitro and that the degraded LtxA is unable to kill HL-60 cells [94], suggesting that the LtxA defect in QPS003 may reflect LtxA degradation by endogenous ROS. Since LtxA is one of the major virulence factors of *A. actinomycetemcomitans*, QPS003 is expected to be exhibit reduced pathogenesis. Hence, QPO would be considered a target for drugs that aim to reduce the pathogenicity of *A. actinomycetemcomitans*.

8.5.6 QPO Inhibition Prevents Secretion of LtxA

We screened approximately 300 chemical compounds, comprising metabolites of bacteria and fungi, in the Kitasato Institute for Life Science Chemical Library, for inhibitors of QPO activity [148, 149]. The strongest hit was ascofuranone, a prenylphenol that has been previously identified as a strong inhibitor of trypanosome alternative oxidase, a terminal oxidase of the respiratory chain of *Trypanosoma brucei brucei* [150]. Ascofuranone has been proposed as a drug candidate for treatment of trypanosomiasis and has been shown to cure mice infected with the trypanosome by intraperitoneal injection of ascofuranone [151].

Investigation of ascofuranone inhibition of QPO suggests a mixed-type mechanism of inhibition that can be described by equilibrium kinetics, with a K_i value for ubiquinone-1 of 9.56 nM. Addition of 20 μg mL^{-1} ascofuranone reduced the growth rate of IDH781, consistent with the growth-impaired phenotype of *qpo* mutant strain QPS003. Addition of 20 μg mL^{-1} ascofuranone did not affect the growth rate of QPS003, suggesting that QPO is the major in vivo target of this inhibitor [148]. These results suggested that treatment with ascofuranone should induce oxidative stress. Western blotting analysis using anti-dinitrophenyl (DNP) antibody showed that ascofuranone induced dose-dependent oxidative modification of proteins in aerobically grown cultures of IDH781. In IDH781 treated with 20 μg mL^{-1} inhibitor, the levels of oxidatively damaged proteins were similar to those seen in (untreated) QPS003, suggesting that the majority of QPO activity in bacteria was inhibited by this concentration of ascofuranone [148]. A previous report showed that OxyR, a global regulator of antioxidant defense [152], is involved in the activation of *E. coli yhjA*, a homolog of *A. actinomycetemcomitans qpo* [145]. Sequence analysis identified a putative OxyR element in the promoter region of the *qpo* gene, and a search of the Oralgen genome database (http://www.oralgen.lanl.gov/) revealed an *A. actinomycetemcomitans* locus (accession number AA01513) that encodes an OxyR homolog. Together, these results suggest that *qpo* might be a member of an *A. actinomycetemcomitans* OxyR regulon. Consistent with this hypothesis, an aerobic culture of *A. actinomycetemcomitans* mutated for AA01513 exhibited an approximately 2.5-fold reduction in QPO activity compared to the wild-type parent strain IDH781 (Konishi, unpublished observation).

To examine the effect of ascofuranone on the secretion of LtxA, *A. actinomycetemcomitans* was cultured aerobically in the presence of different concentrations of inhibitor. SDS-PAGE showed a dose-dependent decrease in LtxA levels in the resulting culture medium. Consistent with decreased LtxA levels, culture media from ascofuranone-exposed *A. actinomycetemcomitans* exhibited reduced cytotoxic activity against HL-60 cells [148]. Cytotoxicity was not detected in the culture medium from the QPS003 mutant strain. Taken together, these data indicate that ascofuranone attenuated the virulence of the supernatant of the *A. actinomycetemcomitans* strains by decreasing LtxA accumulation.

Minimum inhibitory concentration (MIC) values of ascofuranone for *Streptococcus gordonii* DL1 and *E. coli* were >50 μg mL^{-1}, suggesting that

ascofuranone is not an effective bacteriostatic agent at concentrations that would prevent the secretion of LtxA. Thus, ascofuranone would be expected to have a smaller impact on the normal bacterial flora compared to other antibiotics, and therefore may have fewer adverse effects. To our knowledge, there are at present no drugs effective for preventing the onset of LAP. The development of chemical agents for the prevention of LAP would greatly contribute to the periodontal health of adolescents, especially for high-risk individuals, such as those from a Moroccan immigrant family infected with a highly leukotoxic strain of *A. actinomycetemcomitans* [153]. Thus, QPO inhibitors like ascofuranone are promising candidates for the prevention of LAP. Moreover, for individuals who do not respond to conventional therapy, QPO inhibitors may provide an alternative practical approach based on attenuation of *A. actinomycetemcomitans* virulence. Ascofuranone is not significantly cytotoxic for primary culture of rat mesangial cells [154]. The intraperitoneal and oral administration of ascofuranone cures *T. brucei* infected mice and does not demonstrate the toxicity for mice, suggesting that ascofuranone might exhibit in vivo efficacy [155].

References

1. Naqui A, Chance B, Cadenas E (1986) Reactive oxygen intermediates in biochemistry. Annu Rev Biochem 55:137–166
2. Massey V, Strickland A, Mayhew SG et al (1969) The production of superoxide anion radicals in the reaction of reduced flavins and flavoproteins with molecular oxygen. Biochem Biophys Res Commun 36:891–897
3. Messner KR, Imlay JA (2002) Mechanism of superoxide and hydrogen peroxide formation by fumarate reductase, succinate dehydrogenase, and aspartate oxidase. J Biol Chem 277:42563–42571
4. Seaver LC, Imlay JA (2004) Are respiratory enzymes the primary sources of intracellular hydrogen peroxide? J Biol Chem 279:48742–48750
5. Carlioz A, Touati D (1986) Isolation of superoxide dismutase mutants in *Escherichia coli*: is superoxide dismutase necessary for aerobic life? EMBO J 5:623–630
6. Farr SB, D'Ari R, Touani D (1986) Oxygen-dependent mutagenesis in *Escherichia coli* lacking superoxide dismutase. Proc Natl Acad Sci U S A 83:8268–8272
7. Seaver LC, Imlay JA (2001) Alkyl hydroperoxide reductase is the primary scavenger of endogenous hydrogen peroxide in *Escherichia coli*. J Bacteriol 183:7173–7181
8. Hebrard H, Viala JP, Meresse S et al (2009) Redundant hydrogen peroxide scavengers contribute to *Salmonella* virulence and oxidative stress resistance. J Bacteriol 191:4605–4614
9. Park S, You X, Imlay JA (2005) Substantial DNA damage from submicromolar intracellular hydrogen peroxide detected in Hpx-mutants of *Escherichia coli*. Proc Natl Acad Sci U S A 102:9317–9322
10. Jang S, Imlay JA (2007) Micromolar intracellular hydrogen peroxide disrupts metabolism by damaging iron–sulfur enzymes. J Biol Chem 282:929–937
11. Sobata JM, Imlay JA (2011) Iron enzyme ribulose-5-phosphate 3-epimerase in *Escherichia coli* is rapidly damaged by hydrogen peroxide but can be protected by manganese. Proc Natl Acad Sci U S A 108:5402–5407
12. Anjem A, Imlay JA (2012) Mononuclear iron enzymes are primary targets of hydrogen peroxide stress. J Biol Chem 287:15544–15556

13. Mishra S, Imlay JA (2012) Why do bacteria use so many enzymes to scavenge hydrogen peroxide? Arch Biochem Biophys 525:145–160
14. Seaver LC, Imlay JA (2001) Hydrogen peroxide fluxes and compartmentalization inside growing *Escherichia coli*. J Bacteriol 183:7182–7189
15. Winterbourn CC, Hampton MB, Livesey JH et al (2006) Modeling the reactions of superoxide and myeloperoxidase in the neutrophil phagosome: implications for microbial killing. J Biol Chem 281:39860–39869
16. Pericone CD, Overweg K, Hermans PW et al (2000) Inhibitory and bactericidal effects of hydrogen peroxide production by *Streptococcus pneumoniae* on other inhabitants of the upper respiratory tract. Infect Immun 68:3990–3997
17. Imlay JA (2009) In: Bock A, Curtiss R III, Kaper JB, Karp PD, Neidhart FC, Nystrom T, Slauch JM, Sqires CL, Ussery D (eds) EcoSal-*Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, DC. Available from http://www.ecosal.org
18. Loew O (1900) A new enzyme of general occurrence in organisms. Science 11:701–702
19. Zheng L, Cash VL, Flint DH et al (1998) Assembly of iron–sulfur clusters. Identification of an iscSUA-hscBA-fdx gene cluster from *Azotobacter vinelandii*. J Biol Chem 273: 13264–13272
20. Lee C, Lee SM, Mukhopadhyay P et al (2004) Redox regulation of OxyR requires specific disulfide bond formation involving a rapid kinetic reaction path. Nat Struct Mol Biol 11:1179–1185
21. Herbig AF, Helmann JD (2001) Roles of metal ions and hydrogen peroxide in modulating the interaction of the *Bacillus subtilis* PerR peroxide regulon repressor with operator DNA. Mol Microbiol 41:849–859
22. Lee JW, Helmann JD (2006) The PerR transcription factor senses H_2O_2 by metal-catalysed histidine oxidation. Nature 440:363–367
23. Gaballa A, Helmann JD (2002) A peroxide-induced zinc uptake system plays an important role in protection against oxidative stress in *Bacillus subtilis*. Mol Microbiol 45:997–1005
24. Herbert D, Pinsent J (1948) Crystalline bacterial catalase. Biochem J 43:193–202
25. Clayton RK (1959) Purified catalase from *Rhodopseudomonas spheroides*. Biochim Biophys Acta 36:40–47
26. Bravo J, Verdaguer N, Tormo J et al (1995) Crystal structure of catalase HPII from *Escherichia coli*. Structure 3:491–502
27. Bravo J, Mate MJ, Schneider T et al (1999) Structure of catalase HPII from *Escherichia coli* at 1.9 A resolution. Proteins 34:155–166
28. Gouet P, Jouve HM, Dideberg O (1995) Crystal structure of *Proteus mirabilis* PR catalase with and without bound NADPH. J Mol Biol 249:933–954
29. Murshudov GN, Melik-Adamyan WR, Grebenko AI et al (1992) Three-dimensional structure of catalase from *Micrococcus lysodeikticus* at 1.5 A resolution. FEBS Lett 312:127–131
30. Loewen PC, Carpena X, Rovita C (2004) Structure of *Helicobacter pylori* catalase, with and without formic acid bound, at 1.6 A resolution. Biochemistry 43:3089–3103
31. Carpena X, Perez R, Ochoa WF (2001) Crystallization and preliminary X-ray analysis of clade I catalases from *Pseudomonas syringae* and *Listeria seeligeri*. Acta Crystallogr D Biol Crystallogr 57:1184–1186
32. Wada K, Tada T, Nakamura Y et al (2002) Crystallization and preliminary X-ray diffraction studies of catalase-peroxidase from *Synechococcus* PCC 7942. Acta Crystallogr D Biol Crystallogr 58:157–159
33. Bertrand T, Eady NA, Jones JN et al (2004) Crystal structure of *Mycobacterium tuberculosis* catalase-peroxidase. J Biol Chem 279:38991–38999
34. Carpena X, Loprasert S, Mongkolsuk S et al (2003) Catalase-peroxidase KatG of *Burkholderia pseudomallei* at 1.7A resolution. J Mol Biol 327:475–489
35. Carpena X, Melik-Adamya W, Loewen PC et al (2004) Structure of the C-terminal domain of the catalase-peroxidase KatG from *Escherichia coli*. Acta Crystallogr D Biol Crystallogr 60:1824–1832
36. Amara P, Andreoletti P, Jouve HM et al (2001) Ligand diffusion in the catalase from *Proteus mirabilis*: a molecular dynamics study. Protein Sci 10:1927–1935

37. Chelikani P, Fita I, Loewen PC (2004) Diversity of structures and properties among catalases. Cell Mol Life Sci 61:192–208
38. Hillar A, Peters B, Pauls R et al (2000) Modulation of the activities of catalase-peroxidase HPI of *Escherichia coli* by site-directed mutagenesis. Biochemistry 59:5868–5875
39. Regelsberger G, Jakopitsch C, Engleder M et al (1999) Spectral and kinetic studies of the oxidation of monosubstituted phenols and anilines by recombinant *Synechocystis* catalase-peroxidase compound I. Biochemistry 38:10480–10488
40. Singh R, Wiseman B, Deemagam T (2008) Comparative study of catalase-peroxidases (KatGs). Arch Biochem Biophys 471:207–214
41. Christman MF, Stolz G, Ames BN (1989) OxyR, a positive regulator of hydrogen peroxide-inducible genes in *Escherichia coli* and *Salmonella typhimurium*, is homologous to a family of bacterial regulatory proteins. Proc Natl Acad Sci U S A 86:3484–3488
42. Bsat N, Herbig A, Casillas-Martinez L et al (1998) *Bacillus subtilis* contains multiple Fur homologues: identification of the iron uptake (Fur) and peroxide regulon (PerR) repressors. Mol Microbiol 29:189–198
43. Bol DK, Yasbin RE (1994) Analysis of the dual regulatory mechanisms controlling expression of the vegetative catalase gene of *Bacillus subtilis*. J Bacteriol 176:6744–6748
44. Hahn JS, Oh SY, Chater KF et al (2000) H_2O_2-sensitive fur-like repressor CatR regulating the major catalase gene in *Streptomyces coelicolor*. J Biol Chem 275:38254–38260
45. Zou P, Borovok I, Ortiz de Orué Lucana D, Muller D et al (1999) The mycelium-associated *Streptomyces reticuli* catalase-peroxidase, its gene and regulation by FurS. Microbiology 145:549–559
46. LeBlanc JJ, Brassinga AK, Ewann F et al (2008) An ortholog of OxyR in *Legionella pneumophila* is expressed postexponentially and negatively regulates the alkyl hydroperoxide reductase (*ahp*C2D) operon. J Bacteriol 190:3444–3455
47. Loewen PC, Switala J (1988) Purification and characterization of spore-specific catalase-2 from *Bacillus subtilis*. Biochem Cell Biol 66:707–714
48. Engelmann S, Lindner C, Hecker M (1995) Cloning, nucleotide sequence, and regulation of katE encoding a sigma B-dependent catalase in *Bacillus subtilis*. J Bacteriol 177:5598–5605
49. Brunder W, Schmidt H, Karch H (1996) KatP, a novel catalase-peroxidase encoded by the large plasmid of enterohaemorrhagic *Escherichia coli* O157:H7. Microbiology 142 (Pt 11):3305–3315
50. Sha Z, Stabel TJ, Mayfield JE (1994) *Brucella abortus* catalase is a periplasmic protein lacking a standard signal sequence. J Bacteriol 176:7375–7377
51. Whittenbury R (1964) Hydrogen peroxide formation and catalase activity in the lactic acid bacteria. J Gen Microbiol 35:13–26
52. Johnston MA, Delwiche EA (1965) Distribution and characteristics of the catalases of *Lactobacillaceae*. J Bacteriol 90:347–351
53. Kono Y, Fridovich I (1983) Inhibition and reactivation of Mn-catalase. Implications for valence changes at the active site manganese. J Biol Chem 258:13646–13648
54. Allgood GS, Perry JJ (1986) Characterization of a manganese-containing catalase from the obligate thermophile *Thermoleophilum album*. J Bacteriol 168:563–567
55. Jacquamet L, Michaud-Soret I, Debaecker-Petit N et al (1997) Magnetization studies of the reduced active form of the catalase from *Thermus thermophilus*. Angew Chem Int Ed Engl 36:1626–1628
56. Robbe-Saule V, Coynault C, Ibanez-Ruiz M et al (2001) Identification of a non-haem catalase in *Salmonella* and its regulation by RpoS (sigmaS). Mol Microbiol 39:1533–1545
57. Amo T, Atomi H, Imanaka T (2002) Unique presence of a manganese catalase in a hyperthermophilic archaeon, *Pyrobaculum calidifontis* VA1. J Bacteriol 184:3305–3312
58. Barynin VV, Whittaker MM, Antonyuk SV et al (2001) Crystal structure of manganese catalase from *Lactobacillus plantarum*. Structure 9:725–738

59. Antonyuk SV, Melik-Adamyan VR, Popov AN et al (2000) Three-dimensional structure of dimanganese catalase from *Thermus thermophilus* at 1 A resolution. Kristallografiya 45:111–122
60. Beyer WF Jr, Fridovich I (1985) Pseudocatalase from *Lactobacillus plantarum*: evidence for a homopentameric structure containing two atoms of manganese per subunit. Biochemistry 24:6460–6467
61. Shank M, Barynin V, Dismukes GC (1994) Protein coordination to manganese determines the high catalytic rate of dimanganese catalases. Comparison to functional catalase mimics. Biochemistry 33:15433–15436
62. Passardi F, Theiler G, Zamocky M et al (2007) PeroxiBase: the peroxidase database. Phytochemistry 68:1605–1611
63. Kono Y, Fridovich I (1983) Functional significance of manganese catalase in *Lactobacillus plantarum*. J Bacteriol 155:742–746
64. Martin JE, Imlay JA (2011) The alternative aerobic ribonucleotide reductase of *Escherichia coli*, NrdEF, is a manganese-dependent enzyme that enables cell replication during periods of iron starvation. Mol Microbiol 80:319–334
65. Patzer SI, Hantke K (2001) Dual repression by Fe^{2+}-Fur and Mn^{2+}-MntR of the mntH gene, encoding an NRAMP-like Mn^{2+} transporter in *Escherichia coli*. J Bacteriol 183:4806–4813
66. Tardat B, Touati D (1991) Two global regulators repress the anaerobic expression of MnSOD in *Escherichia coli*::Fur (ferric uptake regulation) and Arc (aerobic respiration control). Mol Microbiol 5:455–465
67. Kim K, Kim IH, Lee KY et al (1988) The isolation and purification of a specific "protector" protein which inhibits enzyme inactivation by a thiol/Fe(III)/O_2 mixed-function oxidation system. J Biol Chem 263:4704–4711
68. Cha MK, Kim HK, Kim IH et al (1995) Thioredoxin-linked "thiol peroxidase" from periplasmic space of *Escherichia coli*. J Biol Chem 270:28635–28641
69. Baker LM, Poole LB (2003) Catalytic mechanism of thiol peroxidase from *Escherichia coli.* Sulfenic acid formation and overoxidation of essential CYS61. J Biol Chem 278: 9203–9211
70. Choi J, Choi S, Cha MK et al (2003) Crystal structure of *Escherichia coli* thiol peroxidase in the oxidized state: insights into intramolecular disulfide formation and substrate binding in atypical 2-Cys peroxiredoxins. J Biol Chem 278:49478–49486
71. Hall A, Sankaran B, Poole LB et al (2009) Structural changes common to catalysis in the Tpx peroxiredoxin subfamily. J Mol Biol 393:867–881
72. Zhou Y, Wan XY, Wang HL et al (1997) Bacterial scavengase p20 is structurally and functionally related to peroxiredoxins. Biochem Biophys Res Commun 233:848–852
73. Cha MK, Kim W, Lim CJ et al (2004) *Escherichia coli* periplasmic thiol peroxidase acts as lipid hydroperoxide peroxidase and the principal antioxidative function during anaerobic growth. J Biol Chem 279:8769–8778
74. Tao K (2008) Subcellular localization and in vivo oxidation-reduction kinetics of thiol peroxidase in *Escherichia coli*. FEMS Microbiol Lett 289:41–45
75. Atack JM, Harvey P, Jones MA et al (2008) The *Campylobacter jejuni* thiol peroxidases Tpx and Bcp both contribute to aerotolerance and peroxide-mediated stress resistance but have distinct substrate specificities. J Bacteriol 190:5279–5290
76. Comtois SL, Gidley MD, Kelly DJ (2003) Role of the thioredoxin system and the thiol-peroxidases Tpx and Bcp in mediating resistance to oxidative and nitrosative stress in *Helicobacter pylori*. Microbiology 149:121–129
77. La Carbona S, Sauvageot N, Giard JC et al (2007) Comparative study of the physiological roles of three peroxidases (NADH peroxidase, alkyl hydroperoxide reductase and thiol peroxidase) in oxidative stress response, survival inside macrophages and virulence of *Enterococcus faecalis*. Mol Microbiol 66:1148–1163
78. Tartaglia LA, Storz G, Ames BN (1989) Identification and molecular analysis of oxyR-regulated promoters important for the bacterial adaptation to oxidative stress. J Mol Biol 210:709–719

79. Jacobson FS, Morgan RW, Christman MF et al (1989) An alkyl hydroperoxide reductase from *Salmonella typhimurium* involved in the defense of DNA against oxidative damage. Purification and properties. J Biol Chem 264:1488–1496
80. Neidhardt FC, Varghn V, Phillips TA et al (1983) Gene-protein index of *Escherichia coli* K-12. Microbiol Rev 47:231–284
81. Andrews SC, Harrison PM, Guest JR (1991) A molecular analysis of the 53.3 minute region of the *Escherichia coli* linkage map. J Gen Microbiol 137:61–367
82. Clarke DJ, Mackay CL, Campopiano DJ et al (2009) Interrogating the molecular details of the peroxiredoxin activity of the *Escherichia coli* bacterioferritin comigratory protein using high-resolution mass spectrometry. Biochemistry 48:3904–3914
83. Liao SJ, Yang CY, Chin KH et al (2009) Insights into the alkyl peroxide reduction pathway of *Xanthomonas campestris* bacterioferritin comigratory protein from the trapped intermediate-ligand complex structures. J Mol Biol 390:951–966
84. Clarke DJ, Ortega XP, Mackay CL et al (2010) Subdivision of the bacterioferritin comigratory protein family of bacterial peroxiredoxins based on catalytic activity. Biochemistry 49:1319–1330
85. Wang G, Adriana A, Olczak AA et al (2005) Contribution of the *Helicobacter pylori* thiol peroxidase bacterioferritin comigratory protein to oxidative stress resistance and host colonization. Infect Immun 73:378–384
86. Jeong W, Cha MK, Kim IH (2000) Thioredoxin-dependent hydroperoxide peroxidase activity of bacterioferritin comigratory protein (BCP) as a new member of the thiol-specific antioxidant protein (TSA)/alkyl hydroperoxide peroxidase C (AhpC) family. J Biol Chem 275:2924–2930
87. Wang G, Hong Y, Johnson MK et al (2006) Lipid peroxidation as a source of oxidative damage in *Helicobacter pylori*: protective roles of peroxiredoxins. Biochim Biophys Acta 1760:1596–1603
88. Johnson NA, McKenzie RM, Fletcher HM (2011) The *bcp* gene in the *bcp-recA-vimA-vimE-vimF* operon is important in oxidative stress resistance in *Porphyromonas gingivalis* W83. Mol Oral Microbiol 26:62–77
89. Foumier M, Aubert C, Dermoun Z et al (2006) Response of the anaerobe *Desulfovibrio vulgaris* Hildenborough to oxidative conditions: proteome and transcript analysis. Biochimie 88:85–94
90. Lynch RE, Fridovich I (1978) Permeation of the erythrocyte stroma by superoxide radical. J Biol Chem 253:4697–4699
91. Korshunov SS, Imlay JA (2002) A potential role for periplasmic superoxide dismutase in blocking the penetration of external superoxide into the cytosol of Gram-negative bacteria. Mol Microbiol 43:95–106
92. Korshunov S, Imlay JA (2006) Detection and quantification of superoxide formed within the periplasm of *Escherichia coli*. J Bacteriol 188:6326–6334
93. De Groote MA, Granger D, Xu Y et al (1995) Genetic and redox determinants of nitric oxide cytotoxicity in a *Salmonella typhimurium* model. Proc Natl Acad Sci U S A 92:6399–6403
94. Balashova NV, Park DH, Patel JK et al (2007) Interaction between leukotoxin and Cu, Zn superoxide dismutase in *Aggregatibacter actinomycetemcomitans*. Infect Immun 75: 4490–4497
95. Cattoir V, Lemenand O, Avril JL et al (2006) The *sodA* gene as a target for phylogenetic dissection of the genus *Haemophilus* and accurate identification of human clinical isolates. Int J Med Microbiol 296:531–540
96. Zambon JJ (1985) Actinobacillus actinomycetemcomitans in human periodontal disease. J Clin Periodontol 12:1–20
97. Scannapieco FA, Bush RB, Paju S (2003) Periodontal disease as a risk factor for adverse pregnancy outcomes. A systematic review. Ann Periodontol 8:70–78
98. Roos D, van Bruggen R, Meischl C (2003) Oxidative killing of microbes by neutrophiles. Microbes Infect 5:1307–1315

99. Dongari AI, Miyasaki KT (1991) Sensitivity of *Actinobacillus actinomycetemcomitans* and *Haemophyllus aphrophilus* to oxidative killing. Oral Microbiol Immunol 6:363–372
100. Imlay JA (2002) How oxygen damages microbes: oxygen tolerance and obligate anaerobiosis. Adv Microb Physiol 46:111–153
101. Smith AH, Imlay JA, Mackie RI (2003) Increasing the oxidative stress response allows *Escherichia coli* to overcome inhibitory effects of condensed tannins. Appl Environ Microbiol 69:3406–3411
102. Imlay JA (2003) Pathway of oxidative damage. Annu Rev Microbiol 57:395–418
103. Soballe B, Poole RK (2000) Ubiquinone limits oxidative stress in *Escherichia coli*. Microbiology 146:787–796
104. Messner KR, Imlay JA (1999) The identification of primary sites of superoxide and hydrogen peroxide formation in the aerobic respiratory chain and sulfide reductase complex of *Escherichia coli*. J Biol Chem 274:10119–10128
105. Gonzalez-Flecha B, Demple B (1995) Metabolic sources of hydrogen peroxide in aerobically growing *Escherichia coli*. J Biol Chem 270:13681–13687
106. Imlay JA, Fridovich I (1991) Assay of metabolic superoxide production in *Escherichia coli*. J Biol Chem 266:6957–6965
107. Imlay JA (1995) A metabolic enzyme that rapidly produces superoxide, fumarate reductase of *Escherichia coli*. J Biol Chem 270:19767–19777
108. Thomson VJ, Bhattacharjee MK, Fine DH et al (1999) Direct selection of IS*903* transposon insertions by use of broad-host range vector: isolation of catalase-deficient mutants of *Actinobacillus actinomycetemcomitans*. J Bacteriol 181:7298–7307
109. Ellfolk N, Soininen R (1970) *Pseudomonas* cytochrome *c* peroxidase. I. Purification procedure. Acta Chem Scand 24:2126–2136
110. Alves T, Besson S, Duarte LC et al (1999) A cytochreome *c* peroxidase from *Pseudomonas nautica* 617 active at high ionic strength: expression, purification and characterization. Biochim Biophys Acta 1434:248–259
111. Pettigrew GW (1991) The cytochrome *c* peroxidase of *Paracoccus denitrificans*. Biochim Biophys Acta 1058:25–27
112. Hu W, De Smet L, Van Beeumen J et al (1998) Characterization of cytochreome *c*-556 from the purple phototrophic bacterium *Rhodobacter capsulatus* as a cytochrome *c* peroxidase. Eur J Biochem 258:29–36
113. Arciero DM, Hooper AB (1994) A di-heme cytochrome *c* peroxidase from *Nitrosomonas europaea* catalytically active in both the oxidized and half-reduced states. J Biol Chem 269:11878–11886
114. Zahn JA, Arciero DM, Hooper AB et al (1997) Cytochrome *c* peroxidase from *Methylococcus capsulatus* Bath. Arch Microbiol 168:362–372
115. Fulop V, Ridout CJ, Greenwood C et al (1995) Crystal structure of the di-haem cytochrome *c* peroxidase from *Pseudomonas aeruginosa*. Structure 3:1225–1233
116. Dias JM, Alves T, Bonifacio C et al (2004) Structural basis for the mechanism of Ca^{2+} activation of the di-heme cytochrome *c* peroxidase from *Pseudomonas nautica* 617. Structure 12:961–973
117. Shimizu H, Schuller DJ, Lanzilotta WN et al (2001) Crystal structure of *Nitrosomonas europaea* cytochrome *c* peroxidase and the structural basis for ligand switching in bacterial di-heme peroxidases. Biochemistry 40:13483–13490
118. De Smetl L, Savvides SN, Van Horen E et al (2006) Structural and mutagenesis studies on the cytochrome *c* peroxidase from *Rhodobacter capsulatus* provide new insights into structure-function relationship of bacterial di-heme peroxidases. J Biol Chem 281:4371–4379
119. Echalier A, Goodhew CF, Pettigrew GW et al (2006) Activation and catalysis of the di-heme cytochrome *c* peroxidase from *Paracoccus pantotrophus*. Structure 14:107–117
120. Gilmour R, Goodhew CF, Pettigrew GW et al (1994) The kinetics of the oxidation of cytochrome *c* by *Paracoccus* cytochrome *c* peroxidase. Biochem J 300:907–914

121. Hu W, Van Driessche G, Devreese B et al (1997) Structural characterization of *Paracoccus denitrificans* cytochrome c peroxidase and assignment of the low and high potential heme sites. Biochemistry 36:7958–7966
122. McGinnity DF, Devreese B, Prazeres S et al (1996) A single histidine is required for activity of cytochrome *c* peroxidase from *Paracoccus denitrificans*. J Biol Chem 271:11126–11133
123. Samyn B, Van Craenenbroeck K, De Smet L et al (1995) A reinvestigation of the covalent structure of *Pseudomonas aeruginosa* cytochrome *c* peroxidase. FEBS Lett 377:145–149
124. Foote N, Turner R, Brittain T et al (1992) A quantitative model for the mechanism of action of the cytochrome *c* peroxidase of *Pseudomonas aeruginosa*. Biochem J 283:839–843
125. Ellfolk N, Ronnberg M, Osterlund K (1991) Structural and functional features of *Pseudomonas* cytochrome *c* peroxidase. Biochim Biophys Acta 1080:68–77
126. Goodhew CF, Wilson IB, Hunter DJ et al (1990) The cellular location and specificity of bacterial cytochrome *c* peroxidases. Biochem J 271:707–712
127. Foote N, Peterson J, Gadsby PM et al (1985) Redox-linked spin-state changes in the di-haem cytochrome *c*-551 peroxidase from *Pseudomonas aeruginosa*. Biochem J 230:227–237
128. Ellfolk N, Ronnberg M, Aasa R et al (1983) Properties and function of the two hemes in *Pseudomonas* cytochrome *c* peroxidase. Biochim Biophys Acta 743:23–30
129. Ronnberg M, Ellfolk N (1979) Heme-linked properties of *Pseudomonas* cytochrome *c* peroxidase. Evidence for non-equivalence of the hemes. Biochim Biophys Acta 581:325–333
130. Foote N, Peterson J, Gadsby PM et al (1984) A study of the oxidized form of *Pseudomonas aeruginosa* cytochrome *c*-551 peroxidase with the use of magnetic circular dichroism. Biochem J 223:369–378
131. Bradley AL, Chobot SE, Arciero DM et al (2004) A distinctive electrocatalytic response from the cytochrome *c* peroxidase of *Nitrosomonas europaea*. J Biol Chem 279:13297–13300
132. Unden G, Bongaerts J (1997) Alternative respiratory pathways of *Escherichia coli*: energetics and transcriptional regulation in response to electron acceptors. Biochim Biophys Acta 1320:217–234
133. Kita K, Konishi K, Anraku Y (1984) Terminal oxidases of *Escherichia coli* aerobic respiratory chain. I. Purification and properties of cytochrome b_{562}-*o* complex from cells in the early exponential phase of aerobic growth. J Biol Chem 259:3368–3374
134. Yamada H, Takashima E, Konishi K (2007) Molecular characterization of the membrane-bound quinol peroxidase functionally connected to the respiratory chain. FEBS J 274:853–866
135. Gilmour R, Prazeres S, McGinnity DF et al (1995) The affinity and specificity of Ca^{2+}-binding sites of cytochrome *c* peroxidase from *Paracoccus denitrificans*. Eur J Biochem 234:878–886
136. Hirokawa T, Boon-Chieng S, Mitaku S (1998) SOSUI: classification and secondary structure prediction system for membrane proteins. Bioinformatics 14:378–379
137. Kita K, Konishi K, Anraku Y (1984) Terminal oxidases of *Escherichia coli* aerobic respiratory chain. II. Purification and properties of cytochrome b_{558}-*d* complex from cells grown with limited oxygen and evidence of branched electron-carrying systems. J Biol Chem 259:3375–3381
138. Takashima E, Konishi K (2008) Characterization of a quinol peroxidase mutant in *Aggregatibacter actinomycetemcomitans*. FEMS Microbiol Lett 286:66–70
139. Slots J, Ting M (2002) Systemic antibiotics in the treatment of periodontal disease. Periodontol 2000 28:106–176
140. Van Winkelhoff AT, Slots J (1999) *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in nonoral infections. Periodontol 2000 20:122–135
141. Kachlany SC, Fine DH, Figurski DH (2000) Secretion of RTX leukotoxin by *Actinobacillus actinomycetemcomitans*. Infect Immun 68:6094–6100
142. Lally ET, Hill RB, Kieba IR et al (1999) The interaction between RTX toxins and target cells. Trends Microbiol 7:356–361
143. Takashima E, Yamada H, Konishi K (2007) Bacterial multi-heme peroxidase. In: Mohan RM (ed) Research advances in biochemistry, vol 1. Global Research Network, Kerala, pp 21–27

144. Herren CD, Rocha ER, Smith CJ (2003) Genetic analysis of an important oxidative stress locus in the anaerobe *Bacteroides fragilis*. Gene 316:167–175
145. Partridge JD, Poole RK, Green J (2007) The *Escherichia coli yhjA* gene, encoding a predicted cytochrome *c* peroxidase, is regulated by FNR and OxyR. Microbiology 153:1499–1507
146. Charoensuk K, Irie A, Lertwattanasakul N et al (2011) Physiological importance of cytochrome *c* peroxidase in ethanologenic thermotoletant *Zymomonas mobilis*. J Mol Microbiol Biotechnol 20:70–82
147. Poole LB (2005) Bacterial defenses against oxidants: mechanistic features of cysteine-based peroxidases and their flavoprotein reductases. Arch Biochem Biophys 433:240–254
148. Takashima E, Yamada H, Yajima A et al (2010) A quinol peroxidase inhibitor prevents secretion of a leukotoxin from *Aggregatibacter actinomycetemcomitans*. J Periodontal Res 45:123–128
149. Ui H, Ishiyama A, Sekiguchi H et al (2007) Selective and potent in vitro antimalarial activities found in four microbial metabolites. J Antibiot (Tokyo) 60:220–222
150. Minagawa N, Yabu Y, Kita K et al (1997) An antibiotic, ascofuranone, specifically inhibits respiration and in vitro growth of long slender bloodstream forms of *Trypanosoma brucei brucei*. Mol Biochem Parasitol 84:271–280
151. Yabu Y, Yoshida A, Suzuki T et al (2003) The efficacy of ascofuranone in a consecutive treatment on *Trypanosoma brucei brucei* in mice. Parasitol Int 52:155–164
152. Toledano MB, Kullik I, Trinh F et al (1994) Redox-dependent shift of OxyR-DNA contacts along an extended DNA-binding site: a mechanism for differential promoter selection. Cell 78:897–909
153. Haubek D, Ennibi OK, Abdellaoui L et al (2002) Attachment loss in Moroccan early onset periodontitis patients and infection with the JP2-type of *Actinobacillus actinomycetemcomitans*. J Clin Periodontol 29:657–660
154. Cho HJ, Kang JH, Kwak JY et al (2007) Ascofuranone suppresses PMA-mediated matrix metalloproteinase-9 gene activation through the Ras/Raf/MEK/ERK and Ap1-dependent mechanisms. Carcinogenesis 28:1104–1110
155. Yabu Y, Minagawa N, Kita K et al (1998) Oral and intraperitoneal treatment of *Trypanosoma brucei brucei* with a combination of ascofuranone and glycerol, in mice. Parasitol Int 47: 131–137

Part II
Oxidative Stress in Periodontal Diseases (Clinical Studies)

Chapter 9
Periodontitis and Oxidative Stress: Human Studies

Luigi Nibali, Brian Henderson, Giovanni Li Volti, and Nikos Donos

9.1 Introduction

Periodontal diseases (PD) are common inflammatory diseases of the supporting apparatus of the teeth. Clinically, two main forms of periodontitis are differentially diagnosed by the rapidity of progression and other contributing factors. While aggressive periodontitis (AgP) affects healthy individuals, is associated with a positive family history of the condition and has a rapid progression [1, 2], chronic periodontitis (CP) usually affects adults, may be associated with other predisposing systemic conditions and shows a slow progression of periodontal attachment and alveolar bone loss [3].

The mechanisms linking periodontal disease pathogenesis and oxidative stress have been thoroughly discussed in previous chapters. This chapter will discuss the so far poorly researched role of redox proteins in periodontitis and will review the clinical evidence for changes in the redox balance in periodontal diseases, with an emphasis on measures of oxidative stress and oxidative damage, both locally in the periodontium and systemically. Published studies on this topic will be critically appraised and a set of overall conclusions and suggested future research developments will be proposed.

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9.2 Oxidative and Non-oxidative Actions of Redox Proteins

A growing number of proteins are being discovered which control redox events within the cell and its environments, and are now well recognized for their role in systemic pathology in conditions such as cardiovascular diseases [4]. These proteins are generally members of the thioredoxin superfamily and include proteins such as the thioredoxins, glutaredoxins, peroxiredoxins and protein disulfide isomerases (PDIs) [5] and also proteins associated with the function of these redox proteins [6]. Human thioredoxin-1 (Trx-1) is part of a system of proteins which include a truncated form of Trx termed Trx80, thioredoxin reductase (TrxR) and a natural inhibitory protein known as thioredoxin-interacting protein (TXNIP). It is now recognized that this system is essential for maintaining cellular redox status and redox signaling and is linked to many aspects of normal cell and organismal control including apoptosis, growth promotion, and control of inflammation and immunity. Over the past 20 years, evidence has accumulated to support the hypothesis that mis-regulation of this thioredoxin signaling is involved in a variety of cardiovascular conditions including the sequelae of atherosclerosis, heart failure, immune/inflammatory conditions, metabolic syndrome, arthritis, cancer, and neurodegenerative disease [7]. However, also interaction with other members of the thioredoxin superfamily including the PDIs [8], peroxiredoxins [9], and glutaredoxins [10] is implicated in controlling tissue homeostasis in health and disease. In addition to functioning as redox proteins, the proteins mentioned also play roles in the intracellular folding of proteins and come under the heading of protein-folding catalysts which have actions similar to those of molecular chaperones [11]. It is important to appreciate that in addition to having a redox role, a growing number of the proteins described above are also being found to have the capacity to exhibit more than one unique biological activity, defined as protein moonlighting activity. For example, human thioredoxin was actually discovered as a cytokine which stimulated T lymphocytes to synthesize interleukin (IL)-2 [12]. Indeed, human Trx-1 has a wide range of biological functions and there is now substantial animal model data to suggest that this protein, if administered to animals with various human disease states, can have potent therapeutic activity [13, 14]. There is now good evidence for the hypothesis that many molecular chaperones and protein-folding catalysts have moonlighting actions which are relevant to human disease conditions. Indeed, these proteins can be divided into those that have pathological properties and those that have therapeutic actions and a number of molecular chaperones are currently in clinical trial for various human diseases [15].

In periodontitis, both host and microbial redox proteins could contribute to tissue pathology. It is established that a small number of molecular chaperones in a large number of bacteria can act as virulence factors with key roles in a range of human infections [16]. At the time of writing it is recognized that bacterial molecular chaperones can contribute to tissue pathology in periodontitis—largely through immunological cross-reactivity [17]. However, to date very little attention has been paid to this group of redox proteins in periodontitis. The small literature on this area is

largely devoted to these redox proteins from the bacteria implicated in periodontitis, such as antioxidant protein (AOP)2, also known as peroxiredoxin 5, which was suspected to be predisposing to atherosclerosis in the mouse [18] and which is differentially expressed in response to oxidative stress [19]. This protein is one of a small number of epithelial cell cross-reactive autoantigens (cross-reacting with periodontal bacteria) in patients with periodontitis [20]. Other workers have found the thiol peroxidase of periodontopathogen *Porphyromonas gingivalis* a key T cell immunogen in mice with periapical lesions induced by this organism [21]. The 35 kDa hemin-binding protein of *P. gingivalis* (HBP35) is a member of the thioredoxin family of proteins and has been shown to have a growth promoting activity and an ability to monitor the organism redox state. Of interest, a monoclonal antibody to this protein can inhibit bacterial growth [22]. There is also evidence for the peroxiredoxin of periodontopathogen *Aggregatibacter actinomycetemcomitans* as a virulence factor for this bacterium [23].

A possible role for redox proteins in periodontitis can be deduced from the finding that thioredoxin transfection into the mouse monocyte cell line RAW264.7 accentuates the formation of osteoclasts from these cells. In contrast, transfection with glutathione peroxidase-1 or peroxiredoxin-1 inhibited osteoclast formation [24]. This suggests that osteoclast formation, a key parameter in periodontal bone loss, could be controlled by the state of the osteoclast's redox environment and the redox proteins it produces. Thus one of the future directions that research into redox status in periodontitis needs to take is to pay more attention to the redox and non-redox actions of the large number of redox proteins that are involved in controlling cellular homeostasis and are clearly intimately involved in inflammation and immunity [25].

9.3 Measures of Oxidative Stress

As recently reviewed [26], three subgroups of oxidative stress measures associated with periodontitis can be identified: (1) free radicals/oxidants, (2) antioxidants, and (3) measures of oxidative damage.

9.3.1 Redox Balance (Oxidants/Antioxidants Balance)

Different methods have been employed for measuring free radicals in biological fluids and tissues, including UV–visible spectroscopy (colorimetry), fluorescence, chemiluminescence, and electron paramagnetic resonance (EPR) spectroscopy. However, direct measurement of free radical levels is difficult because of their short half-life [27]. In the periodontal literature, free radicals have mainly been measured as: (1) Diacron Reactive Oxygen Metabolites (D-ROM), using an assay [28–30] which measures whole oxidant capacity of serum against

N,*N-diethylparaphenylendiamine* in acidic buffer using a spectrophotometer (this evaluates the ability of transition metals to catalyze, in the presence of peroxides, the formation of free radicals, which are trapped by an alkylamine) [31–33]. The validity of this method has been under question, as the alkylamine is also a substrate for the ceruloplasmin oxidase, whose activity can account for the results observed [34]; (2) total oxidant status (TOS) [35], using oxidation of ferric ion to ferrous ion and the measurement of ferric ion by xylenol orange (colorimetric) [36–38].

Total antioxidant levels can be measured in the plasma, usually by the decolorization technique, where the radical cation 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) is generated by reaction with potassium persulfate. In the periodontal literature, total antioxidant capacity (TAOC or TAC) has been measured: (1) based on reduction of ABTS cation by antioxidants [37, 39–42]; (2) by inhibition of ABTS oxidation by metmyoglobin [43]; (3) plasma biological antioxidant potential, testing agents able to reduce the iron from its ferric to ferrous form measured with the BAP test [44, 45] or with the ferric reducing antioxidant power (FRAP) assay [46, 47]; (4) enhanced chemiluminescent (ECL) assay [48] based upon the inhibition by a test plasma sample of light output generated by the horseradish-peroxidase catalyzed oxidation of luminal, depending on the constant production of free radical intermediates and therefore sensitive to interference by scavenging water-soluble antioxidants [49–51]. Further antioxidant measures have been reported in the periodontal literature such as: superoxide dismutase (SOD) measured by reduction of nitroblue tetrazolium (NBT) by xanthine–xanthine oxidase system [41, 52–54]; catalase activity by measuring the decay of peroxide solution [55, 56]; glutathione (GSH) by spectrophotometric assay [57] or by high performance liquid chromatography (HPLC) using a fluorometric detector after derivatization with dansyl chloride [58, 59]; glutathione peroxidase (GPx) through the glutathione/NADPH/glutathione reductase system by the dismutation of *tert-butyl* hydroperoxide [56]. One study [60] reported total blood antioxidant capacity, taking into account also the antioxidant capacity of erythrocytes, measured with the NBT test [61]. It is worth to notice that while both serum and plasma measurements have been reported, serum is claimed to be more prone to oxidation, therefore plasma redox measures are thought to be more reliable [60].

9.3.1.1 Clinical Studies on Redox Balance in Periodontitis

Local (Periodontal) Redox Balance

Local levels of oxidative stress markers have been measured in saliva or in gingival crevicular fluid (GCF), which is a transudate resulting from an increase in the permeability of the vessels underlying junctional and sulcular epithelium or derived by a gradient between a capillary filtrate and lymphatic uptake [62]. A Turkish group collected GCF and gingival tissue samples in 26 patients with chronic periodontitis and 18 healthy controls and measured SOD activities spectrophotometrically. They found similar levels of SOD in the GCF, but increased SOD activity in the gingival

tissues in periodontitis patients, suggesting this might be due to increased need for antioxidant protection during disease [63]. Reductions in TAOC and SOD in the GCF were observed in another sample of chronic periodontitis patients compared to healthy controls by the same group [41]. Comparing both the saliva and GCF of pregnant women with periodontitis, compared to non-pregnant periodontitis patients and healthy subjects, similar reductions were observed, particularly pronounced in the third trimester of pregnancy [39]. The same investigators observed higher TOS measured by a colorimetric method based on the oxidation of ferrous iron to ferric iron in a separate cohort [36]. Reductions in TAOC from the above-mentioned studies are consistent with what was observed in studies on UK patients [49, 50], but they are in contrast with an increase in TAOC reported in the saliva of periodontitis patients by ABTS assay by Su et al. [43]. In a cross-sectional study on 120 individuals ranging from periodontal health to advanced periodontitis, lower antioxidant levels were detected in the GCF of patients with worse clinical periodontal scores [47]. Esen et al. [37] found higher TOS and oxidative stress index (balance between free radicals and antioxidants) in the GCF of patients with chronic periodontitis and rheumatoid arthritis. An abnormal glutathione-redox balance, with a potential effect in regulating cellular immune responses, was detected in the GCF of 20 non-smoking periodontitis patients [59]. Glutathione peroxidase (eGPx) levels were investigated in the GCF of ten healthy, ten gingivitis and ten periodontitis patients and were found to increase according to periodontal disease severity [64].

Systemic Redox Balance

The local shift in the redox balance in the inflamed periodontium may have an effect on systemic redox balance. In addition to a series of studies in animal models [65, 66], which have been covered in an earlier chapter, human epidemiological studies have also reported association between parameters of oxidative stress and presence of periodontitis.

Several of the above-described methods for the measurement of circulating oxygen species have been used for comparing oxidative stress measures between periodontitis and healthy controls. Serum TOS was found to be higher in periodontitis patients compared to healthy individuals in two separate studies [36, 54]. An association was observed between reactive oxygen metabolites and presence of periodontitis in Japanese populations [31] and in a large cohort of severe periodontitis patients including CP and AgP, compared to healthy subjects [45, 67]. Smokers were included in these studies, with the potential residual confounding despite adjustment in the analysis.

In a Syrian sample of 30 non-smoking chronic periodontitis, plasma TAOC levels measured with the ABTS assay were found to be lower than in the same number of healthy controls [40]. TAC measured as serum concentrations of mainly uric acid, vitamin A, vitamin C, and vitamin E, was found to be inversely associated with the presence of periodontitis when data from the large NHANES III survey on 11,480 U.S. adults were examined [68]. The same group, in a smaller cross-sectional

study, had observed no difference in plasma TAOC between chronic periodontitis and healthy subjects as measured by enhanced chemiluminescence [50]. Systemic reductions in TAOC were also detected in the above-mentioned studies by Masi et al. [45] and Baltacioglu et al. [41]. Using the same assay, TAC (renamed small molecule antioxidant capacity) was found to be lower in the plasma of diabetic patients with periodontitis compared to diabetic patients with healthy periodontia [51]. On the other hand, the antioxidant capacity of venous blood measured by reduction of NBT test was found to be higher in periodontitis patients compared to healthy subjects [60]. Serum SOD levels measured by the reduction of NBT by xanthine–xanthine oxidase system, were found to be higher in periodontitis patients compared to healthy individuals [54], in contrast with a Turkish study [41].

9.4 Oxidative Stress Fingerprints

Consequences of oxidative stress include cell adaptation, damage, or death [69]. Efforts in periodontal research have recently moved towards attempting to estimate DNA damage due to oxidative stress. Owing to the difficulty in reliably assessing total oxidative stress [27, 69], measures of oxidant damage have acquired an important role in the epidemiological research in this field. Oxidative stress leaves measurable "fingerprints" in the form of damage to several molecules such as carbohydrates, lipids, proteins, and DNA. Table 9.1 reports a summary of measures of oxidative stress damage in periodontal research.

Table 9.1 Summary of measures of oxidative stress damage in periodontal research, divided by protein, lipid, and DNA damage

	Oxidative stress damage	Measurable compounds/assays used in the periodontal literature
Proteins	• Folding or unfolding • Fragmentation and polymerization reactions • Degradation • Formation of protein radicals, protein-bound ROS • Formation of carbonyl compounds	• Protein carbonyl assay
Lipids	• Oxidation • Nitration • Chlorination	• Oxidized LDL • Lipid peroxidation (TBARS assay) • Malondialdehyde • F-2 isoprostanes • Serum-8 isoprostanes
DNA	• Mutations • Insertions • Deletions • Conversion of guanine to 8-hydroxyguanine • Strand breaks	• 8-OHdG • Leukocyte telomere lengths

9.4.1 Lipid Oxidative Fingerprints

Lipids can be oxidized, chlorinated, and nitrated by reactive oxygen species. In the periodontal literature, the most frequently measured lipid oxidative stress damage markers are (1) oxidized LDL, (2) colorimetric assay for measurement of levels of thiobarbituric acid reactive substances (TBARS) to estimate lipid peroxidation [70, 71], (3) serum 8-isoprostane, and (4) malondialdehyde (MDA), an end product of lipid peroxidation by ROS, measured by HPLC [72]. In this assay, samples are reacted with thiobarbituric acid (TBA) by heating under acidic conditions. Preformed MDA in the sample reacts with TBA to form a TBA–MDA adduct consisting of two molecules of TBA and one of MDA [36, 54].

Oxidized LDL (oxLDL) levels and antibodies against oxidized LDL in blood were found to be higher in periodontitis patients than in healthy controls in two separate investigations [33, 73]. Levels of MDA were found to be higher in periodontitis cases [54], while in another study they did not significantly differ between periodontitis and healthy subjects but showed a positive correlation with periodontal parameters in periodontitis patients [36]. MDA in plasma was found to be associated with severity of periodontal disease in 120 Brazilian subjects including diabetics and healthy individuals [74]. Among other lipid peroxidation markers, serum 8-isoprostane was found to be associated with periodontal disease severity and with decreased IgG antibodies to oral bacteria in 4,717 participants in the ARIC survey [75]. The same association was observed examining data from the Oral Conditions and Pregnancy study in North Carolina including 791 pregnant women [76]. In this study, moderate to severe periodontal disease was associated with an elevated serum 8-isoprostane level (O.R. 2.9 after adjustments). The authors suggested that maternal periodontal disease is associated with oxidative stress during pregnancy. TBARS levels were found to be higher in periodontitis patients, suggesting higher lipid peroxidation processes, associated with higher enzymatic antioxidant activities and lower levels of non-enzymatic antioxidants [71]. When oxidative stress markers were analyzed in gingival biopsies from periodontitis patients and healthy subjects [56], individuals with periodontal disease exhibited a significant increase in the activities of myeloperoxidase activity (MPO), glutathione peroxidase (GPx), glutathione S-transferase (GST), and TBARS and GSSG levels, indicating disturbances in the endogenous antioxidant defense system due to the over-production of lipid peroxidation products. Other reliable markers of lipid peroxidation such as F2-isoprostanes may be used to assess oxidative stress in various biological fluids. The measurement of F2-isoprostanes by methods utilizing mass spectrometry is widely regarded as the best currently available biomarker of lipid peroxidation. F2-isoprostanes and their metabolites can be measured accurately in plasma, urine, and other body fluids using mass spectrometric techniques [77]. The importance of such biomarkers is underscored by a previous study demonstrating that salivary F2-isoprostanes can reliably assess the degree of oxidative stress and suggesting that isoprostanes might be increased in the saliva of periodontitis patients, especially in smokers [78].

9.4.2 Protein Oxidative Fingerprints

Oxygen metabolites can cause protein folding or unfolding, fragmentation and polymerization reactions, degradation, formation of protein radicals, protein-bound ROS, and formation of carbonyl compounds [79]. The most stable and measurable, although not very specific, markers of protein oxidative damage are the carbonyl compounds. The carbonyl assay measures carbonyls generated as a result of protein glycation by sugars, by the binding of aldehydes to proteins and by the direct oxidation of amino-acid chains [69]. Protein carbonyl assays have been used in the periodontal literature to measure protein oxidative damage [47, 51, 80] and were found to be increased in the GCF of periodontitis patients [43, 47]. Protein carbonyl levels were also found to be higher in periodontitis cases compared with healthy controls [41, 47, 51].

9.4.3 DNA Damage Caused by Oxidative Stress

DNA subjected to attack by free radicals generates base and sugar modification products [81], ranging from mutations, insertions, deletions, conversion of guanine to 8-hydroxyguanine, and strand breaks. One report exists of mitochondrial DNA damage (accumulation of DNA mutations) in human gingival tissues possibly due to oxidative stress [82]. 8-Hydroxy-2′-deoxyguanosine (8-OHdG, a marker of DNA damage derived from conversion of guanine to 8-hydroxyguanine) levels can be measured by enzyme linked immunosorbent assay (ELISA) [83, 84]. 8-Hydroxydeoxyguanosine was found to be higher in samples from subjects with chronic periodontitis compared with periodontally healthy controls in saliva, but no data on serum levels were reported [83, 85]. Another study by Konopka et al. [42] analyzed oxidative stress markers by puncture and blood collection from a gingival papilla and found increased 8-OHdG in aggressive and chronic periodontitis. This measure of DNA oxidative damage in GCF was consistently higher also in studies from Japanese populations [43, 83, 84].

Telomeres are repetitive nucleoprotein complexes at the ends of chromosomes, with a “capping role” which protects chromosomes from degradation. Telomere ends become shorter following each cell division, representing a sort of cellular clock which will eventually signal senescence and apoptosis [86]. Telomere lengths are affected by number of cell divisions over a life-span but also by cumulative exposure to inflammatory and oxidative stressors [87, 88]. Epidemiological studies have identified associations between telomere lengths and cardiovascular disease [89], cancer [90, 91], and diabetes [92]. However, whether telomere shortening is cause or consequence of aging and chronic disease is still not clear. Leukocytes telomere lengths have been investigated in case–control studies involving periodontitis cases. The first report was published by Takahashi et al. [93], who studied by

Southern blotting telomere lengths of genomic DNA extracted peripheral leukocytes in 21 aggressive periodontitis (localized and generalized) and 50 healthy individuals. No telomere length reduction was detected in aggressive periodontitis subjects in this study compared with healthy subjects, rejecting the hypothesis of premature cellular aging in this group of patients. A similar case–control study from our group included 356 periodontitis patients and 207 healthy individuals [45]. In these subjects, the ratio of telomere repeats to single-copy gene (SCG) copies was obtained by PCR. A significant reduction in telomere length was detected in patients with CP (but not AgP) independent of age, gender, ethnicity, and smoking differences. The difference between cases and controls for LTL was estimated to be 641 base pairs. LTL showed a reverse association with total levels of reactive oxidative metabolites and severity of periodontitis. The lack of association between AgP and telomere length is in agreement with the above-mentioned Japanese study [93], and suggests that such association may become evident only with the presence of disease over a longer period of time in adulthood. However, the association with chronic periodontitis suggests that exposure to chronic periodontal inflammation and consequent oxidative stress may leave irreversible fingerprints in the form of reduced telomere length, which is in turn associated with higher mortality rates and shorter life-span, as observed in epidemiological studies in periodontitis cases [94].

9.5 Summary

Overall, the evidence from the literature suggests an increase in reactive oxygen metabolites measured in GCF, saliva, and serum of periodontitis patients compared with healthy controls. The magnitude of the elevation in oxidative stress markers is more pronounced locally than systemically, confirming that the source of production of oxygen metabolites is mainly from defensive cells in the periodontal lesion. Antioxidant levels have consistently been found to be low in periodontitis cases, with a clear shift in the redox balance both locally and systemically. Furthermore, studies have almost unanimously reported an increase in oxidative stress fingerprints, measured as damage to proteins, lipids, and DNA in periodontitis cases both locally (gingival tissues, GCF) and systemically (plasma). Generally most preliminary treatment studies point towards a re-balancing of the local and systemic redox balance in periodontitis cases following successful non-surgical therapy. Collectively, these observations lead to the concept that the local microbial trigger leads to an excessive local production of oxygen metabolites associated with systemic propagation of the redox response. However, the limitations of these studies include the low reliability of some of the tests used [69], the inclusion of smokers in some studies with a high chance of residual confounding effects (even despite adjustments), usually small sample sizes and unknown blind status of the personnel dedicated to the laboratory analyses, which could have introduced bias.

9.6 Conclusions and Future Steps

The phagocytic response to oral bacteria leads to a shift in the redox balance, with an excessive local production of reactive oxygen metabolites and a depletion of the antioxidant capacity, measurable in the GCF. As well as contributing to tissue damage in periodontal diseases, this is associated with systemic redox balance modifications and with oxidative stress fingerprints systemically, such as reduced leukocyte telomere lengths. The redox balance in periodontitis cases appears to follow the same pattern and kinetics of the associated inflammatory response. Future studies should aim to clarify mechanisms for redox balance alterations locally and systemically in periodontal disease and to identify whether interventions directed to specifically affect the redox balance could bring significant clinical benefits.

References

1. Lang NP, Bartold M, Cullinan M, Jeffcoat M, Mombelli A, Murakami S (1999) Consensus report: aggressive periodontitis. Ann Periodontol 4:53
2. Nibali L, Donos N, Brett PM, Parkar M, Ellinas T, Llorente M et al (2008) A familial analysis of aggressive periodontitis – clinical and genetic findings. J Periodontal Res 43(6):627–634
3. Lindhe J, Lamster RI, Charles A, Chung CP, Flemmig T, Kinane D, Listgarten M, Löe H, Schoor R, Seymour G, Somerman M (1999) Consensus report: chronic periodontitis. Ann Periodontol 4:38
4. Ahsan MK, Lekli I, Ray D, Yodoi J, Das DK (2009) Redox regulation of cell survival by the thioredoxin superfamily: an implication of redox gene therapy in the heart. Antioxid Redox Signal 11(11):2741–2758
5. Pedone E, Limauro D, D'Ambrosio K, De Simone G, Bartolucci S (2010) Multiple catalytically active thioredoxin folds: a winning strategy for many functions. Cell Mol Life Sci 67(22):3797–3814
6. Maulik N, Das DK (2008) Emerging potential of thioredoxin and thioredoxin interacting proteins in various disease conditions. Biochim Biophys Acta 1780(11):1368–1382
7. Mahmood DF, Abderrazak A, El Hadri K, Simmet T, Rouis M (2013) The thioredoxin system as a therapeutic target in human health and disease. Antioxid Redox Signal 19:1266–1303
8. Laurindo FR, Pescatore LA, Fernandes DC (2012) Protein disulfide isomerase in redox cell signaling and homeostasis. Free Radic Biol Med 52(9):1954–1969
9. Yamada S, Ding Y, Sasaguri Y (2012) Peroxiredoxin 4: critical roles in inflammatory diseases. J UOEH 34(1):27–39
10. Lillig CH, Berndt C, Holmgren A (2008) Glutaredoxin systems. Biochim Biophys Acta 1780(11):1304–1317
11. Wallis AK, Freedman RB (2013) Assisting oxidative protein folding: how do protein disulphide-isomerases couple conformational and chemical processes in protein folding? Top Curr Chem 328:1–34
12. Tagaya Y, Maeda Y, Mitsui A, Kondo N, Matsui H, Hamuro J, Brown N, Arai K, Yokota T, Wakasugi H et al (1989) ATL-derived factor (ADF), an IL-2 receptor/Tac inducer homologous to thioredoxin; possible involvement of dithiol-reduction in the IL-2 receptor induction. EMBO J 8(3):757–764
13. Holmgren A, Lu J (2010) Thioredoxin and thioredoxin reductase: current research with special reference to human disease. Biochem Biophys Res Commun 396(1):120–124

14. Matsushima S, Zablocki D, Sadoshima J (2011) Application of recombinant thioredoxin1 for treatment of heart disease. J Mol Cell Cardiol 51(4):570–573
15. Henderson B, Pockley AG (2010) Molecular chaperones and protein-folding catalysts as intercellular signaling regulators in immunity and inflammation. J Leukoc Biol 88(3):445–462
16. Henderson B, Martin A (2011) Bacterial virulence in the moonlight: multitasking bacterial moonlighting proteins are virulence determinants in infectious disease. Infect Immun 79(9):3476–3491
17. Goulhen F, Grenier D, Mayrand D (2003) Oral microbial heat-shock proteins and their potential contributions to infections. Crit Rev Oral Biol Med 14(6):399–412
18. Phelan SA, Beier DR, Higgins DC, Paigen B (2002) Confirmation and high resolution mapping of an atherosclerosis susceptibility gene in mice on Chromosome 1. Mamm Genome 13(10):548–553
19. Sparling NE, Phelan SA (2003) Identification of multiple transcripts for antioxidant protein 2 (Aop2): differential regulation by oxidative stress and growth factors. Redox Rep 8(2):87–94
20. Ye P, Simonian M, Nadkarni MA, Decarlo AA, Chapple CC, Hunter N (2005) Identification of epithelial auto-antigens associated with periodontal disease. Clin Exp Immunol 139(2): 328–337
21. Gonçalves RB, Leshem O, Bernards K, Webb JR, Stashenko PP, Campos-Neto A (2006) T-cell expression cloning of *Porphyromonas gingivalis* genes coding for T helper-biased immune responses during infection. Infect Immun 74(7):3958–3966
22. Shibata Y, Okano S, Shiroza T, Tahara T, Nakazawa K, Kataoka S, Ishida I, Kobayashi T, Yoshie H, Abiko Y (2011) Characterization of human-type monoclonal antibodies against reduced form of hemin binding protein 35 from *Porphyromonas gingivalis*. J Periodontal Res 46(6):673–681
23. Maeda T, Maeda H, Yamabe K, Mineshiba J, Tanimoto I, Yamamoto T, Naruishi K, Kokeguchi S, Takashiba S (2010) Highly expressed genes in a rough-colony-forming phenotype of *Aggregatibacter actinomycetemcomitans*: implication of a mip-like gene for the invasion of host tissue. FEMS Immunol Med Microbiol 58(2):226–236
24. Lean HJ, Kirstein B, Urry Z, Chambers T, Fuller K (2004) Thioredoxin-1 mediates osteoclast stimulation by reactive oxygen species. Biochem Biophys Res Commun 321:845–850
25. Ishikawa S, Kuno A, Tanno M, Miki T, Kouzu H, Itoh T, Sato T, Sunaga D, Murase H, Miura T (2012) Role of connexin-43 in protective PI3K-Akt-GSK-3β signaling in cardiomyocytes. Am J Physiol Heart Circ Physiol 302:H2536–H2544
26. Nibali L, Donos N (2013) Periodontitis and redox status: a review. Curr Pharm Des 19: 2687–2697
27. Buico A, Cassino C, Ravera M, Betta PG, Osella D (2009) Oxidative stress and total antioxidant capacity in human plasma. Redox Rep 14(3):125–131
28. Parmigiani S, Payer C, Massari A, Bussolati G, Bevilacqua G (2000) Normal values of reactive oxygen metabolites on the cord-blood of full-term infants with a colorimetric method. Acta Biomed Ateneo Parmense 71(1–2):59–64
29. Carratelli M, Porcaro L, Ruscica M, De Simone E, Bertelli AA, Corsi MM (2001) Reactive oxygen metabolites and prooxidant status in children with Down's syndrome. Int J Clin Pharmacol Res 21(2):79–84
30. Iamele L, Fiocchi R, Vernocchi A (2002) Evaluation of an automated spectrophotometric assay for reactive oxygen metabolites in serum. Clin Chem Lab Med 40(7):673–676
31. Tamaki N, Tomofuji T, Maruyama T, Ekuni D, Yamanaka R, Takeuchi N et al (2008) Relationship between periodontal condition and plasma reactive oxygen metabolites in patients in the maintenance phase of periodontal treatment. J Periodontol 79(11):2136–2142
32. Tamaki N, Tomofuji T, Ekuni D, Yamanaka R, Yamamoto T, Morita M (2009) Short-term effects of non-surgical periodontal treatment on plasma level of reactive oxygen metabolites in patients with chronic periodontitis. J Periodontol 80(6):901–906
33. Tamaki N, Tomofuji T, Ekuni D, Yamanaka R, Morita M (2011) Periodontal treatment decreases plasma oxidized LDL level and oxidative stress. Clin Oral Investig 15(6):953–958

34. Harma MI, Harma M, Erel O (2006) D-ROM test detects ceruloplasmin, not oxidative stress. Chest 130(4):1276
35. Erel O (2005) A new automated colorimetric method for measuring total oxidant status. Clin Biochem 38(12):1103–1111
36. Akalin FA, Baltacioglu E, Alver A, Karabulut E (2007) Lipid peroxidation levels and total oxidant status in serum, saliva and gingival crevicular fluid in patients with chronic periodontitis. J Clin Periodontol 34(7):558–565
37. Esen C, Alkan BA, Kirnap M, Akgül O, Işıkoğlu S, Erel O (2012) The effects of chronic periodontitis and rheumatoid arthritis on serum and gingival crevicular fluid total antioxidant/oxidant status and oxidative stress index. J Periodontol 83(6):773–779
38. Wei D, Zhang XL, Wang YZ, Yang CX, Chen G (2010) Lipid peroxidation levels, total oxidant status and superoxide dismutase in serum, saliva and gingival crevicular fluid in chronic periodontitis patients before and after periodontal therapy. Aust Dent J 55(1):70–78
39. Akalin FA, Baltacioğlu E, Alver A, Karabulut E (2009) Total antioxidant capacity and superoxide dismutase activity levels in serum and gingival crevicular fluid in pregnant women with chronic periodontitis. J Periodontol 80(3):457–467
40. Abou Sulaiman AE, Shehadeh RMH (2010) Assessment of total antioxidant capacity and the use of vitamin c in the treatment of non-smokers with chronic periodontitis. J Periodontol 81(11):1547–1554
41. Baltacioglu E, Akalin FA, Alver A, Balaban F, Unsal M, Karabulut E (2006) Total antioxidant capacity and superoxide dismutase activity levels in serum and gingival crevicular fluid in post-menopausal women with chronic periodontitis. J Clin Periodontol 33(6):385–392
42. Konopka T, Krol K, Kopec W, Gerber H (2007) Total antioxidant status and 8-hydroxy-2′-deoxyguanosine levels in gingival and peripheral blood of periodontitis patients. Arch Immunol Ther Exp (Warsz) 55(6):417–422
43. Su HX, Gornitsky M, Velly AM, Yu HL, Benarroch M, Schipper HM (2009) Salivary DNA, lipid, and protein oxidation in nonsmokers with periodontal disease. Free Radic Biol Med 46(7):914–921
44. Komatsu F, Kagawa Y, Ishiguro K, Kawabata T, Purvee B, Otgon J et al (2009) The association of very high hair manganese accumulation and high oxidative stress in Mongolian people. Curr Aging Sci 2:28–42
45. Masi S, Salpea KD, Li KW, Parkar M, Nibali L, Donos N et al (2011) Oxidative stress, chronic inflammation, and telomere length in patients with periodontitis. Free Radic Biol Med 50(6):730–735
46. Benzie IFF, Strain JJ (1996) The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal Biochem 239(1):70–76
47. Sculley DV, Langley-Evans SC (2003) Periodontal disease is associated with lower antioxidant capacity in whole saliva and evidence of increased protein oxidation. Clin Sci 105(2):167–172
48. Chapple ILC, Mason GI, Garner I, Matthews JB, Thorpe GH, Maxwell SRJ et al (1997) Enhanced chemiluminescent assay for measuring the total antioxidant capacity of serum, saliva and crevicular fluid. Ann Clin Biochem 34:412–421
49. Brock GR, Butterworth CJ, Matthews JB, Chapple ILC (2004) Local and systemic total antioxidant capacity in periodontitis and health. J Clin Periodontol 31(7):515–521
50. Chapple ILC, Brock GR, Milward MR, Ling N, Matthews JB (2007) Compromised GCF total antioxidant capacity in periodontitis: cause or effect? J Clin Periodontol 34(2):103–110
51. Allen EM, Matthews JB, O'Halloran DJ, Griffiths HR, Chapple ILC (2011) Oxidative and inflammatory status in type 2 diabetes patients with periodontitis. J Clin Periodontol 38(10):894–901
52. Sun Y, Oberley LW, Li Y (1988) A Simple method for clinical assay of superoxide-dismutase. Clin Chem 34(3):497–500
53. Akalin FA, Isiksal E, Baltacioglu E, Renda N, Karabulut E (2008) Superoxide dismutase activity in gingiva in type-2 diabetes mellitus patients with chronic periodontitis. Arch Oral Biol 53(1):44–52

54. Wei PF, Ho KY, Ho YP, Wu YM, Yang YH, Tsai CC (2004) The investigation of glutathione peroxidase, lactoferrin, myeloperoxidase and interleukin-1 beta in gingival crevicular fluid: implications for oxidative stress in human periodontal diseases. J Periodontal Res 39(5):287–293
55. Aebi H (1984) Catalase in vitro. Methods Enzymol 105:121–126
56. Borges I, Moreira EAM, Wilhem D, de Oliveira TB, da Silva MBS, Froede TS (2007) Proinflammatory and oxidative stress markers in patients with periodontal disease. Mediators Inflamm 2007:45794
57. Tsai CC, Chen HS, Chen SL, Ho YP, Ho KY, Wu YM et al (2005) Lipid peroxidation: a possible role in the induction and progression of chronic periodontitis. J Periodontal Res 40(5):378–384
58. Chapple ILC, Brock G, Eftimiadi C, Matthews JB (2002) Glutathione in gingival crevicular fluid and its relation to local antioxidant capacity in periodontal health and disease. Mol Pathol 55(6):367–373
59. Grant MM, Brock GR, Matthews JB, Chapple ILC (2010) Crevicular fluid glutathione levels in periodontitis and the effect of non-surgical therapy. J Clin Periodontol 37(1):17–23
60. Zilinskas J, Kubilius R, Zekonis G, Zekonis J (2011) Total antioxidant capacity of venous blood, blood plasma, and serum of patients with periodontitis, and the effect of Traumeel S on these characteristics. Medicina 47(4):193–199
61. Demehin AA, Abugo OO, Rifkind JM (2001) The reduction of nitroblue tetrazolium by red blood cells: a measure of red cell membrane antioxidant capacity and hemoglobin-membrane binding sites. Free Radic Res 34(6):605–620
62. Griffiths GS (2003) Formation, collection and significance of gingival crevice fluid. Periodontol 2000 31:32–42
63. Akalin FA, Toklu E, Renda N (2005) Analysis of superoxide dismutase activity levels in gingiva and gingival crevicular fluid in patients with chronic periodontitis and periodontally healthy controls. J Clin Periodontol 32(3):238–243
64. Patel SP, Pradeep AR, Chowdhry S (2009) Crevicular fluid levels of plasma glutathione peroxidase (eGPx) in periodontal health and disease. Arch Oral Biol 54(6):543–548
65. Ekuni D, Tomofuji T, Irie K, Kasuyama K, Umakoshi M, Azuma T et al (2010) Effects of periodontitis on aortic insulin resistance in an obese rat model. Lab Invest 90(3):348–359
66. Herrera BS, Martins-Porto R, Maia-Dantas A, Campi P, Spolidorio LC, Costa SKP et al (2011) iNOS-derived nitric oxide stimulates osteoclast activity and alveolar bone loss in ligature-induced periodontitis in rats. J Periodontol 82(11):1608–1615
67. D'Aiuto F, Nibali L, Parkar M, Patel K, Suvan J, Donos N (2010) Oxidative stress, systemic inflammation, and severe periodontitis. J Dent Res 89(11):1241–1246
68. Chapple ILC, Milward MR, Dietrich T (2007) The prevalence of inflammatory periodontitis is negatively associated with serum antioxidant concentrations. J Nutr 137(3):657–664
69. Halliwell B, Whiteman M (2004) Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? Br J Pharmacol 142(2):231–255
70. Yagi K (1978) Lipid peroxide and human disease. Chem Phys Lipids 45:337–357
71. Panjamurthy K, Manoharan S, Ramachandran CR (2005) Lipid peroxidation and antioxidant status in patients with periodontitis. Cell Mol Biol Lett 10(2):255–264
72. Young IS, Trimble ER (1991) Measurement of malondialdehyde in plasma by high-performance liquid-chromatography with fluorometric detection. Ann Clin Biochem 28: 504–508
73. Monteiro AM, Jardini MAN, Alves S, Giarnpaoli V, Aubin ECQ, Neto AMF et al (2009) Cardiovascular disease parameters in periodontitis. J Periodontol 80(3):378–388
74. Bastos AS, Graves DT, Loureiro AP, Rossa Junior C, Abdalla DS, Faulin TD et al (2012) Lipid peroxidation is associated with the severity of periodontal disease and local inflammatory markers in patients with type 2 diabetes. J Clin Endocrinol Metab 97:1353–1362

75. Singer RE, Moss K, Beck JD, Offenbacher S (2009) Association of systemic oxidative stress with suppressed serum IgG to commensal oral biofilm and modulation by periodontal infection. Antioxid Redox Signal 11(12):2973–2983
76. Hickman MA, Boggess KA, Moss KL, Beck JD, Offenbacher S (2011) Maternal periodontal disease is associated with oxidative stress during pregnancy. Am J Perinatol 28(3):247–251
77. Halliwell B, Lee CY (2010) Using isoprostanes as biomarkers of oxidative stress: some rarely considered issues. Antioxid Redox Signal 13(2):145–156. doi:10.1089/ars.2009.2934
78. Wolfram RM, Budinsky AC, Eder A, Presenhuber C, Nell A, Sperr W, Sinzinger H (2006) Salivary isoprostanes indicate increased oxidation injury in periodontitis with additional tobacco abuse. Biofactors 28(1):21–31
79. Dean RT, Fu SL, Stocker R, Davies MJ (1997) Biochemistry and pathology of radical-mediated protein oxidation. Biochem J 324:1–18
80. Carty JL, Bevan R, Waller H, Mistry N, Cooke M, Lunec J et al (2000) The effects of vitamin C supplementation on protein oxidation in healthy volunteers. Biochem Biophys Res Commun 273(2):729–735
81. Dizdaroglu M, Jaruga P, Birincioglu M, Rodriguez H (2002) Free radical-induced damage to DNA: mechanisms and measurement. Free Radic Biol Med 32(11):1102–1115
82. Sugano N, Kawamoto K, Numazaki H, Murai S, Ito K (2000) Detection of mitochondrial DNA mutations in human gingival tissues. J Oral Sci 42:221–223
83. Takane M, Sugano N, Iwasaki H, Iwano Y, Shimizu N, Ito K (2002) New biomarker evidence of oxidative DNA damage in whole saliva from clinically healthy and periodontally diseased individuals. J Periodontol 73(5):551–554
84. Sawamoto Y, Sugano N, Tanaka H, Ito K (2005) Detection of periodontopathic bacteria and an oxidative stress marker in saliva from periodontitis patients. Oral Microbiol Immunol 20(4): 216–220
85. Sezer U, Cicek Y, Canakci CF (2012) Increased salivary levels of 8-hydroxydeoxyguanosine may be a marker for disease activity for periodontitis. Dis Markers 32(3):165–172
86. Fyhrquist F, Saijonmaa O (2012) Telomere length and cardiovascular aging. Ann Med 44(Suppl 1):S138–S142. doi:10.3109/07853890.2012.660497
87. von Zglinicki T (2002) Oxidative stress shortens telomeres. Trends Biochem Sci 27(7): 339–344
88. Oikawa S, Kawanishi S (1999) Site-specific DNA damage at GGG sequence by oxidative stress may accelerate telomere shortening. FEBS Lett 453(3):365–368
89. Matthews C, Gorenne I, Scott S, Figg N, Kirkpatrick P, Ritchie A, Goddard M, Bennett M (2006) Vascular smooth muscle cells undergo telomere-based senescence in human atherosclerosis: effects of telomerase and oxidative stress. Circ Res 99(2):156–164
90. Skinner HG, Gangnon RE, Litzelman K, Johnson RA, Chari ST, Petersen GM, Boardman LA (2012) Telomere length and pancreatic cancer: a case–control study. Cancer Epidemiol Biomarkers Prev 21(11):2095–2100
91. Cui Y, Cai Q, Qu S, Chow WH, Wen W, Xiang YB, Wu J, Rothman N, Yang G, Shu XO, Gao YT, Zheng W (2012) Association of leukocyte telomere length with colorectal cancer risk: nested case–control findings from the Shanghai Women's Health Study. Cancer Epidemiol Biomarkers Prev 21(10):1807–1813
92. Shen J, Terry MB, Liao Y, Gurvich I, Wang Q, Senie RT, Santella RM (2012) Genetic variation in telomere maintenance genes, telomere length and breast cancer risk. PLoS One 7(9):e44308
93. Takahashi K, Nishida H, Takeda H, Shin K (2004) Telomere length in leukocytes and cultured gingival fibroblasts from patients with aggressive periodontitis. J Periodontol 75(1):84–90
94. Söder B, Jin LJ, Klinge B, Söder PO (2007) Periodontitis and premature death: a 16-year longitudinal study in a Swedish urban population. J Periodontal Res 42(4):361–366

Chapter 10
Effects of Periodontal Therapy on Circulating Oxidative Stress

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10.1 Introduction

Periodontal disease is a chronic inflammatory disease of the supporting tissues of teeth, leading to tooth loss [1, 2]. Subgingival plaque biofilm is responsible for the initiation and progression of periodontitis [3, 4]. Abnormal host responses to bacterial pathogens also play a crucial role in the progression of periodontal disease [5].

Reactive oxygen species (ROS), including superoxide, hydrogen peroxide, and hydroxyl anions, are products of normal cellular metabolism. When stimulated by oral bacterial pathogens, host cells such as polymorphonuclear leukocytes produce ROS as part of the immune response [6, 7]. However, as ROS are not target-specific, overproduced ROS oxidize DNA, lipids, and proteins, contributing to tissue damage (oxidative stress) [8]. Studies have indicated that oxidative stress is involved in the pathogenesis of periodontitis. For instance, clinical studies have demonstrated that periodontitis is positively correlated with increased lipid, DNA, and protein oxidation in gingival crevicular fluid (GCF) and saliva [9–13]. In addition, animal studies have shown that oxidative DNA damage [14, 15], and increased hydrogen peroxide [16, 17], protein nitration [18], and lipid peroxidation [19] are present in inflamed periodontal tissue.

With the progression of periodontitis, ROS produced by periodontal inflammation diffuse into the blood stream [20, 21]. This induces oxidation of various molecules in the blood, leading to circulating oxidative stress that may gradually damage

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multiple organs [22–24]. Therefore, increases in circulating oxidative stress after periodontitis may negatively affect systemic health.

Clinical studies have demonstrated that periodontal therapy decreases oxidative stress in GCF and saliva [25, 26]. If periodontitis causes circulating oxidative stress, improvement of periodontitis by periodontal therapy would result in a decrease in both oral and blood oxidative stress. In this chapter, we summarized the effects of periodontal therapy on circulating oxidative stress. We also discuss the possibility of periodontal therapy decreasing the risk of systemic diseases in periodontitis patients.

10.2 Periodontitis as a Risk Factor for Circulating Oxidative Stress

Various studies have examined the relationship between periodontitis and circulating oxidative stress. For example, it has been reported that plasma levels of lipid peroxidation [27] and reactive oxygen metabolites (ROM) [28] are associated with severity of periodontitis. It is also known that plasma or serum levels of total oxidative status [9, 29], protein oxidation [12], ROM [30, 31], and oxidized low-density lipoprotein (LDL) [32] are significantly higher in chronic periodontitis patients than in control subjects. Furthermore, a recent clinical study found that the mean plasma glutathione peroxidase increased from healthy status to gingivitis, and then to periodontitis in serum [33]. These findings indicate that periodontitis can lead to increased oxidative stress at the systemic level. However, some studies have found no significant differences in serum lipid peroxidation [9, 33], serum oxidative stress index [34], and oxidative DNA damage in venous blood [35] between subjects with and without periodontitis. Therefore, further studies are necessary to investigate the factors that affect the relationship between periodontitis and circulating oxidative stress.

Information regarding blood levels of antioxidants is also important to assess the relationship between periodontitis and circulating oxidative stress. Some clinical studies have found that plasma or serum levels of total antioxidant capacity in subjects with periodontitis are lower than those in subjects with healthy gingiva [36, 37], while others have suggested that there were no significant differences in serum or plasma antioxidant concentration between the group with chronic periodontitis and controls [10, 38]. Thus, there is no consensus regarding the relationship between periodontitis and blood antioxidant status. In addition, a previous study using plasma samples has demonstrated that the activities of superoxide dismutase, catalase, and glutathione peroxide were significantly higher, whereas the levels of vitamin C, vitamin E, and reduced glutathione were significantly lower in periodontitis patients than in controls [39]. The relationship between periodontitis and blood antioxidant status may differ depending on the antioxidant examined.

10.3 Changes in GCF and Salivary Oxidative Stress After Periodontal Therapy

The effects of periodontal therapy on oral oxidative stress have been reported. Clinical studies have found that successful periodontal therapy can decrease oxidative DNA damage in saliva [40] and GCF [41], plasma glutathione peroxidase in GCF [33], and lipid peroxidation in saliva [25] and GCF [29]. It has also been reported that locally delivered lycopene reduced oxidative DNA damage in GCF with an improvement of periodontitis [42]. These findings show that improvement of periodontitis by periodontal therapy could contribute to decrease in oxidative stress within the oral cavity. Such a condition would result in decreased oxidative stress at the systemic level.

On the other hand, clinical studies also showed that periodontal therapy increases total antioxidant capacity [10] and reduced/oxidized glutathione ratio [26] in GCF. These findings indicate that periodontal therapy can improve GCF antioxidant status. In contrast, salivary levels of total antioxidants and superoxide dismutase activity in periodontitis patients decreased after non-surgical periodontal therapy [29, 43]. The mechanisms of fluctuations in salivary antioxidant status following periodontal therapy may differ from those in GCF.

10.4 Effects of Periodontal Therapy on Circulating Oxidative Stress in Periodontitis Patients

If periodontitis causes circulating oxidative stress, periodontal therapy would result in a decrease in circulating oxidative stress. To clarify this issue, studies have evaluated the effects of periodontal therapy on circulating oxidative stress (Table 10.1).

To the best of our knowledge, five clinical studies have shown that periodontal therapy reduces oxidative parameters in blood [29, 32, 44–46]. Sonoki et al. [44] reported that non-surgical therapy decreased plasma levels of lipid peroxidation and anti-malondialdehyde modified LDL cholesterol in periodontitis patients with diabetes at 6 months. In our previous studies, plasma levels of oxLDL [32] and ROM [45] in subjects with chronic periodontitis decreased after periodontal therapy for 2 months. Furthermore, reductions in serum total oxidant status have been observed in periodontitis patients at 6 weeks after non-surgical periodontal therapy [46]. These observations indicate that periodontal therapy offers clinical benefits in decreasing circulating oxidative stress in periodontitis patients. We also found a positive association between the degrees of changes in circulating oxidative stress and the percentage of sites with bleeding on probing (BOP) after periodontal therapy [32, 45]. This suggests that circulating oxidative stress showed a dose–response type decrease, depending on the degree of improvement in BOP. As BOP reflects disease activity in the periodontium [47], the decrease in circulating oxidative stress may be due to the reduction in disease activity in the periodontium. On the other

Table 10.1 Main studies about effects of periodontal therapy on circulating oxidative stress

Reference	Study population	Age ranges (years)	Intervention	Oxidative parameters	Major results
Christgau et al. [48]	Persons with moderate to severe periodontitis: 20 diabetes patients 20 systemically healthy persons	30–67	Non-surgical periodontal therapy	Oxidative burst response of polymorphonuclear leukocytes to inflammatory and bacterial stimulation	Periodontal therapy modified oxidative burst response in both groups
Sonoki et al. [44]	Persons with periodontitis: 5 type-2 diabetes patients 6 systemically healthy persons	>40	Non-surgical periodontal therapy	1. Plasma lipid peroxidation	1. Periodontal therapy decreased lipid peroxidation in diabetes patients, but not in control persons
				2. Serum anti-malondialdeyde modified low-density lipoprotein cholesterol (anti-MDA-LDL-C)	2. Periodontal therapy decreased anti-MDA-LDL-C in both groups
Matthews et al. [49]	19 persons with chronic periodontitis	36–61	Non-surgical periodontal therapy	ROS production from peripheral blood neutrophils	Periodontal therapy decreased Fcγ-receptor-stimulated ROS production, but not unstimulated extracellular radical release
Tamaki et al. [45]	19 persons with chronic periodontitis	21–75	Non-surgical periodontal therapy	Plasma reactive oxygen metabolites (ROM)	Periodontal therapy decreased ROM
D'Aiuto et al. [30]	145 persons with severe generalized periodontitis	47.3 ± 8.3 (mean ± SD)	Non-surgical periodontal therapy	Plasma ROM	Acute increases of ROM were observed following periodontal therapy
Tamaki et al. [32]	22 persons with chronic periodontitis	44.0 ± 19.2 (mean ± SD)	Non-surgical periodontal therapy	Plasma oxLDL	Periodontal therapy decreased oxLDL
Wei et al. [29]	48 persons with chronic periodontitis	40.1 ± 7.3 (mean ± SD)	Non-surgical periodontal therapy	1. Serum lipid peroxidation	1. Periodontal therapy did not alter lipid peroxidation
				2. Serum total oxidant status	2. Periodontal therapy decreased total oxidant status
Akpinar et al. [46]	15 smokers with chronic periodontitis	Smokers: 38.4 ± 5.5 (mean ± SD)	Non-surgical periodontal therapy	Serum total oxidant status	Periodontal therapy decreased total oxidant status in both groups
	14 non-smokers with chronic periodontitis	Non-smokers: 37.7 ± 5.9 (mean ± SD)			

hand, in severe periodontitis patients, it has been reported that sustained increases in plasma ROM levels are observed up to 5 days from a single session of initial periodontal therapy [30]. In patients with severe periodontitis, the changes in circulating oxidative stress after periodontal therapy may differ from those with mild or moderate periodontitis.

Periodontal therapy may also modify immunological reactions in the blood. Christgau et al. [48] compared the oxidative burst response of polymorphonuclear granulocytes to tumor necrosis factor (TNF)-α and bacterial peptide *N*-formyl-methionyl-leucyl-phenylalanin (FMLP) in venous blood samples before and after periodontal therapy. They found that the burst reaction following combined stimulation with TNF-α and FMLP was significantly increased at 4 months after periodontal therapy, as compared to the first and second examinations ($p<0.05$). Matthews et al. [49] also demonstrated that the well-characterized hyper-reactive peripheral blood neutrophil phenotype relative to total radical production after Fcγ-receptor stimulation was reduced, but not removed, by periodontal therapy. These observations suggest that periodontal therapy influences circulating oxidative stress by changing reactions of polymorphonuclear granulocytes and neutrophils in venous blood.

In addition, four studies investigated the effects of periodontal therapy on circulating antioxidant status (Table 10.2). One study showed that plasma total antioxidant levels at 1 month post-therapy increased significantly when compared with baseline levels ($p<0.001$) [36]. However, two studies indicate that periodontal therapy had no effect on circulating total antioxidant status [10, 46]. Furthermore, the remaining study states that there was a significant reduction in plasma glutathione peroxidase concentration in serum after periodontal therapy ($p<0.05$) [33]. Further studies are necessary in order to clarify how periodontal therapy affects circulating antioxidant status in periodontitis patients.

10.5 Periodontal and Systemic Health: Is Circulating Oxidative Stress a Common Link?

Oxidative stress plays an important etiological role in a number of diseases: diabetes mellitus, heart disease, liver disease, stroke, acquired immunodeficiency syndrome, Alzheimer's disease, Parkinson's disease, and alcoholism [50–53]. Therefore, circulating oxidative stress could be a common mechanism in the development of several features related to both systemic diseases and periodontitis. This notion is supported by clinical studies investigating the relationships between periodontitis and type 2 diabetes mellitus [54] and between periodontitis and hepatocellular carcinoma [55], as well as a review summarizing the relationship between periodontitis and metabolic syndrome [56]. However, it remains unclear whether periodontal therapy has beneficial effects on systemic health by decreasing circulating oxidative stress. Further studies are required to clarify this point.

Table 10.2 Main studies about effects of periodontal therapy on circulating antioxidant status

Reference	Study population	Age ranges (years)	Intervention	Antioxidant parameters	Major results
Chapple et al. [10]	17 persons with chronic periodontitis	23–60	Non-surgical periodontal therapy	Plasma total antioxidant capacity	Periodontal therapy did not alter total antioxidant capacity
Abou Sulaiman and Shehadeh [36]	30 persons with chronic periodontitis	23–65	1. Non-surgical periodontal therapy alone 2. Non-surgical periodontal therapy with adjunctive dose of vitamin C	Plasma total antioxidant capacity	1. Periodontal therapy increased total antioxidant capacity in both groups 2. Adjunctive dose of vitamin C did not offer additional effect
Patel et al. [33]	30 persons: group 1-healthy group 2-gingivitis individuals group 3-chronic periodontitis patients group 4-chronic periodontitis patients after therapy	30–38	Non-surgical periodontal therapy	Serum glutathione peroxidase	Periodontal therapy decreased glutathione peroxidase
Akpinar et al. [46]	15 smokers with chronic periodontitis 14 non-smokers with chronic periodontitis	Smokers: 38.4 ± 5.5 (mean ± SD) Non-smokers: 37.7 ± 5.9 (mean ± SD)	Non-surgical periodontal therapy	Serum total antioxidant status	Periodontal therapy did not alter total antioxidant status in both groups

10.6 Recommendations for Future Research

Clinical studies have indicated that periodontitis and circulating oxidative stress are positively associated, and that periodontal therapy may provide beneficial effects on circulating oxidative stress. However, the evidence could be further strengthened by future studies incorporating the following design features:

1. Randomized, multicenter studies that are properly powered;
2. Development of large cohorts to assess the impact of periodontal condition on circulating oxidative stress;
3. Inclusion of populations with differing characteristics, such as overweight/obese populations, the elderly and persons with unhealthy eating habits;
4. Recording nutrition and exercise habits;
5. Accounting for periodontitis definitions that include extent, severity and definitions of cases.

10.7 Conclusion

Several studies have indicated that periodontitis causes circulating oxidative stress in humans. In periodontitis patients, periodontal therapy may be useful for maintaining systemic health, as well as improving periodontal health, by decreasing circulating oxidative stress.

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References

1. Page RC, Engel LD, Narayanan AS, Clagett JA (1978) Chronic inflammatory gingival and periodontal disease. JAMA 11:545–550
2. Pihlstrom BL, Michalowicz BS, Johnson NW (2005) Periodontal disease. Lancet 366: 1809–1820
3. Madianos PN, Bobetsis YA, Kinane DF (2005) Generation of inflammatory stimuli: how bacteria set up inflammatory responses in the gingiva. J Clin Periodontol 32:57–71
4. Komiya-Ito A, Ishihara K, Tomita S, Kato T, Yamada S (2010) Investigation of subgingival profile of periodontopathic bacteria using polymerase chain reaction. Bull Tokyo Dent Coll 51:139–144
5. Page RC, Kornman K (1997) The pathogenesis of human periodontitis: an introduction. Periodontol 2000 14:9–11
6. Hyslop PA, Hinshaw DB, Scraufstatter IU, Cochrane CG, Kunz S, Vosbeck K (2007) Hydrogen peroxide as a potent bacteriostatic antibiotic: implications for host defense. Free Radic Biol Med 19:31–37
7. Fialkow L, Wang Y, Downey GP (2007) Reactive oxygen and nitrogen species as signaling molecules regulating neutrophil function. Free Radic Biol Med 42:153–164

8. Halliwell B (1994) Free radicals, antioxidants and human disease: curiosity, cause or consequence. Lancet 344:721–724
9. Akalın FA, Baltacioğlu E, Alver A, Karabulu E (2007) Lipid peroxidation levels and total oxidant status in serum, saliva and gingival crevicular fluid in patients with chronic periodontitis. J Clin Periodontol 34:558–565
10. Chapple IL, Brock GR, Milward MR, Ling N, Matthews JB (2007) Compromised GCF total antioxidant capacity in periodontitis: cause or effect? J Clin Periodontol 34:103–110
11. Tsai CC, Chen HS, Chen SL, Ho YP, Ho KY, Wu YM, Hung CC (2005) Lipid peroxidation: a possible role in the induction and progression of chronic periodontitis. J Periodontal Res 40:378–384
12. Baltacıoğlu E, Akalın FA, Alver A, Değer O, Karabulut E (2007) Protein carbonyl levels in serum and gingival crevicular fluid in patients with chronic periodontitis. Arch Oral Biol 53:716–722
13. Sezer U, Çiçek Y, Canakçi CF (2012) Increased salivary levels of 8-hydroxydeoxyguanosine may be a marker for disease activity for periodontitis. Dis Markers 32:165–172
14. Tomofuji T, Azuma T, Kusano H, Sanbe T, Ekuni D, Tamaki N, Yamamoto T, Watanabe T (2006) Oxidative damage of periodontal tissue in the rat periodontitis model: effects of a high-cholesterol diet. FEBS Lett 580:3601–3604
15. Canakci CF, Cicek Y, Yildirim A, Sezer U, Canakci V (2009) Increased levels of 8-hydroxydeoxyguanosine and malondialdehyde and its relationship with antioxidant enzymes in saliva of periodontitis patients. Eur J Dent 3:100–106
16. Ekuni D, Tomofuji T, Tamaki N, Sanbe T, Azuma T, Yamanaka R, Yamamoto T, Watanabe T (2008) Mechanical stimulation of gingiva reduces plasma 8-OHdG level in rat periodontitis. Arch Oral Biol 53:324–329
17. Ekuni D, Firth JD, Nayer T, Tomofuji T, Sanbe T, Irie K, Yamamoto T, Oka T, Liu Z, Vielkind J, Putnins EE (2009) Lipopolysaccharide-induced epithelial monoamine oxidase mediates alveolar bone loss in a rat chronic wound model. Am J Pathol 175:1398–1409
18. Lohinai Z, Stachlewitz R, Virág L, Székely AD, Haskó G, Szabó C (2001) Evidence for reactive nitrogen species formation in the gingivomucosal tissue. J Dent Res 80:470–475
19. Ekuni D, Tomofuji T, Sanbe T, Irie K, Azuma T, Maruyama T, Tamaki N, Murakami J, Kokeguchi S, Yamamoto T (2009) Periodontitis-induced lipid peroxidation in rat descending aorta is involved in the initiation of atherosclerosis. J Periodontal Res 44:434–442
20. Tomofuji T, Ekuni D, Yamanaka R, Kusano H, Kusano H, Azuma T, Sanbe T, Tamaki N, Yamamoto T, Watanabe T, Miyauchi M, Takata T (2007) Chronic administration of lipopolysaccharide and proteases induces periodontal inflammation and hepatic steatosis in rats. J Periodontol 78:1999–2006
21. Sobaniec H, Sobaniec-Lotowska ME (2000) Morphological examinations of hard tissues of periodontium and evaluation of selected processes of lipid peroxidation in blood serum of rats in the course of experimental periodontitis. Med Sci Monit 6:875–881
22. Yamamoto T, Tomofuji T, Tamaki N, Ekuni D, Azuma T, Sanbe T (2010) Effects of topical application of lipopolysaccharide and proteases on hepatic injury induced by high-cholesterol diet in rats. J Periodontal Res 45:129–135
23. Tomofuji T, Ekuni D, Irie K, Azuma T, Tamaki N, Maruyama T, Yamamoto T, Watanabe T, Morita M (2011) Biomed Res 32:343–349
24. Herrera BS, Martins-Porto R, Campi P, Holzhausen M, Teixeira SA, Mendes GD, Costa SK, Gyurko R, Van Dyke TE, Spolidório LC, Muscará MN (2011) Local and cardiorenal effects of periodontitis in nitric oxide-deficient hypertensive rats. Arch Oral Biol 56:41–47
25. Guentsch A, Preshaw PM, Bremer-Streck S, Klinger G, Glockmann E, Sigusch BW (2008) Lipid peroxidation and antioxidant activity in saliva of periodontitis patients: effect of smoking and periodontal treatment. Clin Oral Investig 12:345–352
26. Grant MM, Brock GR, Matthews JB, Chapple IL (2010) Crevicular fluid glutathione levels in periodontitis and the effect of non-surgical therapy. J Clin Periodontol 37:17–23
27. Bastos AS, Graves DT, Loureiro AP, Rossa Júnior C, Abdalla DS, Faulin Tdo E, Olsen Câmara N, Andriankaja OM, Orrico SR (2011) Lipid peroxidation is associated with the severity of

periodontal disease and local inflammatory markers in patients with type 2 diabetes. J Clin Endocrinol Metab 97:E1353–E1362
28. Tamaki N, Tomofuji T, Maruyama T, Ekuni D, Yamanaka R, Takeuchi N, Yamamoto T (2008) Relationship between periodontal condition and plasma reactive oxygen metabolites in patients in the maintenance phase of periodontal treatment. J Periodontol 79:2136–2142
29. Wei D, Zhang XL, Wang YZ, Yang CX, Chen G (2010) Lipid peroxidation levels, total oxidant status and superoxide dismutase in serum, saliva and gingival crevicular fluid in chronic periodontitis patients before and after periodontal therapy. Aust Dent J 55:70–78
30. D'Aiuto F, Nibali L, Parkar M, Patel K, Suvan J, Donos N (2010) Oxidative stress, systemic inflammation, and severe periodontitis. J Dent Res 89:1241–1246
31. Masi S, Salpea KD, Li K, Parkar M, Nibali L, Donos N, Patel K, Taddei S, Deanfield JE, D'Aiuto F, Humphries SE (2011) Oxidative stress, chronic inflammation, and telomere length in patients with periodontitis. Free Radic Biol Med 50:730–735
32. Tamaki N, Tomofuji T, Ekuni D, Yamanaka R, Morita M (2011) Periodontal treatment decreases plasma oxidized LDL level and oxidative stress. Clin Oral Investig 15:953–958
33. Patel SP, Rao NS, Pradeep AR (2012) Effect of nonsurgical periodontal therapy on crevicular fluid and serum glutathione peroxidase levels. Dis Markers 32:1–7
34. Esen C, Alkan BA, Kırnap M, Akgül O, Işıkoğlu S, Erel O (2012) The effects of chronic periodontitis and rheumatoid arthritis on serum and gingival crevicular fluid total antioxidant/oxidant status and oxidative stress index. J Periodontol 83:773–779
35. Konopka T, Król K, Kopeć W, Gerber H (2007) Total antioxidant status and 8-hydroxy-2′-deoxyguanosine levels in gingival and peripheral blood of periodontitis patients. Arch Immunol Ther Exp (Warsz) 55:417–422
36. Abou Sulaiman AE, Shehadeh RM (2010) Assessment of total antioxidant capacity and the use of vitamin C in the treatment of non-smokers with chronic periodontitis. J Periodontol 81:1547–1554
37. Žilinskas J, Kubilius R, Žekonis G, Žekonis J (2011) Total antioxidant capacity of venous blood, blood plasma, and serum of patients with periodontitis, and the effect of Traumeel S on these characteristics. Medicina (Kaunas) 47:193–199
38. Brock GR, Butterworth CJ, Matthews JB, Chapple IL (2004) Local and systemic total antioxidant capacity in periodontitis and health. J Clin Periodontol 31:515–521
39. Panjamurthy K, Manoharan S, Ramachandran CR (2005) Lipid peroxidation and antioxidant status in patients with periodontitis. Cell Mol Biol Lett 10:255–264
40. Takane M, Sugano N, Iwasaki H, Iwano Y, Shimizu N, Ito K (2002) New biomarker evidence of oxidative DNA damage in whole saliva from clinically healthy and periodontally diseased individuals. J Periodontol 73:551–554
41. Ongöz Dede F, Otan Özden F, Avcı B (2013) 8-OHdG levels in gingival crevicular fluid and saliva from patients with chronic periodontitis during initial periodontal treatment. J Periodontol 84:821–828
42. Chandra RV, Sandhya YP, Nagarajan S, Reddy BH, Naveen A, Murthy KR (2012) Efficacy of lycopene as a locally delivered gel in the treatment of chronic periodontitis: smokers vs non-smokers. Quintessence Int 43:401–411
43. Kim SC, Kim OS, Kim OJ, Kim YJ, Chung HJ (2010) Antioxidant profile of whole saliva after scaling and root planing in periodontal disease. J Periodontal Implant Sci 40:164–171
44. Sonoki K, Nakashima S, Takata Y, Naito T, Fujisawa K, Ootsubo T, Wakisaka M, Iwase M, Iida M, Yokota M (2006) Decreased lipid peroxidation following periodontal therapy in type 2 diabetic patients. J Periodontol 77:1907–1913
45. Tamaki N, Tomofuji T, Ekuni D, Yamanaka R, Yamamoto T, Morita M (2009) Short-term effects of non-surgical periodontal treatment on plasma level of reactive oxygen metabolites in patients with chronic periodontitis. J Periodontol 80:901–906
46. Akpinar A, Toker H, Ozdemir H, Bostanci V, Aydin H (2013) The effects of non-surgical periodontal therapy on oxidant and anti-oxidant status in smokers with chronic periodontitis. Arch Oral Biol 58:717–723

47. Greenstein G (1984) The role of bleeding upon probing in the diagnosis of periodontal disease. A literature review. J Periodontol 55:684–688
48. Christgau M, Palitzsch KD, Schmalz G, Kreiner U, Frenzel S (1998) Healing response to non-surgical periodontal therapy in patients with diabetes mellitus: clinical, microbiological, and immunologic results. J Clin Periodontol 25:112–124
49. Matthews JB, Wright HJ, Roberts A, Ling-Mountford N, Cooper PR, Chapple IL (2007) Neutrophil hyper-responsiveness in periodontitis. J Dent Res 86:718–722
50. McCord JM (2000) The evolution of free radicals and oxidative stress. Am J Med 108: 652–659
51. Rizzo MR, Barbieri M, Marfella R, Paolisso G (2012) Reduction of oxidative stress and inflammation by blunting daily acute glucose fluctuations in patients with type 2 diabetes: role of dipeptidyl peptidase-IV inhibition. Diabetes Care 35:2076–2082
52. Stadler K (2012) Oxidative stress in diabetes. Adv Exp Med Biol 771:272–287
53. Sánchez-Valle V, Chávez-Tapia NC, Uribe M, Méndez-Sánchez N (2012) Role of oxidative stress and molecular changes in liver fibrosis: a review. Curr Med Chem 19:4850–4860
54. Allen EM, Matthews JB, O'Halloran DJ, Griffiths HR, Chapple IL (2011) Oxidative and inflammatory status in type 2 diabetes patients with periodontitis. J Clin Periodontol 38: 894–901
55. Tamaki N, Takaki A, Tomofuji T, Endo Y, Kasuyama K, Ekuni D, Yasunaka T, Yamamoto K, Morita M (2011) Stage of hepatocellular carcinoma is associated with periodontitis. J Clin Periodontol 38:1015–1020
56. Bullon P, Morillo JM, Ramirez-Tortosa MC, Quiles JL, Newman HN, Battino M (2009) Metabolic syndrome and periodontitis: is oxidative stress a common link? J Dent Res 88: 503–518

Chapter 11
Role of HMGB1 in Periodontal Disease

Noriko Ebe, Miki Hara-Yokoyama, and Yuichi Izumi

Abbreviations

BA	Butyric acid
Ca9-22	Gingival epithelial cells
CHX	Cycloheximide
CP	Chronic periodontitis
Cys	Cysteine
GCF	Gingival crevicular fluid
HDAC	Histone deacetylase
HMGB1	High-mobility group box-1
IL	Interleukin
LPS	Lipopolysaccharide
PDL	Periodontal ligament
RAGE	Receptor for advanced glycation end products
ROS	Reactive oxygen species
TNF-α	Tumor necrosis factor

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11.1 Introduction

11.1.1 Role of HMGB1 in Disease

HMGB1 is one of the most abundant nuclear non-histone proteins and is expressed in all eukaryotic cells [1]. HMGB1 has intracellular and extracellular functions. In the nucleus, HMGB1 binds to DNA and act as a DNA chaperone, facilitating rate-limiting DNA distortion during nucleosome sliding and regulating transcription. HMGB1 knockout mice die shortly after birth because of hypoglycemia resulting from a defect in transcription activation by the glucocorticoid receptor [2]. HMGB1 can also serve as a cytokine when released in the extracellular milieu upon tissue injury or inflammation [3]. Extracellular HMGB1 promotes tissue repair and proliferation of cells, and induces the maturation of dendritic cells [4], recruitment of neutrophils [5], or activation of monocytes/macrophages [6]. Therefore, HMGB1 is considered to be a danger signal (alarmin) that triggers repair and defense programs (Fig. 11.1).

HMGB1 induces autoimmune responses and becomes pathogenic when released chronically and/or in the absence of infection. HMGB1 has been found to act as a late mediator of endotoxin lethality in mice [7]. HMGB1 administrated in vivo induces rheumatoid arthritis [8] and acute lung injury [9]. Because administration of antibodies to HMGB1 attenuates endotoxin lethality [7], HMGB1 is currently considered a new potential therapeutic target.

In most cells, HMGB1 is located in the nucleus, where it acts in replication, recombination, transcription, and DNA repair processes. In all cells, HMGB1 shuttles actively between the nucleus and cytoplasm. In lipopolysaccharide (LPS)-stimulated monocytes and macrophages, HMGB1 is relocated to the cytoplasm, possibly via hyperacetylation [10], and subsequently accumulates in secretory lysosomes and is then actively released [11]. In the case of tissue injury, it is considered that HMGB1, transiently existing in the cytoplasm during the course of nucleocytoplasmic shuttling, is passively released when the cells lose integrity of the plasma membrane as a result of necrosis [3]. The relocation of HMGB1 from the nucleus occurs during some types of necrosis, such as induction by DNA-alkylating damage [12].

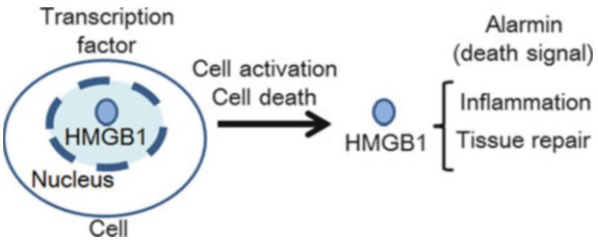

Fig. 11.1 Schematic summary of the HMGB1 release and action. HMGB1 is a nuclear protein and acts as a transcription factor. Upon cell activation and cell death, HMGB1 is released to the extracellular space and functions as an alarmin. Extracellular HMGB1 acts as a trigger of inflammation and tissue repair

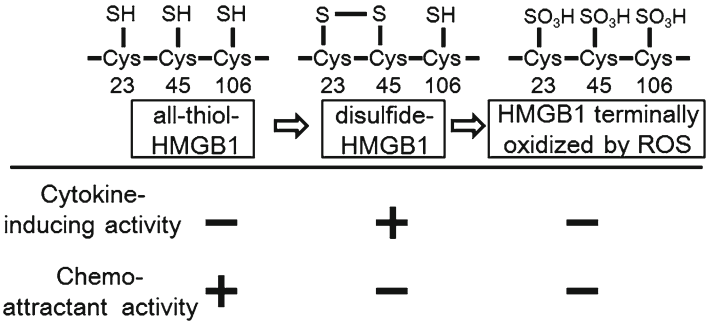

Fig. 11.2 Activity of HMGB1 depends on its reduced or oxidized form [18]

In HeLa cells, HMGB1 is retained in the nucleus by condensed chromatin during apoptosis. Therefore, the release of HMGB1 does not occur, even when plasma membranes are impaired after secondary necrosis [6]. However, some contradictory results have been demonstrated [13], implying that the release of HMGB1 from apoptotic cells is context-dependent.

11.1.2 Oxidative Stress and HMGB1

HMGB1 has several functions, and the function of HMGB1 changes with the oxidation state. In liver grafts, oxidation of HMGB1 that is induced during prolonged liver ischemia and by reoxygenation during reperfusion in vivo might also attenuate its proinflammatory activity [14]. As a redox-sensitive protein, HMGB1 contains three cysteines (Cys23, 45, and 106). In the setting of oxidative stress, HMGB1 can form a Cys23–Cys45 disulfide bond. The oxidation of Cys106 does not occur in mild oxidative conditions [15]. The oxidation of Cys106 abolishes the ability of HMGB1 to activate dendritic cells. Therefore, oxidation of Cys106 is necessary and sufficient to inactivate the immunostimulatory activity of HMGB1 [16]. However, in necrotic cells, HMGB1 is released from dying cells possessing full immunogenic activity, including dendritic cells [17].

Recently, Venereau et al. have found that activity of HMGB1 depends on its reduced or oxidized form. All-thiol-HMGB1 plays a role as a chemoattractant, whereas a disulfide bond results in HMGB1 being a proinflammatory cytokine, and further cysteine oxidation to sulfonates by reactive oxygen species (ROS) abrogates both activities. They used the human acute monocytic leukemia cell line THP-1 and measured the redox status of intracellular and extracellular HMGB1. HMGB1 from cells treated with or without LPS and from the supernatant of cells mechanically necrotized with freeze–thaw cycles was analyzed. Intracellular HMGB1 had all-thiol-HMGB1, whereas extracellular HMGB1 contained all-thiol-HMGB1 and disulfide HMGB1. HMGB1 orchestrates leukocyte recruitment and their induction to secrete inflammatory cytokines by redox states (Fig. 11.2) [18].

Tang et al. proposed that HMGB1 is a redox-sensitive regulator of the balance between autophagy and apoptosis. In pancreatic and colon cancer cells, anticancer agents enhance autophagy and apoptosis, as well as HMGB1 release. Reduced HMGB1 binds to the receptor for advanced glycation end products (RAGE) and promotes tumor resistance to anticancer agents. Conversely, oxidized HMGB1 increases the cytotoxicity of anticancer agents and induces apoptosis mediated by the caspase-9/-3 intrinsic pathway [19].

11.2 HMGB1 and Periodontal Disease

11.2.1 Gingival Soft Tissues and HMGB1 in Periodontal Disease

Periodontal disease is the chronic inflammation of periodontal tissues, mainly caused by gram-negative bacteria populating the gingival sulcus and periodontal pocket. *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* are considered as major periodontal pathogens involved in periodontal disease [20]. These bacteria produce an elaborate variety of virulence factors, such as proteases, LPS, and short-chain fatty acids, such as butyric acid and propionic acid, which are major by-products of anaerobic metabolism that are released into the microenvironment at the infection site. The pathogenesis of periodontal disease is characterized by complex interaction between pathogens and host responses, through cytokines and inflammatory mediators in periodontal tissues [21]. Acute inflammation of periodontal tissues induces alveolar bone to be dissolved and leads to loss of teeth. Previous studies have demonstrated high levels of HMGB1 in gingival crevicular fluid (GCF) from periodontal patients and have reported the release of HMGB1 from gingival epithelial cells (Ca9-22) by tumor necrosis factor (TNF-α) [22]. Luo et al. suggested that the expression of HMGB1 is increased in gingival tissues and GCF in chronic periodontitis (CP), and in generalized aggressive periodontitis and peri-implant crevicular fluid of peri-implantitis. HMGB1 expression is highest in gingival tissues and GCF from CP patients and is accompanied by increased concentrations of interleukin (IL)-1β, IL-6, and IL-8 (proinflammatory cytokines) [23]. Feghali et al. [24] observed active secretion of HMGB1 from human gingival fibroblasts stimulated by LPS. IL-1β promotes HMGB1 production in human gingival epithelial cells and fibroblast cells in a nitric oxide-dependent manner, and RAGE, which is a ligand of HMGB1, is increased [25]. These studies showed active release of HMGB1 from periodontal tissues by TNF-α, LPS, and IL-1β. They suggested a role of HMGB1 in inflammatory periodontal disease, and gingival epithelial cells were the main origin of HMGB1 in periodontal tissues (Fig. 11.3a).

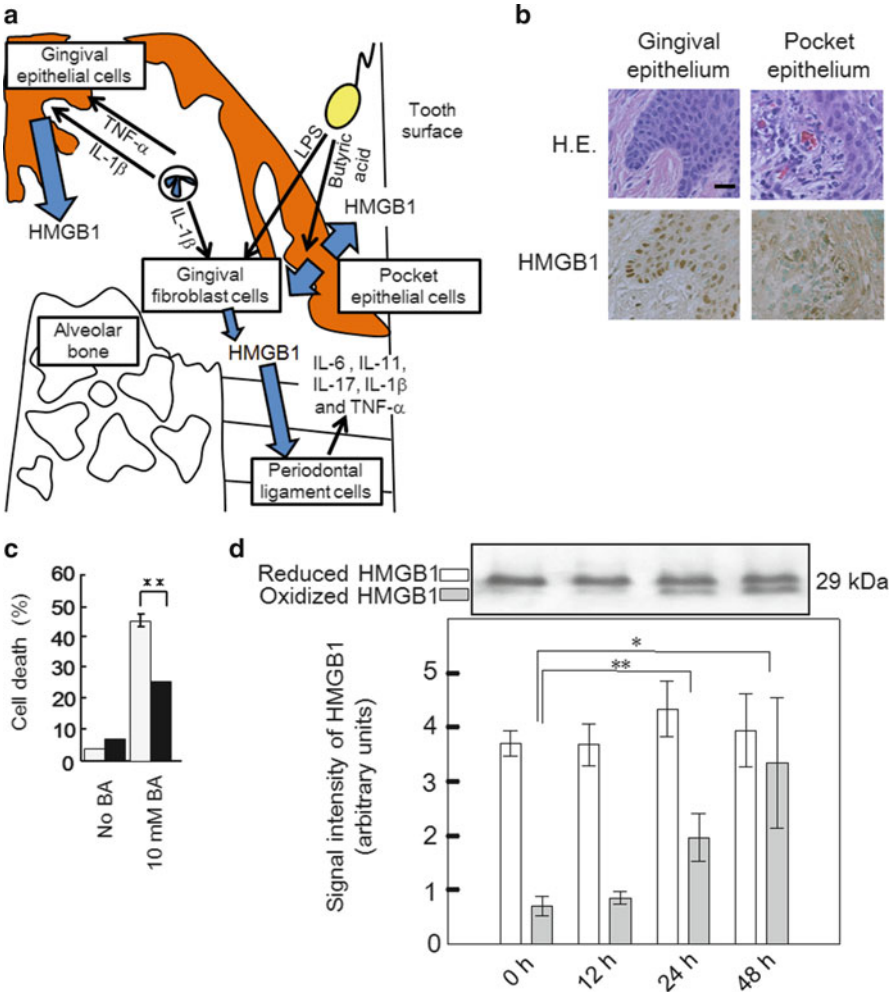

Fig. 11.3 (**a**) Schematic diagram showing the release of HMGB1 from various periodontal cells. (**b**) Immunohistochemical localization of HMGB1 in gingival tissue from a patient with chronic periodontitis (pocket depth: 10 mm). The section was stained with H.E. (*upper panels*) or anti-HMGB1 antibody (HMGB1) (*lower panels*). Areas corresponding to gingival epithelium (*left panels*) and pocket epithelium (*right panels*) are magnified. Scale bar: (*upper left panel*), 20 μm. (**c**) Ca9-22 cells were incubated without (*gray*) and with (*black*) 10 μg/ml CHX for 48 h in the absence or presence of 10 mM butyric acid, and cell death was evaluated. Values are means ± s.d. ($n = 3$). BA = butyric acid. $^{**}p < 0.01$. (**d**) Ca9-22 cells were incubated with 10 mM butyric acid for the indicated time and the conditioned medium was subjected to 11 % SDS-polyaclylamidegel electrophoresis (SDS-PAGE) in non-reducing conditions. Band intensities of HMGB1 in the slow- and fast-migrating forms (*white* and *gray*, respectively) are shown. Densitometric analysis of the band intensity was carried out using ImageJ software (http://rsbweb.nih.gov/ij/). It is necessary to concentrate the culture medium to detect released HMGB1 by immunoblotting using a currently available anti-HMGB1 antibody. Therefore, to minimize oxidation of HMGB1 during such a preparation step, a stable transfectant expressing HMGB1 with a triple FLAG tag at the N-terminus was used. Values are means ± s.d. ($n = 4$). $^{**}p < 0.01$; $^{*}p < 0.05$

11.2.2 Cytokine-Dependent Expression of HMGB1 in Periodontal Ligament Cells

Periodontal ligament (PDL) cells are a heterogeneous population, mainly consisting of fibroblasts and osteoblasts. In an in vivo study, HMGB1 was found in periodontal cells and expression was increased in the healing process after orthodontic treatment in rat PDL [26]. Wolf et al. indicated a regulatory role for HMGB1 in the response of PDL cells to tissue damage induced by mechanical loading and in the initiation of the subsequent repair processes.

In vitro, human PDL cells express RAGE, TLR2, and TLR4 m-RNA, and produce IL-6 and IL-11 in response to HMGB1 via RAGE, TLR2, and TLR4 [27]. HMGB1 up-regulates the expression of proinflammatory and osteoclastogenic cytokines, such as TNF-α IL-1β, IL-6, IL-17, and receptor activator of nuclear factor kappa-B ligand (RANKL) [28]. These results suggest that PDL releases inflammatory cytokines by secreting HMGB1, and HMGB1 may enhance the progression and development of periodontal disease (Fig. 11.3a).

11.2.3 HMGB1 Localization in the Periodontal Pocket

In our previous study, we hypothesized that a periodontal pocket causes a unique pathological setting to induce HMGB1 release [29]. We demonstrated the distribution of HMGB1 around the periodontal pocket by immunohistochemistry. Hematoxylin and eosin (H.E.) staining (Fig. 11.3b upper panels) showed infiltration of inflammatory cells in the pocket epithelium (Fig. 11.3b right upper panel). In the gingival epithelium, HMGB1 was mainly localized in the nucleus (Fig. 11.3b left lower panel). In contrast, in the pocket epithelium, the localization of HMGB1 was exclusively cytoplasmic (Fig. 11.3b right lower panel). Immunohistochemical staining of gingiva showed that HMGB1 is dislocated from the nucleus to the cytoplasm of inflamed epithelial cells in pocket epithelium, whereas it is mainly present in the nucleus in the gingival epithelium. Furthermore, infiltration of leukocytes did not result in localization of HMGB1 in the nucleus (Fig. 11.3b right lower panel). The nature of these leukocyte cells was not identified in our study.

Butyric acid, an extracellular metabolite from periodontopathic bacteria populating the periodontal pocket, also known as a histone deacetylase (HDAC) inhibitor, is a virulence factor common to *P. gingivalis*, *Prevotella loescheii*, *Fusobacterium nucleatum*, *T. denticola*, and *T. forsythia* [30]. The concentration of butyric acid reaches 14–20 mM in subgingival plaques at the site of periodontitis [31]. When periodontal disease treatment is effective, butyrate concentrations in the GCF are low [32]. Therefore, butyric acid could be used as an indicator for the development and progression of periodontitis.

Butyric acid is involved in the pathogenesis of periodontal diseases via the induction of ROS production and the impairment of cell growth, cell cycle progression,

and expression of cell cycle-related genes in gingival fibroblasts [33]. Pretreatment of cells with the antioxidant *N*-acetyl-cysteine or 3-aminobenzamide attenuates butyric acid-induced apoptosis through a reduction of ROS generation in human Jurkat cells [34]. A previous study showed that during incubation with butyric acid, a human gingival epithelial cell line (Ca9-22 cells) was detached from the substratum and swelled [35]. In our previous study [29], we investigated the effect of butyric acid (0–10 mM) on the release of HMGB1. We found that butyric acid induces cell death, mainly by inducing necrotic cell death, and promotes generation of ROS in Ca9-22 cells. Butyric acid induces the release of HMGB1 time and dose dependently (Fig. 11.3a).

Butyric acid induces necrotic cell death in Ca9-22 cells. Because butyric acid-induced cell death is suppressed by cycloheximide (CHX), which is a known protein synthesis inhibitor (Fig. 11.3c), new synthesis of proteins due to increased gene expression by HDAC inhibitor activity of butyric acid may be involved in necrosis, such as proteins causing oxidative stress (Ebe et al., unpublished result).

Production of ROS was evident after incubation of Ca9-22 cells for 12 h with butyric acid at 10 mM, which coincided with the release of oxidized HMGB1 (Fig. 11.3d). Therefore, HMGB1 could be a target of oxidative stress induced by butyric acid (Ebe et al., unpublished result).

References

1. Bianchi ME, Beltrame M (1998) Flexing DNA: HMG-box proteins and their partners. Am J Hum Genet 63:1573–1577
2. Bonaldi T, Langst G, Strohner R, Becker PB, Bianchi ME (2002) The DNA chaperone HMGB1 facilitates ACF/CHRAC-dependent nucleosome sliding. EMBO J 21:6865–6873
3. Bianchi ME, Manfredi AA (2007) High-mobility group box 1 (HMGB1) protein at the crossroads between innate and adaptive immunity. Immunol Rev 220:35–46
4. Lotze MT, Tracey KJ (2005) High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. Nat Rev Immunol 5:331–342
5. Orlova VV, Choi EY, Xie C et al (2007) A novel pathway of HMGB1-mediated inflammatory cell recruitment that requires Mac-1-integrin. EMBO J 26:1129–1139
6. Scaffidi P, Misteli T, Bianchi ME (2002) Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 418:191–195
7. Wang H, Bloom O, Zhang M et al (1999) HMG-1 as a late mediator of endotoxin lethality in mice. Science 285:248–251
8. Pullerits R, Jonsson IM, Verdrengh M et al (2003) High mobility group box chromosomal protein 1, a DNA binding cytokine, induces arthritis. Arthritis Rheum 48:1693–1700
9. Abraham E, Arcaroli J, Carmody A, Wang H, Tracey KJ (2000) HMG-1 as a mediator of acute lung inflammation. J Immunol 165:2950–2954
10. Bonaldi T, Talamo F, Scaffidi P et al (2003) Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion. EMBO J 22:5551–5560
11. Gardella S, Andrei C, Ferrera D et al (2002) The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway. EMBO Rep 3:995–1001
12. Ditsworth D, Zong WX, Thompson CB (2007) Activation of poly(ADP)-ribose polymerase (PARP-1) induces release of the pro-inflammatory mediator HMGB1 from the nucleus. J Biol Chem 282:17845–17854

13. Bell CW, Jiang W, Reich CF 3rd, Pisetsky DS (2006) The extracellular release of HMGB1 during apoptotic cell death. Am J Physiol Cell Physiol 291:C1318–C1325
14. Liu A, Fang H, Dirsch O, Jin H, Dahmen U (2012) Oxidation of HMGB1 causes attenuation of its pro-inflammatory activity and occurs during liver ischemia and reperfusion. PLoS One. doi:10.1371/journal.pone.0035379
15. Hoppe G, Talcott KE, Bhattacharya SK, Crabb JW, Sears JE (2006) Molecular basis for the redox control of nuclear transport of the structural chromatin protein Hmgb1. Exp Cell Res 312:3526–3538
16. Kazama H, Ricci JE, Herndon JM et al (2008) Induction of immunological tolerance by apoptotic cells requires caspase-dependent oxidation of high-mobility group box-1 protein. Immunity 29:21–32
17. Tang D, Kang R, Zeh HJ III (2011) High-mobility group box 1, oxidative stress, and disease. Antioxid Redox Signal 14:1315–1335
18. Venereau E, Casalgrandi M, Schiraldi M et al (2012) Mutually exclusive redox forms of HMGB1 promote cell recruitment or proinflammatory cytokine release. J Exp Med 209: 1519–1528
19. Tang D, Kang R, Cheh CW et al (2010) HMGB1 release and redox regulates autophagy and apoptosis in cancer cells. Oncogene 29:5299–5310
20. Moore WE, Moore LV (1994) The bacteria of periodontal diseases. Periodontol 2000 5:66–77
21. Page RC (1991) The role of inflammatory mediators in the pathogenesis of periodontal disease. J Periodontal Res 26:230–242
22. Morimoto Y, Kawahara KI, Tancharoen S et al (2008) Tumor necrosis factor-alpha stimulates gingival epithelial cells to release high mobility-group box 1. J Periodontal Res 43:76–83
23. Luo L, Xie P, Gong P et al (2011) Expression of HMGB1 and HMGN2 in gingival tissues, GCF and PICF of periodontitis patients and peri-implantitis. Arch Oral Biol 56:1106–1111
24. Feghali K, Iwasaki K, Tanaka K et al (2009) Human gingival fibroblasts release high-mobility group box-1 protein through active and passive pathways. Oral Microbiol Immunol 24:292–298
25. Ito Y, Bhawal UK, Sasahira T et al (2012) Involvement of HMGB1 and RAGE in IL-1β-induced gingival inflammation. Arch Oral Biol 57:73–80
26. Wolf M, Lossdörfer S, Abuduwali N, Jäger A (2012) Potential role of high mobility group box protein 1 and intermittent PTH (1–34) in periodontal tissue repair following orthodontic tooth movement in rats. Clin Oral Investig. doi:10.1007/s00784-012-0777-2
27. Hasegawa N (2008) Effect of high mobility group box 1 (HMGB1) in cultured human periodontal ligament cells. Kokubyo Gakkai Zasshi 75:155–161
28. Kim YS, Lee YM, Park JS, Lee SK, Kim EC (2010) SIRT1 modulates high-mobility group box 1-induced osteoclastogenic cytokines in human periodontal ligament cells. J Cell Biochem 111:1310–1320
29. Ebe N, Hara-Yokoyama M, Iwasaki K et al (2011) Pocket epithelium in the pathological setting for HMGB1 release. J Dent Res 90:235–240
30. Kurita-Ochiai T, Fukushima K, Ochiai K (1995) Volatile fatty acids, metabolic by-products of periodontopathic bacteria, inhibit lymphocyte proliferation and cytokine production. J Dent Res 74:1367–1373
31. Margolis HC, Duckworth JH, Moreno EC (1988) Composition and buffer capacity of pooled starved plaque fluid from caries-free and caries-susceptible individuals. J Dent Res 67:1476–1482
32. Qiqiang L, Huanxin M, Xuejun G (2012) Longitudinal study of volatile fatty acids in the gingival crevicular fluid of patients with periodontitis before and after nonsurgical therapy. J Periodontal Res 47:740–749
33. Chang MC, Tsai YL, Chen YW et al (2013) Butyrate induces reactive oxygen species production and affects cell cycle progression in human gingival fibroblasts. J Periodontal Res 48:66–73
34. Kurita-Ochiai T, Amano S, Fukushima K, Ochiai K (2003) Cellular events involved in butyric acid-induced T cell apoptosis. J Immunol 171:3576–3584
35. Takigawa S, Sugano N, Nishihara R et al (2008) The effect of butyric acid on adhesion molecule expression by human gingival epithelial cells. J Periodontal Res 43:386–390

Part III
Periodontal Diseases and Systemic Diseases (Possible Link to Other Organs)

Chapter 12
The Inflammation as the Connecting Link Between Systemic Diseases and Periodontal Diseases

Juan Manuel Morillo-Velázquez

12.1 Introduction

Currently, periodontal diseases are considered as an inflammatory condition triggered by some bacterial species living in the gingival sulcus or the periodontal pocket. Numerous epidemiological studies have found associations between periodontitis and conditions such as diabetes, cardiovascular disease (CVD), osteoporosis, respiratory diseases, rheumatoid arthritis, kidney disease, and dementia, among others, but there is a lack of consensus on the nature of these associations [1]. Other approach using the prevalence of cardiovascular and autoimmune diseases among patients attending a dental or periodontal clinic revealed that the prevalence of hypertension, diabetes mellitus, and rheumatoid arthritis is significantly increased in patients with periodontitis. However, when controlled for confounder factors, periodontitis was associated with diabetes only in patients from the dental clinic, whereas hypertension does not seem to be associated with periodontitis. An important finding is that periodontitis may be associated with rheumatoid arthritis, regardless the clinic type [2].

One important challenge to elucidate this relationship is the little evidence about the link connecting periodontitis with other systemic conditions. One interesting hypothesis is that inflammation could be that connecting link between them. In this chapter, our goal will be to show the recent advances regarding:

- systemic inflammatory markers in periodontitis patients, either systemically healthy or suffering from other systemic conditions;
- the effect of periodontal therapy over several systemic inflammatory markers, either systemically healthy or suffering from other systemic conditions;

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- putative pathogenic mechanisms that could be shared with other conditions, such as cross-reactivity to *Porphyromonas gingivalis* (*Pg*) heat shock proteins (HSP) or citrullination of host proteins.

12.2 Systemic Inflammatory Markers and Periodontitis

When the terms *systemic inflammation* or *systemic inflammatory response* are used, we refer to the occurrence of some inflammatory markers in serum, released from relevant organs, such as liver, in response to a local inflammatory challenge as occurs in periodontitis and other oral conditions. To assess this systemic response, it is necessary to determine which of those markers are more relevant, and the vast majority of studies consider C-reactive protein (CRP) as one of the main markers, in addition to interleukin 6 (IL-6) and others.

The acute-phase response is a set of biochemical responses to tissue damage, that comprise essentially the synthesis of proteins in hepatocytes and other cell types, under the control of cytokines (IL-6 and others) released by macrophages and other cells at the affected site. CRP is considered the main acute-phase protein, and is found in high levels in the blood in response to inflammation, infection, trauma and tissue necrosis, cancer, and autoimmune disorders. Among their roles, it can bind to phosphocholine expressed on the surface of affected cells and some bacterial species in order to activate the complement system and thus facilitating their clearance [3–5].

12.2.1 Animal Models

In recent years, several animal model-based studies have been published regarding the relationship of periodontitis or the effect of some periodontal species over the serum levels of some inflammatory molecules. In this sense, the studies by the group from the Department of Preventive Dentistry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences are very interesting. Using a rat model, they measured systemic inflammatory markers in obese and lean rats, with and without ligature induced periodontitis. The production of CRP, IL-6, and tumor necrosis factor alpha (TNF-α) in the liver, as well as CRP and IL-6 in the white adipose tissue of the obesity/periodontitis group was significantly higher than in the remaining groups after 4 weeks. Moreover, some serum markers, such as TNF-α in the periodontitis or obesity groups, were significantly higher than in the control group, or serum CRP and TNF-α in the obesity/periodontitis group were significantly higher than in the remaining groups [6]. These data indicate the additive effect of periodontitis on inflammatory markers when other conditions, such as obesity, are present.

The same research group, also using a rat model, studied whether the generation of lipid peroxide in periodontal tissue inflammation could induce tissue injury in the

liver, heart, kidney, and brain. By topical application of bacterial lipopolysaccharide and proteases to the gingival sulcus for 4 weeks they induced periodontal inflammation in a test group that was compared to a control group. The induced periodontal inflammation in test group led to increasing gingival and serum levels of a lipid peroxide marker, hexanoyl-lysine. The level of another oxidative stress marker, 8-hydroxydeoxyguanosine, increased in an important manner in mitochondrial DNA from the liver, heart, kidney, and brain of rats with periodontal inflammation. Thus, the excessive production of lipid peroxide following periodontal inflammation is involved in oxidative DNA damage of other organs, such as brain, heart, liver, and kidney [7].

Other studies have focused on the effect of *P. gingivalis* infection. One of these reports show a murine model in which periodontitis was induced by ligatures previously incubated or not with *Pg*, or by oral gavage with *Pg*. Periodontal tissue destruction and osteoclast number were significantly elevated in the group with ligature incubated with *Pg* compared to the remaining groups. The synthesis of some tissue destruction markers, such as cathepsin B and matrix metalloproteinase 9 (MMP9), was related to bone destruction processes and *Pg* infection. The highest serum levels of IL-6 and IL-1β were observed in the group with ligature incubated with *Pg*, but also this group showed a decrease of IL-6 and an increase of IL-1β serum level with time [8]. Thus, *Pg* infection appears to be able to modify the systemic response to periodontal inflammation. Findings from this one and previously described studies support the role of periodontitis in the onset and progression of systemic inflammation in several animal species.

12.2.2 Observational Studies in Systemically Healthy Humans

The first step to search a link between a putative risk factor and a condition is conducting observational studies in human populations. As previously mentioned, the main systemic inflammatory marker that has been determined in most studies is CRP. This protein has been found in gingival tissue and gingival crevicular fluid in healthy and pathological locations from periodontitis and healthy subjects, although gingival tissue had not detectable amounts of CRP mRNA. This indicates that CRP in gingival locations appears to have a systemic origin [9]. In another study evaluating systemically healthy subjects, either affected by severe periodontitis or without a history of periodontal disease, full-mouth bleeding score was a predictor of CRP levels in periodontitis patients [10]. Another report reveals that patients suffering from severe and moderate periodontitis had higher mean serum CRP levels, with a close association with higher clinical attachment loss. The presence of *Pg* and *Aggregatibacter actinomycetemcomitans* was also associated with elevated CRP levels and poor periodontal status [11]. When evaluating differences in the inflammatory profile of young systemically healthy periodontal patients with chronic or aggressive periodontitis, no statistically significant difference between the two groups was detected for periodontal variables and for CRP or other cytokines [12].

All these findings support the close association among different clinical and microbiological periodontal parameters and serum and local levels of CRP, but always of systemic origin.

In addition to CRP, other inflammatory markers have been studied in humans. Certain genetic polymorphisms or deficiencies in specific complement components appear to predispose to increased susceptibility to periodontitis. Moreover, *Pg* subverts complement receptor 3 and C5a anaphylatoxin receptor signaling, thus allowing its adaptation to host [13]. Another report found that chronic periodontitis patients had higher serum leptin and IL-6 levels than healthy subjects. Moreover, the serum leptin level was associated with mean probing depth, mean clinical attachment level, and mean alveolar bone loss [14]. This hormone has been associated with some conditions, such as obesity or CVD.

Another way to measure inflammation is by using blood leukocyte numbers and differential counts. Higher neutrophil numbers and serum globulin levels have been found in patients with aggressive periodontitis compared to healthy individuals, in such a way that those inflammatory markers were positively correlated with periodontal parameters [15]. When taking account possible confounder factors such as age, gender, smoking, and ethnicity, subjects with severe periodontitis exhibit higher white cell counts, with a trend for an association between the number of periodontal pockets and this inflammatory marker [16].

Our group evaluated adults with or without periodontitis regarding total plasma fatty acids, saturated, n-6 polyunsaturated and monounsaturated fatty acids, peroxidability index or TNF-α, among other markers, and we found these markers were significantly higher in the periodontitis group compared to the non-periodontitis group, thus noting that periodontitis appear to enhance a low-grade inflammatory state that could contribute to the pathogenesis or progression of other systemic conditions [17].

As conclusion derived from these studies, we can note that periodontitis has an enhancing effect of systemic inflammation, in such a way that higher CRP, IL-6, TNF-α, leptin, white cell counts, or lipid parameters could be predicted in periodontitis subjects compared to in periodontally healthy individuals, thus increasing the risk of other systemic conditions sharing an inflammatory component.

12.2.3 Observational Studies in Humans with Systemic Conditions

In recent years, an increasing number of reports regarding inflammatory markers in periodontitis subjects suffering from other systemic conditions have been published. In this sense, it is important to show the relevance of oral health in patients with chronic kidney disease, as one recent review has emphasized [18]. One study has used the National Health and Nutrition Examination Survey 1988–1994 (NHANES III) dataset including individuals with chronic kidney disease, 12.3 % of them with periodontitis. Among these, 41.8 % had serum CRP higher than 0.3 mg/dL

compared with 27.1 % of periodontally healthy individuals. A strong association between extent of periodontitis and serum CRP levels was found after adjusting by confounder factors [19]. On the other hand, patients on chronic dialysis have also been studied. One report found a prevalence of 57.5 % of subjects with periodontitis in its sample. Near 52.2 % of them had CRP levels >1 mg/dL, in comparison to only 29.4 % of subjects with healthy periodontium [20]. With respect to transplant recipients, one study compared patients with renal and cardiac transplant to age-matched controls. The prevalence of severe periodontitis was not statistically significantly different between transplant and control subjects. However, serum IL-6 and CRP were higher in transplant compared to control subjects, but severe periodontitis was not a significant positive predictor of serum IL-6 in transplant group when multivariate analysis was performed [21].

Inflammatory response as a link between metabolic conditions and periodontitis is increasingly studied. Our group wrote a review about metabolic syndrome (MetS) [22], but new findings have been reported. The association of alveolar bone loss and MetS parameters was analyzed using data from subjects participating in a large longitudinal study. Participants with radiographic evidence of moderate to advanced alveolar bone loss were significantly more likely to have MetS than those with minimal or no bone loss. However, there were no significant differences in systemic inflammation measured by using white blood cells count between subjects with or without periodontitis [23]. Another study has found that TNF-α and IL-6 were associated with the periodontitis–MetS coexistence [24]. With respect to CVD, one report compared two groups of patients suffering from chronic periodontitis with or without CVD. CRP levels were significantly higher in the CVD group compared to the control group. With respect to periodontitis, its main finding is a negative correlation between tooth loss and protein C and between CRP and protein C [25]. In subjects with type 2 diabetes and periodontitis, an increased mean probing depth was significantly associated to higher levels of CRP, after taking account confounder factors such as age, gender, body mass index, duration of diabetes mellitus, smoking, regular physical exercise, and alcohol consumption. No significant difference was found among different groups in the levels of serum TNF-α, fasting glucose, and lipid profiles [26].

In conclusion, there is a trend to higher CRP levels in periodontitis subjects affected by chronic kidney disease, dialysis, or type 2 diabetes compared to periodontally healthy, but there is not a clear additional rising of inflammatory markers in subjects with MetS, other CVDs, or transplant recipients when affected by periodontitis.

12.2.4 Rheumatoid Arthritis and Periodontitis: A Strong Link Between Two Inflammatory Conditions

Rheumatoid arthritis (RA) is an autoimmune condition characterized by a progressive destruction of joint structures [27]. We think that this disease is a good model

to understand possible pathogenic mechanisms linking periodontitis and other inflammatory conditions. Recent years have plenty of findings enhancing the association between rheumatoid arthritis and periodontitis [28]. It is worth noting the importance of *Pg*, either indirectly through the host immune responses or directly through virulence factors from this bacterial species [29].

Prevalence of severe periodontitis appears to be higher in rheumatoid arthritis subjects (27 %) in comparison to healthy individuals (12 %) [28], although another study reported a prevalence of 12.5 % for mild periodontitis and 75 % for moderate cases [30]. Patients with RA and severe periodontitis have higher activity scores than those with RA but no or moderate periodontitis, in addition to higher anti-*Pg* antibody titers than subjects with non-RA severe periodontitis. Interestingly, subgingival occurrence of *Pg* was similar, so a key fact is the distinct response to the same microbial load in RA subjects [28]. An association between occurrence of RA and some periodontal parameters, such as bleeding on probing or clinical attachment loss when comparing a group of RA cases to another control group has been noted [31], in addition to a positive correlation between severity of periodontal disease and rheumatoid arthritis [30]. In a long-term longitudinal study that evaluated more than 9,000 participants, those with periodontitis or five or more missing teeth showed higher prevalence and incidence of RA, but without reaching statistical significance [32]. Nevertheless, a recent review also noted no consistent differences in periodontal parameters and inflammatory biomarkers between RA subjects and healthy adults with periodontitis [33]. Another comparative study determined IL-1β and IL-10 in serum of subjects with either RA under therapy, chronic periodontitis, and systemically/periodontally healthy. The total amount and level of IL-10 was not significantly different between the groups, but IL-1β was significantly lower in the RA group compared to the remaining groups. Nevertheless, internal validity of this study is questionable, as different groups were not properly age and gender matched [34].

In conclusion, whether some studies support the close association between periodontitis and rheumatoid arthritis, either taking account the prevalence of periodontal affectation in rheumatic subjects or other parameters such as immune response to periodontopathogen bacteria, other reports find inconsistent results to support this hypothesis, so future research with better designs are warranted.

12.3 Effect of Periodontal Therapy Over Systemic Inflammatory Markers

Epidemiological studies are very important to understand the possible association between systemic conditions and periodontitis, as previously mentioned. But another approach is to analyze the influence of periodontal therapy over systemic inflammatory markers, in systemically healthy subjects and in subjects suffering other conditions, by means of clinical trials, if possible with randomized controlled trials [35]. In this section, we will show the more recent studies on this topic.

12.3.1 Systemically Healthy Individuals with Periodontitis

A randomized controlled trial with individuals suffering from chronic periodontitis allocated to either initial periodontal treatment or no therapy in a 3-month period, test group showed a no significant decrease in fibrinogen level, in addition to significant increases in hemoglobin and hematocrit [36]. Another interesting report evaluated the variation in inflammatory parameters following nonsurgical and surgical periodontal therapy. Fourteen chronic periodontitis subjects received nonsurgical treatment and at least two surgical sessions after 6 months. In addition to the improvement in periodontal parameters, important increases in the serum levels of CRP and serum amyloid-A were found soon after nonsurgical and surgical therapies, but a greater increase in D-dimer and CRP levels was found following nonsurgical therapy [37]. In relation to renal function evaluated by glomerular filtration rate in systemically healthy subjects with periodontitis, another study found that periodontal therapy appeared to be associated with a significant decrease in cystatin C level along the 180 days of follow-up. In the short term, periodontal therapy was related to greater increases for CRP and serum amyloid-A, while D-dimer and fibrinogen showed only mild variations. After 30 days, inflammatory markers were normalized [38]. One interesting finding in patients with severe periodontitis is that plasma glucose, lipids, and markers of systemic inflammation were not significantly altered following 3 months therapy, but one year after, IL-18 and interferon-gamma levels were lower. There was no variation in plasma levels of IgA, IgG1, IgG2 antibodies against HSP [39]. Another report describes a significant reduction of serum leptin, IL-6, and CRP levels after nonsurgical periodontal therapy [14]. In conclusion, most studies agree in a rising of levels of some acute-phase proteins just after periodontal therapy, especially if nonsurgical, but subsequently there is a trend to the reduction or normalization of these markers.

With respect to oxidative stress, a study compared diacron-reactive oxygen metabolites (D-ROM) levels and total antioxidant capacity in periodontitis and healthy individuals. Patients with severe periodontitis exhibited higher D-ROM levels and lower total antioxidant capacity. There was a positive correlation between D-ROM levels and CRP or periodontal parameters, but an interesting finding was that oxidative stress increased following periodontal therapy, as significant increases of D-ROM were found in treated cases [40]. As previously mentioned, we might hypothesize a long-term reduction of the oxidative stress, once tissues recover from surgical challenge.

In contrast to previous studies, some reports note the variability in inflammatory responses across subjects. In severe periodontitis patients, following treatment completion, some adhesion molecules, defense or tissue destruction markers, such as PAI-1, sE-selectin, sVCAM-1, MMP-9, and myeloperoxidase, were significantly reduced. However, only sE-selectin, sICAM, and serum amyloid P sustained a reduction after 4 weeks. It is surprising the finding that changes in inflammatory markers hardly correlated with clinical, microbiological, and serological parameters of periodontitis, and moreover, the responses were inconsistent across subjects [41].

All these inconsistent findings warrant the conduction of new trials with assessment of systemic inflammatory markers at short and long-term after nonsurgical and surgical therapy.

12.3.2 Rheumatoid Arthritis

One trial compared patients suffering from chronic periodontitis and rheumatoid arthritis, either with moderate to high disease activity or with low disease activity. Erythrocyte sedimentation rate, CRP and TNF-α levels in serum significantly decreased 3 months after the nonsurgical periodontal treatment, without differences between groups. In addition to these findings, there was an improvement of arthritis activity and periodontal parameters [42], results shared by another recent study [30].

12.3.3 Metabolic Syndrome

With respect to MetS, a clinical trial evaluated the effect of nonsurgical periodontal therapy in subjects with chronic generalized periodontitis, either with MetS or systemically healthy subjects. At 2 months after the periodontal therapy, in MetS subjects, a significant decrease was found in mean serum CRP, total leukocyte counts, and serum triglycerides, along with a significant increase in serum high-density lipoprotein (HDL), whereas in systemically healthy periodontal patients, changes in these parameters were not statistically significant [43]. In another study, patients with MetS and periodontitis were randomized to an experimental group that received plaque control and root planing plus amoxicillin and metronidazole or to a control treatment group that received plaque control instructions, supragingival scaling, and two placebos. The periodontal parameters significantly improved in both groups following 3 months therapy and were lower than baseline at 12 months, with better results in the experimental group. CRP levels decreased progressively and were significantly lower following 9 and 12 months after the therapy. Fibrinogen levels significantly decreased only in the experimental group at 6 and 12 months [44].

12.3.4 Hemodialysis

One trial describes a high prevalence of periodontitis in hemodialysis patients (63 %). CRP levels were positively associated with clinical periodontal status before treatment, and decreased significantly following periodontal therapy, in such a way that erythropoietin dosage could be reduced from 8,000 to 6,000 unit/week after treatment. Pre-dialysis blood urea nitrogen and serum albumin level increased after periodontal treatment [45].

12.3.5 Diabetes Mellitus

An interesting trial evaluated 30 periodontitis subjects, 15 of them with type 2 diabetes, either well or poorly controlled, and 15 systemically healthy. HbA1c levels in the poorly controlled group with diabetes decreased significantly following 3 months therapy. However, no significant decreases in TNF-α and CRP levels were noted. IL-6 levels decreased in well-controlled diabetic and healthy, whereas an interesting response in adipokines was found, with higher adiponectin levels in healthy individuals and higher leptin levels in well-controlled diabetic subjects after therapy [46].

12.3.6 Pregnancy

An interesting study analyzed the effect of scaling and root planing in pregnant women with periodontitis, either before 21 weeks of gestation or after delivery. Periodontal treatment didn't modify the level of CRP, PGE2, MMP-9, fibrinogen, endotoxin, and cytokines such as IL-1β, IL-6, IL-8, or TNF-α. Levels of any inflammatory marker were not significantly associated with preterm birth or infant birth weight. However, only the variation in endotoxin was negatively associated with the change in probing depth [47]. In this sense, another study describes a new sensitive assay to determine endotoxin activity that was used with sera from pregnant women with periodontitis in order to detect a low-level bacteremia in chronic periodontitis. These authors found a positive endotoxin activity in 35.5 % of the pregnant women [48].

In conclusion, there is scarcity of studies analyzing the effect of periodontal therapy on systemic inflammatory markers in subjects with other systemic conditions. Nevertheless, there is a trend to a clear improvement in individuals affected by rheumatoid arthritis, MetS, or hemodialysis, but the influence is not clear in diabetic subjects or pregnant women.

12.4 Autoimmune Processes as Inflammatory Link Between Periodontitis and Systemic Conditions

Previous sections in this chapter have focused in the epidemiological association between inflammatory markers and periodontitis, either in systemically healthy or affected individuals. But it is very important to analyze the underlying pathogenic mechanisms that support this association. One interesting area of increasing knowledge is the elicitation of autoimmune responses related to oral pathogens, among them, *Pg* has a preeminent role. Two possible mechanisms related to this species are the cross-reactivity between HSP derived from *Pg* and human cells, and the citrullination enhanced by bacterial enzymes that facilitate the formation of neo-epitopes.

12.4.1 Pg *HSP and Systemic Diseases: Molecular Mimicry as Pathogenic Mechanism*

HSP are groups of proteins whose main role is to protect microorganisms and eukaryotic cells from stress conditions. From an evolutionary viewpoint, these proteins are highly conserved. They act as molecular chaperones in the assembly and folding of proteins, and as proteases when damaged or toxic proteins have to be degraded. Some HSP of oral bacteria have been discovered and characterized regarding location, cytotoxic effects, or sequence homology, and these proteins are important antigens from an immunologic perspective in many human pathogens. Two of these proteins are GroEL and HSP60. The presence of shared epitopes between host proteins and microbial HSP may lead to autoimmune responses that act as mechanism of tissue destruction [49].

Although research has been led to the study of HSP of several periodontopathogen species, our focus will be those from *Pg*. Occurrence of serum antibodies to *Pg* GroEL in periodontitis patients is higher than in healthy subjects, and moreover, these antibodies have been also detected in all samples of gingival tissue extracts. Serum antibodies to human HSP60 are also more prevalent in periodontitis patients, with stronger reactivity. More interesting, this same study demonstrated cross-reactivity of serum antibodies to human HSP60 and *Pg* GroEL [50]. Another relevant finding is the variable response among individuals regarding levels of serum anti-*Pg* GroEL antibody after periodontal therapy, and the apparent independence of anti-human HSP60 antibodies related to periodontal treatment [51]. Another report found showed a significant correlation between a cross-reactive epitope peptide belonging to *Pg* HSP60 and the amount of alveolar bone [52].

In the last decade, several studies have found a strong association between immune response against *Pg* HSP and atherosclerosis. Reactivity of anti-*Pg* antibodies with *Pg* HSP and human HSP has been observed in sera from periodontitis or atherosclerosis subjects, thus emphasizing the importance of cross-reactivity against HSP as pathogenic mechanism in inflammatory conditions [53]. When humoral and cellular responses against human and *Pg* HSP60 have been studied, increasing levels were observed from healthy subjects compared to periodontitis and atherosclerosis patients. With respect to cellular response, the occurrence of human HSP60 and *Pg* GroEL-reactive T-cell populations has been described in the peripheral circulation and atherosclerotic lesions of atherosclerosis subjects [54]. Another interesting study found *Pg* in all of the artery specimens from atherosclerosis patients, and these subjects also showed cross-reactivity between anti-GroEL or anti-*Pg* antibodies with human HSP60 [55]. Human HSP60 expression has been documented on endothelial cells and other cells with the appearance of smooth muscle cells and lymphocytes in the inflammatory cell infiltrate of carotid endarterectomy specimens, which also showed *Pg* occurrence in the 52 % of arteries. Furthermore, GroEL and bacteria were detected within intimal cells [56]. In cardiovascular patients with history of myocardial infarction, increasing anti-human HSP60 antibody was noted as the number of bacterial species increased. There was

a correlation between anti-human HSP60 level and anti-GroEL levels. Moreover, patients with deepest pockets had higher numbers of *Pg* and anti-human HSP60 levels [57].

The peptide 19 is a region of *Pg* and human HSP60 that shows cross-reactivity between them. Sera from 30 % of periodontitis subjects and 100 % of atherosclerosis patients with periodontal disease and previous surgical intervention for atheromatous plaques reacted positively to this peptide from both *Pg* and human HSP60 [58]. Furthermore, sera from periodontal patients with atherosclerosis, type 2 diabetes, rheumatoid arthritis or systemically healthy reacted with the peptide 19 from *Pg* HSP60 as relevant epitope [59].

All these findings support the hypothesis of cross-reactivity between *Pg* and human HSP as a triggering factor of a systemic inflammatory response that could have an effect on the progression of periodontitis and other inflammatory conditions. The putative relationship between anti-HSP antibody levels and inflammatory markers, such as CRP, needs to be explored.

12.4.2 Citrullination of Proteins as Enhancing of Autoimmune Processes

The amino acid citrulline is generated by an enzymatic modification of the amino acid arginine by peptidyl arginine deiminases (PAD). Arginine is an amino acid associated with autoantigenicity in proteins. Their conversion to citrulline in certain self-proteins generates neo-epitope structures that result in reduced self-tolerance, development of autoimmunity, and the production of anti-citrullinated peptide (anti-CCP) antibodies. Citrullination plays a physiologic role in the regulation of protein folding and degradation. Whereas the generation of citrullinated peptides is not unique to rheumatoid arthritis, the development of antibodies against them is quite specific to this condition. In the last years, an important discovery has been the presence of an arginine-specific proteinase in several oral bacteria, with *Pg* among them [60–62]. A recent finding that supports the relevance of this bacterial species is that anti-*Pg* antibody titers in patients with rheumatoid arthritis are associated with the concentration of rheumatoid factor and anti-citrullinated peptides antibodies [63, 64].

As biological process, citrullination has been evaluated in gingival tissues. Citrullinated proteins, PAD-2 and PAD-4 have been detected in gingiva. Whereas expression of both enzymes was detected in both inflamed and non-inflamed gingival tissues, a positive correlation between inflammation and expression of these proteins has been found. Nevertheless, the presence of anti-CCP antibodies in gingival fluid was almost exclusive to a subset of patients with periodontitis [65]. An interesting histological study with polyclonal and monoclonal antibodies against citrullinated proteins found that, in the periodontal epithelium, citrullination is a physiological process, whereas in the periodontal connective tissue, citrullination is

an inflammation-dependent process. The presence of citrullinated protein was higher in periodontitis stroma (80 %) compared to control stroma (33 %) [66]. In an in vitro study, citrullination of proteins within inflamed periodontal tissues was evaluated. *Pg* produces a PAD which can citrullinate extracellular proteins and may increase the citrullinated protein levels in gingival tissues, but it doesn't affect PAD expression or citrullination by host monocytes or macrophages [67].

In the last years, the identification of specific citrullinated antigens has been a focus of research. Fibrinogen, vimentin, collagen type II, and α-enolase, are four main antigens expressed in the joint, and antibodies to citrullinated fibrinogen and collagen type II mediate inflammation by the formation of immune complexes [68]. In this sense, endogenous protein citrullination by *Pg* has been a significant finding. Incubation of *Pg* with fibrinogen or α-enolase generated degradation of the proteins and citrullination of the resulting peptides [69]. Moreover, in a murine model, arthritis was induced by immunization with *Pg* citrullinated and uncitrullinated enolase [70].

As conclusion, there is scarcity of data about the relationship between anti-CCP antibodies and inflammatory markers or clinical parameters in periodontitis subjects, or the relevance of other periodontopathogen species in the triggering of the citrullination process, so more research is warranted about this topic.

12.5 Conclusions

Increasing research is supporting the close link between periodontal and systemic conditions, with inflammatory markers as a main focus of interest. CRP has been the most studied marker, but other cytokines, included adipokines, have also been related to both types of conditions. Moreover, there is evidence of the effect of periodontal therapy on some of these inflammatory markers, and a possible influence of this fact over the progression of the systemic condition.

Rheumatoid arthritis is a good model to study common inflammatory processes with periodontitis. Two pathogenic mechanisms, cross-reactivity to *Pg* HSP and citrullination of host proteins by *Pg*, should be studied thoroughly, either related to periodontal pathogenesis or other systemic conditions, such as atherosclerosis.

References

1. Otomo-Corgel J, Pucher JJ, Rethman MP, Reynolds MA (2012) State of the science: chronic periodontitis and systemic health. J Evid Based Dent Pract 12(Suppl 3):20–28
2. Nesse W, Dijkstra PU, Abbas F, Spijkervet FK, Stijger A, Tromp JA, van Dijk JL, Vissink A (2010) Increased prevalence of cardiovascular and autoimmune diseases in periodontitis patients: a cross-sectional study. J Periodontol 81:1622–1628
3. Hirschfield GM, Pepys MB (2003) C-reactive protein and cardiovascular disease: new insights from an old molecule. QJM 96:793–807

4. Pepys MB, Hirschfield GM (2003) C-reactive protein: a critical update. J Clin Invest 111:1805–1812
5. Thompson D, Pepys MB, Wood SP (1999) The physiological structure of human C-reactive protein and its complex with phosphocholine. Structure 7:169–177
6. Endo Y, Tomofuji T, Ekuni D, Irie K, Azuma T, Tamaki N, Yamamoto T, Morita M (2010) Experimental periodontitis induces gene expression of proinflammatory cytokines in liver and white adipose tissues in obesity. J Periodontol 81:520–526
7. Tomofuji T, Ekuni D, Irie K, Azuma T, Tamaki N, Maruyama T, Yamamoto T, Watanabe T, Morita M (2011) Relationships between periodontal inflammation, lipid peroxide and oxidative damage of multiple organs in rats. Biomed Res 32:343–349
8. Saadi-Thiers K, Huck O, Simonis P, Tilly P, Fabre JE, Tenenbaum H, Davideau JL (2012) Periodontal and systemic responses in various mice models of experimental periodontitis: respective roles of inflammation duration and Porphyromonas gingivalis infection. J Periodontol. doi:10.1902/jop.2012.110540
9. Megson E, Fitzsimmons T, Dharmapatni K, Bartold PM (2010) C-reactive protein in gingival crevicular fluid may be indicative of systemic inflammation. J Clin Periodontol 37:797–804
10. Cairo F, Nieri M, Gori AM, Rotundo R, Castellani S, Abbate R, Pini-Prato GP (2009) Periodontal variables may predict sub-clinical atherosclerosis and systemic inflammation in young adults: a cross-sectional study. Eur J Oral Implantol 2:125–133
11. Pejcic A, Kesic LJ, Milasin J (2011) C-reactive protein as a systemic marker of inflammation in periodontitis. Eur J Clin Microbiol Infect Dis 30:407–414
12. Cairo F, Nieri M, Gori AM, Tonelli P, Branchi R, Castellani S, Abbate R, Pini-Prato GP (2010) Markers of systemic inflammation in periodontal patients: chronic versus aggressive periodontitis. An explorative cross-sectional study. Eur J Oral Implantol 3:147–153
13. Hajishengallis G (2010) Complement and periodontitis. Biochem Pharmacol 80:1992–2001
14. Shimada Y, Komatsu Y, Ikezawa-Suzuki I, Tai H, Sugita N, Yoshie H (2010) The effect of periodontal treatment on serum leptin, interleukin-6, and C-reactive protein. J Periodontol 81:1118–1123
15. Shi D, Meng H, Xu L, Zhang L, Chen Z, Feng X, Lu R, Sun X, Ren X (2008) Systemic inflammation markers in patients with aggressive periodontitis: a pilot study. J Periodontol 79:2340–2346
16. Nibali L, D'Aiuto F, Griffiths G, Patel K, Suvan J, Tonetti MS (2007) Severe periodontitis is associated with systemic inflammation and a dysmetabolic status: a case–control study. J Clin Periodontol 34:931–937
17. Ramirez-Tortosa MC, Quiles JL, Battino M, Granados S, Morillo JM, Bompadre S, Newman HN, Bullon P (2010) Periodontitis is associated with altered plasma fatty acids and cardiovascular risk markers. Nutr Metab Cardiovasc Dis 20:133–139
18. Akar H, Akar GC, Carrero JJ, Stenvinkel P, Lindholm B (2011) Systemic consequences of poor oral health in chronic kidney disease patients. Clin J Am Soc Nephrol 6:218–226
19. Ioannidou E, Swede H, Dongari-Bagtzoglou A (2011) Periodontitis predicts elevated C-reactive protein levels in chronic kidney disease. J Dent Res 90:1411–1415
20. Nadeem M, Stephen L, Schubert C, Davids MR (2009) Association between periodontitis and systemic inflammation in patients with end-stage renal disease. SADJ 64:470–473
21. Shaqman M, Ioannidou E, Burleson J, Hull D, Dongari-Bagtzoglou A (2010) Periodontitis and inflammatory markers in transplant recipients. J Periodontol 81:666–672
22. Bullon P, Morillo JM, Ramirez-Tortosa MC, Quiles JL, Newman HN, Battino M (2009) Metabolic syndrome and periodontitis: is oxidative stress a common link? J Dent Res 88:503–518
23. Nesbitt MJ, Reynolds MA, Shiau H, Choe K, Simonsick EM, Ferrucci L (2010) Association of periodontitis and metabolic syndrome in the Baltimore Longitudinal Study of Aging. Aging Clin Exp Res 22:238–242
24. Han DH, Shin HS, Kim MS, Paek D, Kim HD (2012) Group of serum inflammatory markers and periodontitis-metabolic syndrome coexistence in Koreans. J Periodontol 83:612–620

25. Malali E, Basar I, Emekli-Alturfan E, Elemek E, Oktay S, Ayan F, Emekli N, Noyan U (2010) Levels of C-reactive protein and protein C in periodontitis patients with and without cardiovascular disease. Pathophysiol Haemost Thromb 37:49–54
26. Chen L, Wei B, Li J, Liu F, Xuan D, Xie B, Zhang J (2010) Association of periodontal parameters with metabolic level and systemic inflammatory markers in patients with type 2 diabetes. J Periodontol 81:364–371
27. Culshaw S, McInnes IB, Liew FY (2011) What can the periodontal community learn from the pathophysiology of rheumatoid arthritis? J Clin Periodontol 38(Suppl 11):106–113
28. de Smit MJ, Brouwer E, Vissink A, van Winkelhoff AJ (2011) Rheumatoid arthritis and periodontitis; a possible link via citrullination. Anaerobe 17:196–200
29. Rutger Persson G (2012) Rheumatoid arthritis and periodontitis - inflammatory and infectious connections. Review of the literature. J Oral Microbiol 4, 10.3402/jom.v4i0.11829
30. Ranade SB, Doiphode S (2012) Is there a relationship between periodontitis and rheumatoid arthritis? J Indian Soc Periodontol 16:22–27
31. Torkzaban P, Hjiabadi T, Basiri Z, Poorolajal J (2012) Effect of rheumatoid arthritis on periodontitis: a historical cohort study. J Periodontal Implant Sci 42:67–72
32. Demmer RT, Molitor JA, Jacobs DR Jr, Michalowicz BS (2011) Periodontal disease, tooth loss and incident rheumatoid arthritis: results from the first national health and nutrition examination survey and its epidemiological follow-up study. J Clin Periodontol 38:998–1006
33. Han JY, Reynolds MA (2012) Effect of anti-rheumatic agents on periodontal parameters and biomarkers of inflammation: a systematic review and meta-analysis. J Periodontal Implant Sci 42:3–12
34. Cetinkaya B, Guzeldemir E, Ogus E, Bulut S (2012) Pro- and anti-inflammatory cytokines in gingival crevicular fluid and serum of rheumatoid arthritis and chronic periodontitis patients. J Periodontol. doi:10.1902/jop.2012.110467
35. Soory M (2012) Inflammatory mechanisms and redox status in periodontal and cardiometabolic diseases: effects of adjunctive nutritional antioxidants and statins. Infect Disord Drug Targets 12:301–315
36. Taylor B, Tofler G, Morel-Kopp MC, Carey H, Carter T, Elliott M, Dailey C, Villata L, Ward C, Woodward M, Schenck K (2010) The effect of initial treatment of periodontitis on systemic markers of inflammation and cardiovascular risk: a randomized controlled trial. Eur J Oral Sci 118:350–356
37. Graziani F, Cei S, Tonetti M, Paolantonio M, Serio R, Sammartino G, Gabriele M, D'Aiuto F (2010) Systemic inflammation following non-surgical and surgical periodontal therapy. J Clin Periodontol 37:848–854
38. Graziani F, Cei S, La Ferla F, Vano M, Gabriele M, Tonetti M (2010) Effects of non-surgical periodontal therapy on the glomerular filtration rate of the kidney: an exploratory trial. J Clin Periodontol 37:638–643
39. Buhlin K, Hultin M, Norderyd O, Persson L, Pockley AG, Pussinen PJ, Rabe P, Klinge B, Gustafsson A (2009) Periodontal treatment influences risk markers for atherosclerosis in patients with severe periodontitis. Atherosclerosis 206:518–522
40. D'Aiuto F, Nibali L, Parkar M, Patel K, Suvan J, Donos N (2010) Oxidative stress, systemic inflammation, and severe periodontitis. J Dent Res 89:1241–1246
41. Behle JH, Sedaghatfar MH, Demmer RT, Wolf DL, Celenti R, Kebschull M, Belusko PB, Herrera-Abreu M, Lalla E, Papapanou PN (2009) Heterogeneity of systemic inflammatory responses to periodontal therapy. J Clin Periodontol 36:287–294
42. Erciyas K, Sezer U, Ustün K, Pehlivan Y, Kısacık B, Senyurt S, Tarakçıoğlu M, Onat A (2012) Effects of periodontal therapy on disease activity and systemic inflammation in rheumatoid arthritis patients. Oral Dis. doi:10.1111/odi.12017
43. Acharya A, Bhavsar N, Jadav B, Parikh H (2010) Cardioprotective effect of periodontal therapy in metabolic syndrome: a pilot study in Indian subjects. Metab Syndr Relat Disord 8:335–341
44. Lopez NJ, Quintero A, Casanova PA, Ibieta CI, Baelum V, Lopez R (2012) Effects of periodontal therapy on systemic markers of inflammation in patients with metabolic syndrome: a controlled clinical trial. J Periodontol 83:267–278

45. Siribamrungwong M, Puangpanngam K (2012) Treatment of periodontal diseases reduces chronic systemic inflammation in maintenance hemodialysis patients. Ren Fail 34:171–175
46. Kardesler L, Buduneli N, Cetinkalp S, Kinane DF (2010) Adipokines and inflammatory mediators after initial periodontal treatment in patients with type 2 diabetes and chronic periodontitis. J Periodontol 81:24–33
47. Michalowicz BS, Novak MJ, Hodges JS, DiAngelis A, Buchanan W, Papapanou PN, Mitchell DA, Ferguson JE, Lupo V, Bofill J, Matseoane S, Steffen M, Ebersole JL (2009) Serum inflammatory mediators in pregnancy: changes after periodontal treatment and association with pregnancy outcomes. J Periodontol 80:1731–1741
48. Ebersole JL, Stevens J, Steffen MJ, Dawson Iii D, Novak MJ (2010) Systemic endotoxin levels in chronic indolent periodontal infections. J Periodontal Res 45:1–7
49. Goulhen F, Grenier D, Mayrand D (2003) Oral microbial heat-shock proteins and their potential contributions to infections. Crit Rev Oral Biol Med 14:399–412
50. Tabeta K, Yamazaki K, Hotokezaka H, Yoshie H, Hara K (2000) Elevated humoral immune response to heat shock protein 60 (hsp60) family in periodontitis patients. Clin Exp Immunol 120:285–293
51. Yamazaki K, Ueki-Maruayama K, Honda T, Nakajima T, Seymour GJ (2004) Effect of periodontal treatment on the serum antibody levels to heat shock proteins. Clin Exp Immunol 135:478–482
52. Park CS, Lee JY, Kim SJ, Choi JI (2010) Identification of immunological parameters associated with the alveolar bone level in periodontal patients. J Periodontal Implant Sci 40:61–68
53. Chung SW, Kang HS, Park HR, Kim SJ, Kim SJ, Choi JI (2003) Immune responses to heat shock protein in Porphyromonas gingivalis-infected periodontitis and atherosclerosis patients. J Periodontal Res 38:388–393
54. Yamazaki K, Ohsawa Y, Itoh H, Ueki K, Tabeta K, Oda T, Nakajima T, Yoshie H, Saito S, Oguma F, Kodama M, Aizawa Y, Seymour GJ (2004) T-cell clonality to Porphyromonas gingivalis and human heat shock protein 60s in patients with atherosclerosis and periodontitis. Oral Microbiol Immunol 19:160–167
55. Ford PJ, Gemmell E, Hamlet SM, Hasan A, Walker PJ, West MJ, Cullinan MP, Seymour GJ (2005) Cross-reactivity of GroEL antibodies with human heat shock protein 60 and quantification of pathogens in atherosclerosis. Oral Microbiol Immunol 20:296–302
56. Ford PJ, Gemmell E, Chan A, Carter CL, Walker PJ, Bird PS, West MJ, Cullinan MP, Seymour GJ (2006) Inflammation, heat shock proteins and periodontal pathogens in atherosclerosis: an immunohistologic study. Oral Microbiol Immunol 21:206–211
57. Leishman SJ, Ford PJ, Do HL, Palmer JE, Heng NC, West MJ, Seymour GJ, Cullinan MP (2012) Periodontal pathogen load and increased antibody response to heat shock protein 60 in patients with cardiovascular disease. J Clin Periodontol 39:923–930
58. Choi J, Lee SY, Kim K, Choi BK (2011) Identification of immunoreactive epitopes of the Porphyromonas gingivalis heat shock protein in periodontitis and atherosclerosis. J Periodontal Res 46:240–245
59. Jeong E, Lee JY, Kim SJ, Choi J (2012) Predominant immunoreactivity of Porphyromonas gingivalis heat shock protein in autoimmune diseases. J Periodontal Res 47:811–816
60. Ogrendik M, Kokino S, Ozdemir F, Bird PS, Hamlet S (2005) Serum antibodies to oral anaerobic bacteria in patients with rheumatoid arthritis. Med Gen Med 7(2):2
61. Ogrendik M (2012) Does periodontopathic bacterial infection contribute to the etiopathogenesis of the autoimmune disease rheumatoid arthritis? Discov Med 13(72):349–355
62. Smolik I, Robinson D, El-Gabalawy HS (2009) Periodontitis and rheumatoid arthritis: epidemiologic, clinical, and immunologic associations. Compend Contin Educ Dent 30:188–190, 192, 194 passim; quiz 198, 210
63. Detert J, Pischon N, Burmester GR, Buttgereit F (2010) The association between rheumatoid arthritis and periodontal disease. Arthritis Res Ther 12:218
64. Routsias JG, Goules JD, Goules A, Charalampakis G, Pikazis D (2011) Autopathogenic correlation of periodontitis and rheumatoid arthritis. Rheumatology 50:1189–1193

65. Harvey GP, Fitzsimmons TR, Dhamarpatni AA, Marchant C, Haynes DR, Bartold PM (2012) Expression of peptidylarginine deiminase-2 and −4, citrullinated proteins and anti-citrullinated protein antibodies in human gingiva. J Periodontal Res. doi:10.1111/jre.12002
66. Nesse W, Westra J, van der Wal JE, Abbas F, Nicholas AP, Vissink A, Brouwer E (2012) The periodontium of periodontitis patients contains citrullinated proteins which may play a role in ACPA (anti-citrullinated protein antibody) formation. J Clin Periodontol 39:599–607
67. Marchant C, Smith M, Proudman S, Haynes D, Bartold P (2012) Effect of Porphyromonas gingivalis on citrullination of proteins by macrophages in vitro. J Periodontol. doi:10.1902/jop.2012.120103
68. Wegner N, Lundberg K, Kinloch A, Fisher B, Malmstrom V, Feldmann M, Venables PJ (2010) Autoimmunity to specific citrullinated proteins gives the first clues to the etiology of rheumatoid arthritis. Immunol Rev 233:34–54
69. Wegner N, Wait R, Sroka A, Eick S, Nguyen KA, Lundberg K, Kinloch A, Culshaw S, Potempa J, Venables PJ (2010) Peptidylarginine deiminase from Porphyromonas gingivalis citrullinates human fibrinogen and α-enolase: implications for autoimmunity in rheumatoid arthritis. Arthritis Rheum 62:2662–2672
70. Kinloch AJ, Alzabin S, Brintnell W, Wilson E, Barra L, Wegner N, Bell DA, Cairns E, Venables PJ (2011) Immunization with Porphyromonas gingivalis enolase induces autoimmunity to mammalian α-enolase and arthritis in DR4-IE-transgenic mice. Arthritis Rheum 63: 3818–3823

Chapter 13
Periodontitis and Atherosclerosis

Daisuke Ekuni, Takaaki Tomofuji, and Manabu Morita

13.1 Introduction

Oxidative stress has been implicated in a variety of diseases and syndromes because of a weakening of the antioxidant defense or excess production of radicals that can overwhelm the scavenging capacity of cellular antioxidant systems. Oxidant stress plays an important role in the pathogenesis of atherosclerosis and periodontitis. Several studies have suggested that there is an association between cardiovascular disease (CVD) and periodontitis, and that periodontitis plays an etiological role in CVD, including atherosclerosis [1–6].

In this chapter, we summarize the relationship between periodontitis and atherosclerosis, and the involvement of oxidative stress. Due to the vast nature of this topic, we will review only a single important hypothesis, that is, that oxidative stress by periodontitis may be an initiating or promoting factor leading to inflammatory injury in the pathogenesis of atherosclerosis. In addition, the role of antioxidants for controlling atherosclerosis will be discussed.

13.2 Atherosclerosis and Oxidative Stress

Atherosclerosis is a progressive disease characterized by the accumulation of lipid deposits in macrophages (foam cells) in large and medium arteries [7]. This deposition leads to a proliferation of certain cell types within the arterial wall, which

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gradually influences the vessel lumen and impedes blood flow. These early lesions or fatty streaks are the precursors of more advanced lesions characterized by the accumulation of lipid-rich necrotic debris and smooth muscle cells (SMCs) [8]. In advanced lesions, plaques can become increasingly complex, with calcification, ulceration at the luminal surface, and hemorrhages from small vessels that grow into the lesion from the media of the blood vessel wall [7]. Age, gender, obesity, cigarette smoking, hypertension, diabetes mellitus, dyslipidemia, and periodontitis are known atherogenic risk factors that promote the impairment of endothelial function, smooth muscle function, and vessel wall metabolism [7, 9–15]. These risk factors are associated with an increased production of reactive oxygen species (ROS) [16].

ROS are metabolites of oxygen that are prone to participation in oxidation–reduction reactions. An increasing number of studies have demonstrated that oxidative stress plays a pivotal role in the pathogenesis of atherosclerosis, especially vascular endothelial dysfunction. ROS have detrimental effects on vascular function through several mechanisms: (1) ROS, especially hydroxyl radicals, directly injure cell membranes and nuclei; (2) ROS modulate vasomotion and the atherogenic process; and (3) ROS peroxidize lipid components, leading to the formation of oxidized low-density lipoprotein-cholesterol (ox-LDL), one of the key mediators of atherosclerosis.

Ox-LDL plays a major role in the development and progression of atherosclerosis and its complications [17], although native LDL does not cause cholesterol ester accumulation in macrophages and is not atherogenic. Ox-LDL can damage endothelial cells and induce the expression of adhesion molecules such as P-selectin [18] and chemotactic factors such as monocyte chemoattractant protein-1 and macrophage colony-stimulating factor [19, 20]. These processes lead to the tethering, activation, and attachment of monocytes and T lymphocytes to endothelial cells [21]. Endothelial cells, leukocytes, and SMCs secrete growth factors and chemoattractants, which induce the migration of monocytes and leukocytes into the subendothelial space [22]. Monocytes ingest lipoproteins and morph into macrophages. Macrophages engulf ox-LDL particles and other modified lipoproteins, thus becoming foam cells. Foam cells combine with leukocytes to become the fatty streak, and as the process continues, foam cells secrete growth factors that induce SMC migration into the intima [23].

13.3 Relationship Between Periodontitis and Atherosclerosis

Periodontitis may play an etiological role in CVD, including atherosclerosis [1–5]. An editors' consensus between the American Journal of Cardiology and the Journal of Periodontology was also published in 2009 [24]. This document provides health professionals, especially cardiologists and periodontists, a better understanding of the link between atherosclerosis and periodontitis and, on the basis of current information, an approach to reducing the risk for primary and secondary atherosclerotic CVD events in patients with periodontitis. Systemic reviews suggest that periodontal infections are independently associated with subclinical and clinical atherosclerosis

[1–6, 25, 26]. Analysis of limited data from interventional studies suggests that periodontal treatment generally results in favorable effects on subclinical markers of atherosclerosis, although there are some inconsistent findings. For example, some clinical studies show that periodontal therapy reduced plasma C-reactive protein (CRP) and ox-LDL levels [27] or serum CRP, interleukin (IL)-6, and native LDL levels [28] after 2 months, or serum CRP and IL-6 [29] levels after 6 months of treatment. Other studies [30, 31] reported no significant changes in serum levels of CRP, IL-6, or tumor necrosis factor alpha (TNF-α) 3 months after completion of therapy. A large randomized controlled trial [3] reported no significant differences in post-treatment plasma levels of CRP, IL-6, and plasminogen activator inhibitor-1 (PAI-1) levels between the periodontal treatment and control groups at 6 months, although the treatment group demonstrated improvement of endothelial dysfunction; this effect has also been seen in other small size intervention studies [32–34]. A systematic review of six treatment studies investigating the effects of periodontal therapy (scaling and root planing, with or without adjunctive local or systemic antibiotics) on serum CRP levels [35] concluded that there is modest evidence for a treatment-induced reduction of CRP levels [weighted mean difference of reductions: 0.50 mg/L, 95 % confidence interval (CI): 0.08–0.93]. On the other hand, there is no evidence that they prevent atherosclerotic CVD or modify its outcomes [36]. Thus, further well-designed controlled interventional studies are still required.

13.4 Oxidative Stress by Periodontitis and Pathogenesis of Atherosclerosis

Two prevailing hypotheses may explain the relationship between periodontitis and CVD. First, periodontal bacteria may have a direct effect on the vasculature. Several studies using ApoE-deficient mice, a mouse model prone to accelerated atherosclerosis, evaluated the direct effect of *Porphyromonas gingivalis* (*P. gingivalis*) infection on atherogenesis. Intravenous injection of *P. gingivalis* [37], *P. gingivalis LPS* [38], or repeated oral/anal bacterial applications [39] resulted in enhanced atherosclerosis in infected animals when compared to uninfected controls. Second, local inflammation causes an enhanced inflammatory response at distant sites without the spread of the infectious agent. We focused on the second hypothesis because oxidative stress by periodontitis may be an initiating or promoting factor leading to inflammatory injury in the pathogenesis of atherosclerosis.

13.4.1 Lipid Peroxidation by Periodontitis and the Initial Stage of Atherosclerosis

Because ROS have detrimental effects on vascular function through several mechanisms, including lipid peroxidation, our first study [15] was conducted to investigate

the relationship between lipid peroxidation induced by rat periodontitis and the initial stage of an atherosclerotic lesion and to profile the gene expression pattern in the aorta associated with atherosclerosis. Sixteen rats were randomly divided into two groups. A 3/0 cotton ligature was placed in a sub-marginal position around the mandibular first molars for 4 weeks to induce periodontitis; the control group was left untreated. Periodontitis-induced rats exhibited higher lipid peroxidation in the serum and aorta as well as periodontal tissue than the control rats. The aorta samples in the experimental group showed accumulation of lipids, increased ROS production, and changes of atherosclerosis-related gene expression. The ROS, such as lipid peroxides produced as a result of periodontitis, may diffuse into the blood from the site of inflammation, because diffusion of lipid peroxides in plasma has been reported in rat periodontitis [40]. The level of circulating lipid peroxides increases in atherosclerosis [41]. These data support the hypothesis that periodontitis is a local inflammatory reaction that causes an enhanced inflammatory response at distant sites without the spread of the infectious agent [42], and lipid peroxidation by periodontitis may be an initiating factor leading to inflammatory injury in the early stage of atherosclerosis.

13.4.2 Roles of Ox-LDL Linking Periodontitis and Atherosclerosis

Ox-LDL plays a major role in the development and progression of atherosclerosis and affects the vascular endothelium both directly and indirectly. Direct effects include the induction of cellular activation and apoptosis by interaction with lectin-like oxidized low-density lipoprotein receptor (LOX-1) [43, 44]. Indirect effects include down-regulation of the expression of endothelial nitric oxide synthase (eNOS), which results in increased production of ROS, ongoing LDL oxidation, and endothelial dysfunction [45].

In vitro studies have shown that *P. gingivalis* increases ox-LDL, apolipoprotein M, and cleavage of apolipoprotein B-100 [46]. *P. gingivalis*-modified ox-LDL induces vascular SMC proliferation in vitro, which suggests a potential role in intima–media thickening [47]. In addition, combined effects of *P. gingivalis*-LPS and ox-LDL on activation of the nuclear factor kappa beta pathway in macrophages were observed [48].

In a rat model study, we further investigated the effects of periodontitis on serum ox-LDL levels and oxidative damage in the descending aorta [48]. Twelve 8-week-old male Wistar rats were divided into two groups of six rats: the ligature-induced periodontitis group and no treatment (control) group. After the 4-week experimental period, animals were sacrificed under general anesthesia and blood samples were collected from the heart to measure serum levels of hexanoyl-lysine (HEL) (a marker of early stages of lipid peroxidation), reactive oxygen metabolites (ROM) (whole oxidant capacity of serum against *N*,*N*-diethylparaphenylendiamine in

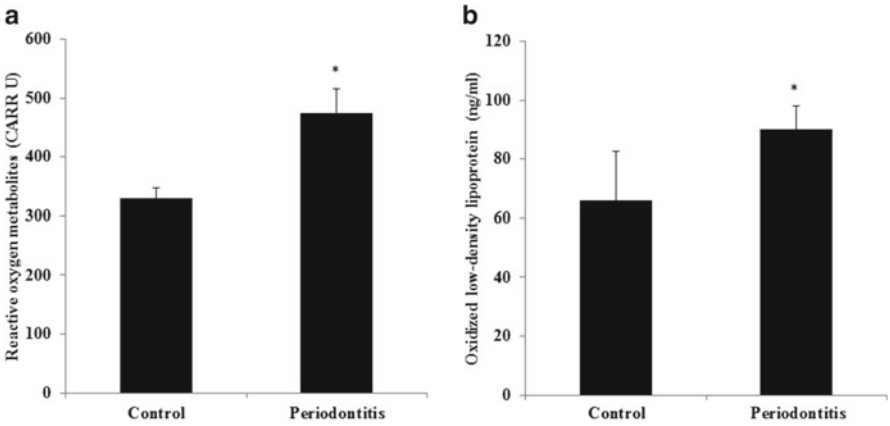

Fig. 13.1 Difference in serum markers between control and periodontitis groups. The serum levels of reactive oxygen metabolites (**a**) and oxidized low-density lipoprotein (**b**) in the periodontitis group were significantly higher than those in the control group (mean ± SD of six rats) (*$p < 0.05$, Mann–Whitney *U*-test). Data are given in terms of Carratelli Units (CARR U), with 1 CARR U corresponding to 0.08 mg/dL hydrogen peroxide (**a**)

acidic buffer, a marker of circulating ROS levels), and ox-LDL. The descending aorta was evaluated by immunohistochemical analysis or enzyme-linked immunosorbent assay (ELISA). The serum levels of ROM and ox-LDL in the periodontitis group were significantly higher than those in the control group (Fig. 13.1). Nitrotyrosine (a marker of protein nitration), 8-hydroxydeoxyguanosine (8-OHdG) (a marker of oxidative DNA damage), and HEL expression of the aortic lumen were significantly higher in the periodontitis group compared to the control group (Fig. 13.2). These results suggest that ROS induced by periodontitis directly injure DNA, protein, and lipid in the aorta, and/or that ROS leads to the formation of ox-LDL, which may induce endothelial dysfunction and contribute to atherogenesis (Fig. 13.3).

Experimental mechanistic studies in vitro and in vivo have established the plausibility of a link between periodontal infections and atherogenesis, and have identified biological pathways by which these effects may be mediated. However, the models used are mostly mono-infections of host cells by a limited number of "model" periodontal pathogens or ligature-induced acute periodontal inflammation. Thus, these models may not adequately portray human periodontitis as a polymicrobial, biofilm-mediated disease [6]. Future research must identify in vivo pathways in humans that may lead to periodontitis-induced atherogenesis or result in treatment-induced reduction of atherosclerosis risk [6]. Based on these studies, further evidence will be provided by determining whether periodontal interventions have a role in the primary or secondary prevention of atherosclerosis.

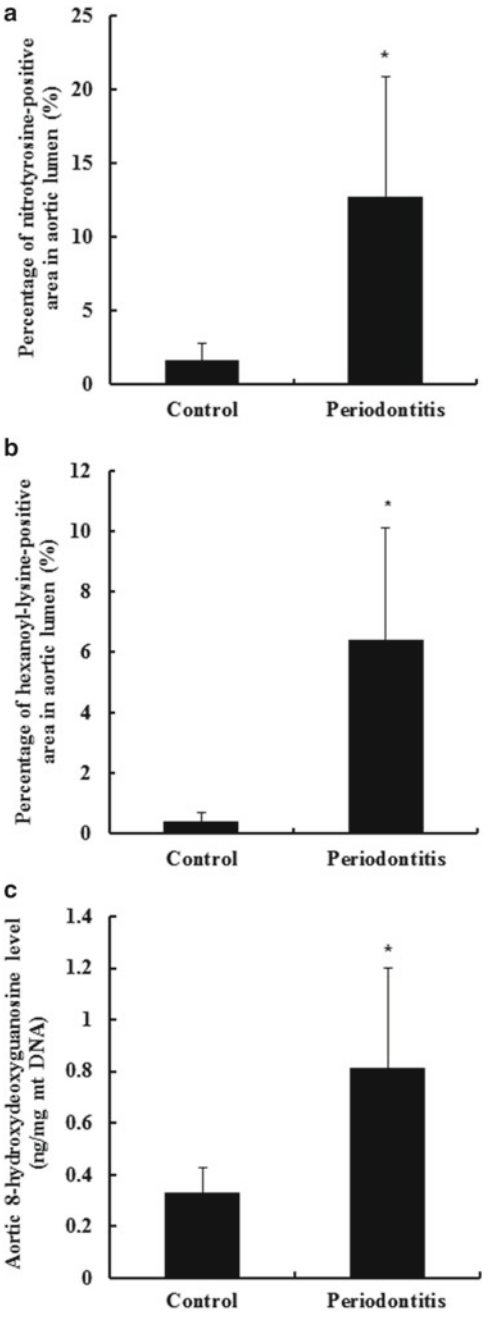

Fig. 13.2 Nitrotyrosine (a marker of protein nitration), hexanoyl-lysine (a marker of early stages of lipid peroxidation), and 8-hydroxydeoxyguanosine (a marker of oxidative DNA damage) expression in rat descending aorta. The percentages of nitrotyrosine-positive lumen (**a**) and hexanoyl-lysine-positive lumen (**b**), and aortic 8-hydroxydeoxyguanosine level (**c**) in the periodontitis group were significantly higher than those in the control group (mean ± SD of six rats) (*$p < 0.05$, Mann–Whitney *U*-test)

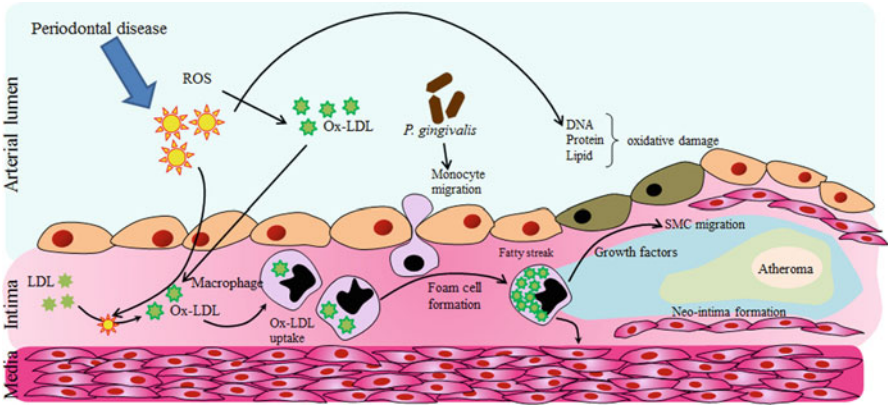

Fig. 13.3 Schematic overview of potential mechanisms linking periodontal oxidative stress and endothelial dysfunction/initial stage of atherosclerosis. The level of circulating reactive oxygen species (ROS) is increased by periodontal disease and leads to increased oxidized low-density lipoprotein (ox-LDL) and oxidative stress in the aorta. Monocytes activated by periodontal pathogens, such as *Porphyromonas gingivalis* (*P. gingivalis*), migrate into the subendothelial space, and transform into foam cells after uptake of ox-LDL. Foam cells secrete growth factors that induce smooth muscle cell (SMC) migration into the intima

13.5 Antioxidants

Vitamin C, vitamin E, and beta-carotene, often referred to as antioxidant vitamins, have been suggested to limit oxidative damage in humans, thereby lowering the risk of certain chronic diseases, such as CVD [49]. Vitamin C is a pivotal redox modulator in many biological reactions.

We previously reported the effects of systemic administration of vitamin C on periodontitis-induced endothelial oxidative stress in the aorta of a rat model [14]. Eighteen rats were divided into three groups and all rats received daily fresh water and powdered food throughout the 6-week study. In the vitamin C and periodontitis groups, periodontitis was ligature-induced for the first 4 weeks. In the vitamin C group, rats were given distilled water containing 1 g/L vitamin C for 2 weeks after removing the ligature. In this study of rats with ligature-induced periodontitis, vitamin C reduced polymorphonuclear leukocyte infiltration in the gingiva, the serum level of HEL, the degree of lipid deposition in the aorta, and the degrees of nitrotyrosine, HEL, and 8-OHdG formation in the aorta. These findings support the hypothesis that vitamin C attenuates not only gingival inflammation, but also the degree of experimental atherosclerosis in the rat periodontitis model with decreased oxidative damage to protein, lipid, and DNA. Aortic nitrotyrosine expression was significantly increased by induction of periodontitis and decreased by vitamin C intake in a previous study [14]. In another model, hypercholesterolemia induced upregulation of nitrotyrosine expression and was inhibited by vitamin C and E

intervention in pig myocardial tissue [50], which may support the effects of vitamin C on nitrotyrosine expression in our results. Studies have demonstrated that protein nitration is evident in human atherosclerotic tissues, associated with different stages of atherosclerosis, and even correlated with plaque instability in patients [51, 52]. Nitrotyrosine also directly increases aortic SMC migration in vitro and may contribute to cardiovascular pathogenesis [53].

Recently, molecular hydrogen, which selectively reduces cytotoxic ROS and oxidative stress, is considered to be a novel antioxidant [54]. Drinking water containing a therapeutic dose of hydrogen (hydrogen-rich water; HW) represents an alternative mode of delivery for molecular hydrogen. A previous animal study demonstrated that HW reduces atherosclerosis in apolipoprotein E knockout mice [55]. Therefore, it is possible that HW is of potential therapeutic value in the prevention of atherosclerosis induced by periodontitis. We reported the effects of systemic administration of HW on periodontitis-induced endothelial oxidative stress in the aorta of a rat model [48]. Rats were allocated randomly using a random number table to one of three groups (one control and two experimental groups). The control group received distilled water instead of active intervention for 4 weeks. In the periodontitis and periodontitis + HW groups, a 3/0 cotton ligature was placed in a sub-marginal position around the mandibular first molars for 4 weeks to induce periodontitis [15]. The rats in the periodontitis group received distilled water during the 4-week study, while the periodontitis + HW group received water containing 800–1,000 μg/L hydrogen for 4 weeks [56]. In the periodontitis group, lipid deposition in the descending aorta was observed. The periodontitis group also showed significantly higher serum levels of ROS and ox-LDL (1.7 and 1.4 times, respectively), and higher aortic expression levels of nitrotyrosine HEL (7.9 and 16.0 times, respectively) compared to the control group. In the periodontitis + HW group, lipid deposition was lower. Significantly lower serum levels of ROS and ox-LDL (0.46 and 0.82 times, respectively) and lower aortic levels of nitrotyrosine and HEL (0.27 and 0.19 times, respectively) were observed in the periodontitis + HW group than in the periodontitis group. These results suggest that HW intake may prevent lipid deposition in the rat aorta induced by periodontitis by decreasing serum ox-LDL levels and aortic oxidative stress.

Effects of antioxidants have also been shown in in vitro studies with *P. gingivalis* as a model organism. Antioxidant treatment (*N*-acetyl-L-cysteine, glutathione, or diphenylene iodonium) of endothelial cells infected with *P. gingivalis* resulted in an attenuated production of monocyte chemoattractant protein-1 [57]. These results suggest an inhibition of monocyte migration into subendothelial spaces, the site where oxidation of LDL primarily takes place, likely due to the limited activity of antioxidants outside of the vessel lumen [58].

These effects of antioxidants may contribute to human periodontitis and/or arthrosclerosis. Antioxidants are currently in wide use in routine general clinical practice. For example, antioxidant therapies for atherosclerosis have been evaluated in placebo-controlled trials involving tens of thousands of patients with atherosclerosis [59]. Despite pathophysiological, epidemiological, and mechanistic data suggesting otherwise, these clinical trial results have yielded mostly negative results in

terms of chronic preventative therapy. However, the lack of benefits seen in clinical trials to date does not disprove the central role of oxidative stress in atherosclerosis. Rather, these results challenge us to re-evaluate antioxidant therapies, patient selection, and trial duration [59]. The Danish Fitness and Nutrition Council has evaluated the basis for recommendations on the intake of antioxidants and has found limited reasons for increasing the recommended intake levels for some antioxidants. The amount of an antioxidant that may offer protection against chronic diseases is unknown and varies among individuals. Antioxidants could be beneficial for people with innate or acquired high baseline levels of ROS, but may be harmful for people with lower innate levels. It is critical to remember that monitoring serum antioxidant levels is necessary for the safe use of antioxidant therapy for patients with periodontitis and/or atherosclerosis.

13.6 Conclusion

Oxidative stress by periodontitis may be an initiating or promoting factor leading to inflammatory injury in the pathogenesis of atherosclerosis. Antioxidants attenuate systemic ox-LDL levels and endothelial dysfunction in in vitro and in vivo models. These results suggest that the effects may contribute to human periodontitis and/or atherosclerosis. However, further evidence will be provided by determining whether periodontal interventions and antioxidants have a role in the primary or secondary prevention of atherosclerosis (Fig. 13.4).

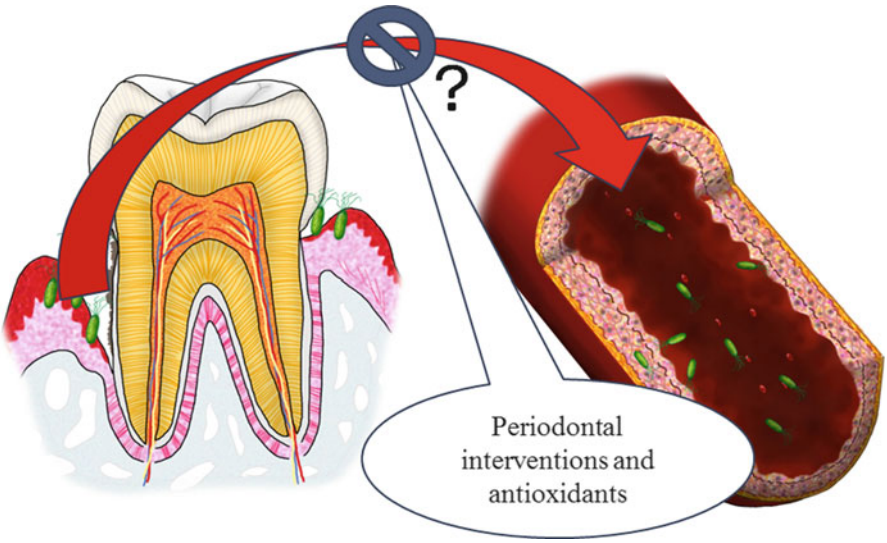

Fig. 13.4 Relationship between periodontitis and atherosclerosis. Periodontal interventions and antioxidants may have a role in the prevention of atherosclerosis

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References

1. Janket SJ, Baird AE, Chuang SK, Jones JA (2003) Meta-analysis of periodontal disease and risk of coronary heart disease and stroke. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 95:559–569
2. Söder PO, Söder B, Nowak J, Jogestrand T (2005) Early carotid atherosclerosis in subjects with periodontal diseases. Stroke 36:1195–1200
3. Tonetti MS, D'Aiuto F, Nibali L, Donald A, Storry C, Parkar M, Suvan J, Hingorani AD, Vallance P, Deanfield J (2007) Treatment of periodontitis and endothelial function. N Engl J Med 356:911–920
4. Cairo F, Castellani S, Gori AM, Nieri M, Baldelli G, Abbate R, Pini-Prato GP (2008) Severe periodontitis in young adults is associated with sub-clinical atherosclerosis. J Clin Periodontol 35:465–472
5. López-Jornet P, Berná-Mestre JD, Berná-Serna JD, Camacho-Alonso F, Fernandez-Millan S, Reus-Pintado M (2011) Measurement of atherosclerosis markers in patients with periodontitis: a case-control study. J Periodontol 83:690–698
6. Kebschull M, Demmer RT, Papapanou PN (2010) "Gum bug, leave my heart alone!"—epidemiologic and mechanistic evidence linking periodontal infections and atherosclerosis. J Dent Res 89:879–902
7. Bonomini F, Tengattini S, Fabiano A, Bianchi R, Rezzani R (2008) Atherosclerosis and oxidative stress. Histol Histopathol 23:381–390
8. Lusis AJ (2000) Atherosclerosis. Nature 407:233–241
9. Bernhard D, Wang XL (2007) Smoking, oxidative stress and cardiovascular diseases—do anti-oxidative therapies fail? Curr Med Chem 14:1703–1712
10. Nicolls MR, Haskins K, Flores SC (2007) Oxidant stress, immune dysregulation, and vascular function in type I diabetes. Antioxid Redox Signal 9:879–889
11. Mügge A, Brandes RP, Böger RH, Dwenger A, Bode-Böger S, Kienke S, Frölich JC, Lichtlen PR (1994) Vascular release of superoxide radicals is enhanced in hypercholesterolemic rabbits. J Cardiovasc Pharmacol 24:994–998
12. Buday A, Orsy P, Godó M, Mózes M, Kökény G, Lacza Z, Koller A, Ungvári Z, Gross ML, Benyó Z, Hamar P (2010) Elevated systemic TGF-beta impairs aortic vasomotor function through activation of NADPH oxidase-driven superoxide production and leads to hypertension, myocardial remodeling and increased plaque formation in apoE(−/−) mice. Am J Physiol Heart Circ Physiol 299:H386–H395
13. Huang A, Sun D, Kaley G, Koller A (1998) Superoxide released to high intra-arteriolar pressure reduces nitric oxide-mediated shear stress- and agonist-induced dilations. Circ Res 83:960–965
14. Ekuni D, Tomofuji T, Sanbe T, Irie K, Azuma T, Maruyama T, Tamaki N, Murakami J, Kokeguchi S, Yamamoto T (2009) Vitamin C intake attenuates the degree of experimental atherosclerosis induced by periodontitis in the rat by decreasing oxidative stress. Arch Oral Biol 54:495–502
15. Ekuni D, Tomofuji T, Sanbe T, Irie K, Azuma T, Maruyama T, Tamaki N, Murakami J, Kokeguchi S, Yamamoto T (2009) Periodontitis-induced lipid peroxidation in rat descending aorta is involved in the initiation of atherosclerosis. J Periodontal Res 44:434–442
16. Antoniades C, Tousoulis D, Tentolouris C, Toutouzas P, Stefanadis C (2003) Oxidative stress, antioxidant vitamins, and atherosclerosis. From basic research to clinical practice. Herz 28:628–638

17. Mitra S, Goyal T, Mehta JL (2011) Oxidized LDL, LOX-1 and atherosclerosis. Cardiovasc Drugs Ther 25:419–429
18. Vora DK, Fang ZT, Liva SM, Tyner TR, Parhami F, Watson AD, Drake TA, Territo MC, Berliner JA (1997) Induction of P-selectin by oxidized lipoproteins. Separate effects on synthesis and surface expression. Circ Res 80:810–818
19. Cushing SD, Berliner JA, Valente AJ, Territo MC, Navab M, Parhami F, Gerrity R, Schwartz CJ, Fogelman AM (1990) Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. Proc Natl Acad Sci U S A 87:5134–5138
20. Rajavashisth TB, Andalibi A, Territo MC, Berliner JA, Navab M, Fogelman AM, Lusis AJ (1990) Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified low-density lipoproteins. Nature 344:254–257
21. McEver RP (1992) Leukocyte-endothelial cell interactions. Curr Opin Cell Biol 4:840–849
22. Ross R (1993) The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 362: 801–809
23. Madamanchi NR, Vendrov A, Runge MS (2005) Oxidative stress and vascular disease. Arterioscler Thromb Vasc Biol 25:29–38
24. Friedewald VE, Kornman KS, Beck JD, Genco R, Goldfine A, Libby P, Offenbacher S, Ridker PM, Van Dyke TE, Roberts WC, American Journal of Cardiology, Journal of Periodontology (2009) The American Journal of Cardiology and Journal of Periodontology editors' consensus: periodontitis and atherosclerotic cardiovascular disease. J Periodontol 80:1021–1032
25. Mustapha IZ, Debrey S, Oladubu M, Ugarte R (2007) Markers of systemic bacterial exposure in periodontal disease and cardiovascular disease risk: a systematic review and meta-analysis. J Periodontol 78:2289–2302
26. Humphrey LL, Fu R, Buckley DI, Freeman M, Helfand M (2008) Periodontal disease and coronary heart disease incidence: a systematic review and meta-analysis. J Gen Intern Med 23:2079–2086
27. Tamaki N, Tomofuji T, Ekuni D, Yamanaka R, Morita M (2011) Periodontal treatment decreases plasma oxidized LDL level and oxidative stress. Clin Oral Investig 15:953–958
28. D'Aiuto F, Nibali L, Parkar M, Suvan J, Tonetti MS (2005) Short-term effects of intensive periodontal therapy on serum inflammatory markers and cholesterol. J Dent Res 84:269–273
29. D'Aiuto F, Parkar M, Andreou G, Suvan J, Brett PM, Ready D, Tonetti MS (2004) Periodontitis and systemic inflammation: control of the local infection is associated with a reduction in serum inflammatory markers. J Dent Res 83:156–160
30. Ide M, McPartlin D, Coward PY, Crook M, Lumb P, Wilson RF (2003) Effect of treatment of chronic periodontitis on levels of serum markers of acute-phase inflammatory and vascular responses. J Clin Periodontol 30:334–340
31. Yamazaki K, Honda T, Oda T, Ueki-Maruyama K, Nakajima T, Yoshie H, Seymour GJ (2005) Effect of periodontal treatment on the C-reactive protein and proinflammatory cytokine levels in Japanese periodontitis patients. J Periodontal Res 40:53–58
32. Mercanoglu F, Oflaz H, Oz O, Gökbuget AY, Genchellac H, Sezer M, Nişanci Y, Umman S (2004) Endothelial dysfunction in patients with chronic periodontitis and its improvement after initial periodontal therapy. J Periodontol 75:1694–1700
33. Elter JR, Hinderliter AL, Offenbacher S, Beck JD, Caughey M, Brodala N, Madianos PN (2006) The effects of periodontal therapy on vascular endothelial function: a pilot trial. Am Heart J 151:47
34. Seinost G, Wimmer G, Skerget M, Thaller E, Brodmann M, Gasser R, Bratschko RO, Pilger E (2005) Periodontal treatment improves endothelial dysfunction in patients with severe periodontitis. Am Heart J 149:1050–1054
35. Paraskevas S, Huizinga JD, Loos BG (2008) A systematic review and meta-analyses on C-reactive protein in relation to periodontitis. J Clin Periodontol 35:277–290
36. Lockhart PB, Bolger AF, Papapanou PN, Osinbowale O, Trevisan M, Levison ME, Taubert KA, Newburger JW, Gornik HL, Gewitz MH, Wilson WR, Smith SC Jr, Baddour LM (2012)

Periodontal disease and atherosclerotic vascular disease: does the evidence support an independent association? A scientific statement from the American Heart Association. Circulation 125:2520–2544
37. Li L, Messas E, Batista EL Jr, Levine RA, Amar S (2002) Porphyromonas gingivalis infection accelerates the progression of atherosclerosis in a heterozygous apolipoprotein E-deficient murine model. Circulation 105:861–867
38. Gitlin JM, Loftin CD (2009) Cyclooxygenase-2 inhibition increases lipopolysaccharide-induced atherosclerosis in mice. Cardiovasc Res 81:400–407
39. Lalla E, Lamster IB, Hofmann MA, Bucciarelli L, Jerud AP, Tucker S, Lu Y, Papapanou PN, Schmidt AM (2003) Oral infection with a periodontal pathogen accelerates early atherosclerosis in apolipoprotein E-null mice. Arterioscler Thromb Vasc Biol 23:1405–1411
40. Tsai CC, Chen HS, Chen SL, Ho YP, Ho KY, Wu YM, Hung CC (2005) Lipid peroxidation: a possible role in the induction and progression of chronic periodontitis. J Periodontal Res 40:378–384
41. Hermans N, Cos P, De Meyer GR, Maes L, Pieters L, Vanden Berghe D, Vlietinck AJ, De Bruyne T (2007) Study of potential systemic oxidative stress animal models for the evaluation of antioxidant activity: status of lipid peroxidation and fat-soluble antioxidants. J Pharm Pharmacol 59:131–136
42. Pouliot M, Clish CB, Petasis NA, Van Dyke TE, Serhan CN (2002) Lipoxin A(4) analogues inhibit leukocyte recruitment to Porphyromonas gingivalis: a role for cyclooxygenase-2 and lipoxins in periodontal disease. Biochemistry 39:4761–4768
43. Li D, Chen H, Romeo F, Sawamura T, Saldeen T, Mehta JL (2002) Statins modulate oxidized low-density lipoprotein-mediated adhesion molecule expression in human coronary artery endothelial cells: role of LOX-1. J Pharmacol Exp Ther 302:601–605
44. Ma FX, Zhou B, Chen Z, Ren Q, Lu SH, Sawamura T, Han ZC (2006) Oxidized low density lipoprotein impairs endothelial progenitor cells by regulation of endothelial nitric oxide synthase. J Lipid Res 47:1227–1237
45. Victor VM, Rocha M, Sola E, Banuls C, Garcia-Malpartida K, Hernandez-Mijares A (2009) Oxidative stress, endothelial dysfunction and atherosclerosis. Curr Pharm Des 15:2988–3002
46. Bengtsson T, Karlsson H, Gunnarsson P, Skoglund C, Elison C, Leanderson P, Lindahl M (2008) The periodontal pathogen Porphyromonas gingivalis cleaves apoB-100 and increases the expression of apoM in LDL in whole blood leading to cell proliferation. J Intern Med 263:558–571
47. Lei L, Li H, Yan F, Li Y, Xiao Y (2011) Porphyromonas gingivalis lipopolysaccharide alters atherosclerotic-related gene expression in oxidized low-density-lipoprotein-induced macrophages and foam cells. J Periodontal Res 46:427–437
48. Ekuni D, Tomofuji T, Endo Y, Kasuyama K, Irie K, Azuma T, Tamaki N, Mizutani S, Kojima A, Morita M (2012) Hydrogen-rich water prevents lipid deposition in the descending aorta in a rat periodontitis model. Arch Oral Biol 57(12):1615–1622
49. Villacorta L, Azzi A, Zingg JM (2007) Regulatory role of vitamins E and C on extracellular matrix components of the vascular system. Mol Aspects Med 28:507–537
50. Zhu XY, Rodriguez-Porcel M, Bentley MD, Chade AR, Sica V, Napoli C, Caplice N, Ritman EL, Lerman A, Lerman LO (2004) Antioxidant intervention attenuates myocardial neovascularization in hypercholesterolemia. Circulation 109:2109–2115
51. Cromheeke KM, Kockx MM, De Meyer GR, Bosmans JM, Bult H, Beelaerts WJ, Vrints CJ, Herman AG (1999) Inducible nitric oxide synthase colocalizes with signs of lipid oxidation/peroxidation in human atherosclerotic plaques. Cardiovasc Res 43:744–754
52. Ivanovski O, Nikolov IG, Drueke BT, Massy AZ (2007) Atherosclerosis and vascular calcification in uraemia: a new experimental model. Prilozi 28:11–24
53. Mu H, Wang X, Lin P, Yao Q, Chen C (1990) Nitrotyrosine promotes human aortic smooth muscle cell migration through oxidative stress and ERK1/2 activation. Biochim Biophys Acta 1783:1576–1584
54. Ohsawa I, Ishikawa M, Takahashi K, Watanabe M, Nishimaki K, Yamagata K, Katsura K, Katayama Y, Asoh S, Ohta S (1990) Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. Nat Med 13:688–694

55. Ohsawa I, Nishimaki K, Yamagata K, Ishikawa M, Ohta S (2008) Consumption of hydrogen water prevents atherosclerosis in apolipoprotein E knockout mice. Biochem Biophys Res Commun 377:1195–1198
56. Kasuyama K, Tomofuji T, Ekuni D, Tamaki N, Azuma T, Irie K, Endo Y, Morita M (2011) Hydrogen-rich water attenuates experimental periodontitis in a rat model. J Clin Periodontol 38:1085–1090
57. Choi EK, Park SA, Oh WM, Kang HC, Kuramitsu HK, Kim BG, Kang IC (2005) Mechanisms of Porphyromonas gingivalis-induced monocyte chemoattractant protein-1 expression in endothelial cells. FEMS Immunol Med Microbiol 44:51–58
58. Verhoye E, Langlois MR (2009) Circulating oxidized low-density lipoprotein: a biomarker of atherosclerosis and cardiovascular risk? Clin Chem Lab Med 47:128–137
59. Steinhubl SR (2008) Why have antioxidants failed in clinical trials? Am J Cardiol 101: 14D–19D

Chapter 14
Periodontitis and Liver Diseases

Koichiro Irie, Daisuke Ekuni, Takaaki Tomofuji, and Manabu Morita

14.1 Introduction

Periodontitis is the inflammation of supporting structures of the tooth caused by chronic bacterial infection [1]. Periodontitis is a risk factor for systemic diseases, including diabetes mellitus [2], hyperlipidemia [3], and coronary heart diseases [4]. The mechanisms by which periodontitis increases the likelihood of these systemic diseases have not been clearly defined, but the prerequisite is believed to be the host response to long-term systemic exposure to bacterial pathogens, such as lipopolysaccharide (LPS), and to oxidative stress. When stimulated by bacterial pathogens, host inflammatory cells produce reactive oxygen species (ROS) as part of the immune response [5]. ROS have a detrimental effect on the cellular antioxidant defense system and induce the oxidation of proteins, lipids, and DNA that contributes to tissue damage [6, 7]. Furthermore, studies have reported that lipid peroxide produced by periodontal inflammation diffuses into the blood stream [8–10], and lipid peroxide is involved in the progression of brain [11, 12], heart [13], kidney [14], and liver diseases [15].

Epidemiological studies have suggested a positive association between periodontal condition and liver diseases [16]. Recently, animal and clinical studies indicated that periodontitis is involved in increased blood ROS levels and that such conditions may be detrimental to hepatic health [17, 18]. In addition, studies on animals have also demonstrated that periodontitis may be a risk factor for the progression of nonalcoholic fatty liver disease (NAFLD) and alcoholic fatty liver disease (AFLD) [18]. These data suggest that periodontitis could damage hepatic health due to the

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increased serum ROS levels. Therefore, maintaining and/or improving periodontal health may offer clinical benefits with respect to hepatic health.

In this chapter, the relationship between periodontitis and liver diseases is reviewed and clarified using the clinical and animal studies as references.

14.2 Pathogenesis of NAFLD/NASH Involves Many Factors

A large number of adults show excessive hepatic fat accumulation. NAFLD is the most common form of chronic liver disease in many countries [19, 20]. The diagnosis of NAFLD is often confirmed after identification of elevated serum alanine aminotransferase (ALT), which is most commonly used for screening of liver diseases. In NAFLD patients, serum ALT levels range from mildly increased to five times those of normal persons [21].

NAFLD means a wide spectrum of conditions ranging from non-alcoholic fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH) [22]. NAFL generally shows a non-progressive clinical course, but NASH is a more serious form of NAFLD and may progress to cirrhosis [23–25].

Although there is controversy about the exact pathogenic mechanism behind NAFLD [26, 27], the following features contribute to the mechanism [28]: (1) adiposopathy involving inflammation, expansion, and increased turnover of adipose tissue leading to excess free fatty acid flux to the liver [29]; (2) impaired hepatic free fatty acid oxidation and decreased levels of protein, such as adiponectin, leading to fat accumulation; and (3) hepatic lipogenesis stimulated by a high carbohydrate intake and hyperinsulinemia, and hepatic insulin resistance leading to further increases in hepatic fat.

NAFLD is often independent of visceral fat-derived insulin resistance [26, 27]. Patients with lipodystrophy or lipoatrophy have severe hepatic insulin resistance but lack visceral fat [30]. Leptin therapy for lipodystrophy improves hepatic insulin resistance and steatosis, and visceral liposuction results in no change in hepatic fat. Patients with microsomal transfer protein or apolipoprotein B mutations associated with abetalipoproteinemia or hypobetalipoproteinemia also show severe hepatic steatosis but lack insulin resistance [31].

Lipid-related genes are intimately involved in hepatic steatosis [28]. In animal models, specific hepatic overexpression of lipoprotein lipase contributes to the development of NAFLD, as does overexpression of the scavenger receptor CD36, whereas knockdown of lipoprotein lipase, CD36, or fatty acid transport protein 1 (FATP1) or the more liver specific FATP2 or FATP5, protects against the development of NAFLD [27]. Plasma lipoproteins also affect hepatic steatosis by regulating the balance between peripheral and hepatic fat stores. In humans, Carriers of the apolipoprotein C3 variant alleles (C-482T, T-455C, or both) had higher levels of plasma apolipoprotein C3 and triglyceride, and higher prevalence of NAFLD, as compared with the wildtype homozygotes [32]. Both PPAR-α and PPAR-γ are involved in the pathogenesis of NAFLD and causally linked to mixed

hyperlipidemia [33]. Mitochondrial myopathies and ceramide and sphingomyelin production are involved in hepatic insulin resistance [28]. A single nucleotide polymorphism in patatin-like phospholipase domain-containing protein 3 (PNPLA3) adiponutrin is also associated with NAFLD but not insulin resistance in humans [34]. Lipids themselves also activate the unfolded protein response [endoplasmic reticulum (ER) stress], and changes in ER stress correlate with changes in liver fat acting through glucose-regulated protein 78, inositol-requiring enzyme-1α, and protein kinase R-like ER kinase [35].

Inflammation also plays a key role in hepatic insulin resistance [36]. Tumor necrosis factor (TNF)-α in adipose tissue induces insulin resistance, and macrophages play a key role driven by expression of chemokine ligand (CCL) 2 and its receptor CCR-2 [28]. The pathways of chemokine activation converge on the activation of c-jun NH_2-kinase (JNK) 1, which is also involved in the ER stress response [27, 37]. Overexpression of JNK-1 induces hepatic steatosis [27].

There are many risk factors for the development of NAFLD, such as obesity, diabetes, insulin resistance, oxidative stress, and inflammation. However, it is still unclear whether any other factors might be involved in the pathogenesis and progression of NASH. Therefore, it would be helpful for the treatment of NASH to identify the factors responsible for its progression.

Because the liver, as the first gate, is exposed to high concentrations of xenobiotics and other chemicals before delivery to the systemic circulation, it is equipped with several defense mechanisms for protection against harmful chemicals and their potentially damaging metabolites [38]. Nevertheless, the liver is highly susceptible to oxidative damage by reactive intermediates. Thus, we have to consider how to protect liver health or control oxidative stress, because oxidative damage has been linked to several types of liver injury and disease, including NASH [39], cirrhosis [40], acute hepatitis [41], and hepatocellular carcinoma [42]. Furthermore, oxidative stress-induced liver injury frequently creates an obstacle in therapeutic development. Therefore, the ability to prevent oxidative stress or decrease hepatic susceptibility to this toxicity seems to be extremely valuable [38].

14.3 Relationship Between Periodontitis and Liver Disease

Epidemiological studies suggest positive correlations between periodontal condition and liver diseases. One of these studies clarified the relationship between periodontitis and hepatic condition in apparently healthy Japanese women [16]. The incidence of periodontitis (deepest probing depth ≥4 mm) in females was significantly increased with elevated serum levels of AST, ALT, and cholinesterase, as well as an AST-to-ALT ratio of less than one [16]. In our study, a higher ALT level was associated with increased risk of periodontitis (PPD ≥ 4 mm) in Japanese university male students aged 18–19 years [43]. Periodontal breakdown and the ALT level in liver cirrhosis patients showed strong positive correlations [44]. Elevation of ALT is associated with liver diseases such as chronic viral hepatitis, autoimmune

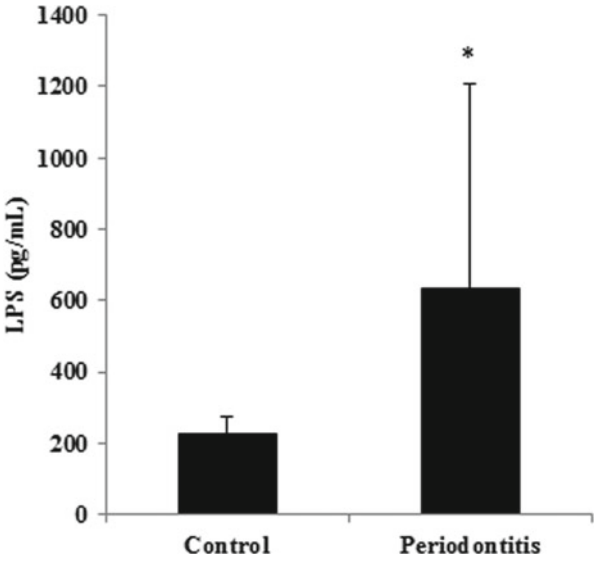

Fig. 14.1 Systemic lipopolysaccharide levels in control and periodontitis groups. The serum levels of lipopolysaccharide are significantly higher in the periodontitis group than that in the control group (mean ± SD of seven rats) (*$p < 0.05$, Mann–Whitney *U*-test)

liver disease, drug hepatotoxicity, liver cirrhosis, and NAFLD [44, 45]. The possibility of a relationship between liver function and periodontal condition has been explained in relation to lipid metabolism [16]. These results suggested that increases in ALT could be a potential risk factor for periodontitis.

Conversely, periodontitis could also contribute to hepatic abnormalities. In the case of carcinoma, hepatocellular carcinoma (HCC) patients with periodontitis also showed higher Japan Integrated Staging (JIS) scores and higher serum levels of total bilirubin than those without periodontitis [46]. Progression of the JIS score was significantly associated with probing pocket depth. Increased serum levels of reactive oxygen metabolites (ROM) were also seen in HCC patients with chronic periodontitis when compared to those without [46]. These findings suggest that oxidative stress may be involved in HCC progression induced by periodontitis. In NAFLD, LPS may lead to its development [47]. In an animal study, chronic administration of LPS and protease in the gingival sulcus caused not only periodontitis but also NAFLD by increasing the serum LPS level (Fig. 14.1) [48]. Other animal studies also indicated that experimental periodontitis can induce oxidative damage in the liver and hepatic inflammation with increasing serum ROS levels [18]. Furthermore, a clinical study has demonstrated that periodontal infection is a potential source of infection in the formation of pyogenic liver abscess [17]. LPS induces the production of various cytokines that affect lipid metabolism, leading to dyslipidemia [48]. LPS itself enters the bloodstream as a result of periodontal pathogens and can directly affect the liver and induce hepatic dyslipidemia [49, 50]. These results indicate that periodontitis can induce liver injury via systemic LPS and ROS.

Another clinical study suggested that *Porphyromonas gingivalis* (*P. gingivalis*) infection was significantly more frequent in NAFLD/NASH patients [51]. The paper concluded that infection with high-virulence *P. gingivalis* might be an

additional risk factor for the development or progression of NAFLD/NASH. Because *P. gingivalis* itself or its LPS can enter the blood circulation easily, the possible mechanism is shown in this study. It has been considered that NASH pathogenesis involves two stages. First, insulin resistance causes lipid accumulation in the hepatocytes. Second, cellular damage due to oxidative stress, lipid toxicity, mitochondrial dysfunction, and bacterial LPS causes hepatic inflammation, resulting in the development of NASH [19–25]. In fact, administration of LPS induces hepatic steatosis [48]; liver samples showed hepatocellular steatosis, ballooning, apoptosis, and inflammatory infiltration in addition to fibrosis. Furthermore, *P. gingivalis* exacerbates high-fat diet (HFD)-induced steatohepatitis via the induction of inflammasomes and inflammatory cytokines and induces inflammation and a fibrogenic response in steatosis [52]. These results indicate that both HFD condition and *P. gingivalis* infection cooperate to increase the risk of the development of NAFLD. LPS might directly affect liver cells through the systemic circulation. An increased number of blood vessels and extension of blood vessels were found in rat gingiva of a group with periodontitis on a normal diet and a combination group of periodontitis and a high-cholesterol diet [53]. These microvascular changes would enable entry of LPS from gingival connective tissue into the systemic circulation. In fact, LPS applied into the gingival sulcus is transferred to blood vessels 2 h after application [54]. Inflammatory cytokines, such as TNF-α, which are also produced in periodontal inflammation, might directly affect liver cells. Steatosis is associated with increased TNF-α [55], the level of which was elevated in the serum of rats fed a high-cholesterol diet and with topical application of a combination of LPS and protease [53]. These results indicated that *P. gingivalis* may generate a large amount of LPS and inflammatory cytokines, and this may result in inflammation of not only the local gingival tissue but also involve other systemic organs [56–58]. In addition, *P. gingivalis* can easily enter the blood stream from the gingival sulcus after several periodontal procedures, including tooth brushing, chewing, subgingival irrigation, and dental extractions [59–61].

Immune mediators originating from infection or severe trauma activate acute-phase protein synthesis, which are secreted by the liver and released into the systemic circulation [62]. C-reactive protein (CRP) is a well-known acute phase reactant produced by the liver in response to inflammation due to various stimuli. A study indicated that there is a significant correlation between attachment loss, probing pocket depth, and CRP levels [63, 64]. They found increased CRP levels in deeper pockets, which could be due to the presence of periodontal Gram-negative pathogens [64]. Our animal study also indicated that serum CRP levels were higher in the periodontitis group than in the control group [48]. Elevation of CRP levels is a risk factor for several systemic diseases, including cardiovascular disease, diabetes mellitus, and obesity [65]. For example, a study investigating the direct and indirect effects of periodontal pathogens on the cardiovascular system suggested that CRP levels were elevated in periodontitis [66, 67]. CRP shows a dose-dependent response to the severity of periodontal inflammation, and CRP concentrations become higher with more extensive disease [68–71]. These studies suggest that CRP is also a key mediator explaining the relationship between liver diseases and periodontitis.

14.4 Relationship Between Periodontal Inflammation and Oxidative Damage of Other Organs

Evidence implies that periodontal inflammation may be a potential risk factor for systemic diseases, although the mechanisms by which periodontitis affects systemic diseases are still unclear. One of the possible mechanisms may be ROS overproduction and oxidative stress.

Polymorphonuclear leukocytes in periodontal tissue produce ROS as the initial host defense against bacterial pathogens. However, excessive ROS production impairs the tissue oxidative/antioxidative balance that contributes to generate oxidative damage. In animal model studies, periodontal inflammation increased not only the gingival level of hexanoyl-lysine (HEL) expression (lipid peroxide) but also the serum level of HEL [72, 73]. Furthermore, increased levels of tissue 8-hydroxydeoxyguanosine (8-OHdG) were found in the brain, heart, liver, and kidney in the periodontal inflammation model [74]. These results suggest that increased blood lipid peroxide caused by periodontal inflammation could induce oxidative DNA damage of the liver, brain, heart, and kidney. The periodontal group showed increased levels of mitochondrial 8-OHdG of 127, 101, 49, and 40 % in the liver, heart, kidney, and brain, respectively (Fig. 14.2). The increase in the tissue 8-OHdG level seemed to be greater in the liver than in the other organs. Furthermore, the pathological changes induced by periodontal inflammation were observed only in the liver tissue. These results suggested that periodontal inflammation damaged the

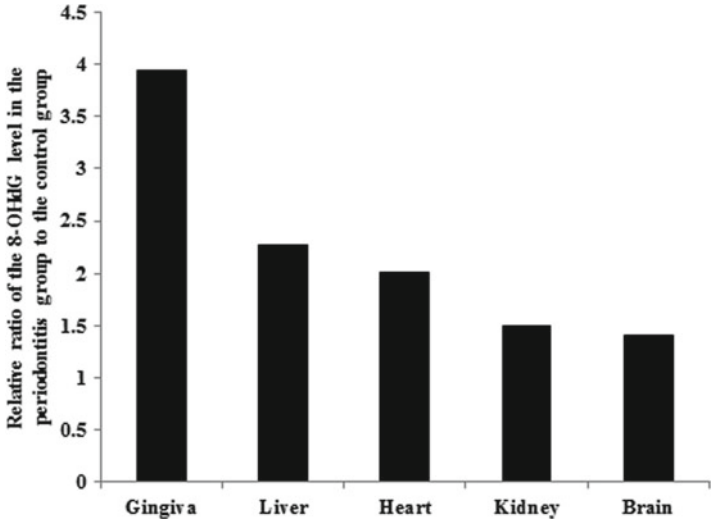

Fig. 14.2 Levels of mitochondrial 8-OHdG in rat gingiva, liver, heart, kidney, and brain. The levels of mitochondrial 8-OHdG in the gingiva, liver, heart, kidney, and brain are 3.94, 2.27, 2.01, 1.49, and 1.40 times higher in the periodontitis group than in the control group ($p<0.05$, using Mann–Whitney *U*-test)

liver more than the brain, heart, and kidney. Since the liver plays a central role in detoxification, it may have a higher sensitivity to circulating lipid peroxide than any other organ. This concept is in agreement with a previous study showing that a long-term hyperglycemic state induced more pronounced oxidative damage in the liver than in the brain, kidney, and heart [75].

Another study also suggested that the submandibular glands in the periodontitis group showed increased vacuolization, 8-OHdG levels, and numbers of apoptosis acinar cells [76]. ROS generation in cases of periodontitis may induce oxidative damage of the submandibular glands and contribute to apoptosis of acinar cells with vacuolization [77]. A relationship between ROS and salivary gland function has been reported [78–80]. Since oxidative stress by periodontitis induces circulating ROS [81], there is a possibility that oxidative stress in periodontitis may lead to salivary gland dysfunction. These results suggested that tissue oxidative damage following increased blood lipid peroxide levels may play a key role in systemic diseases induced by periodontal inflammation.

14.5 Periodontal Treatments in Improving Liver Function

Our study revealed that improvement in periodontitis by tooth brushing decreased plasma 8-OHdG levels [81]. In addition, a decrease in serum LPS by tooth brushing improved oxidative damage of the liver (Fig. 14.3) and could suppress liver injury in the periodontitis model [82]. These results suggested that tooth brushing may decrease LPS within the gingival sulcus and heal inflamed gingival tissue.

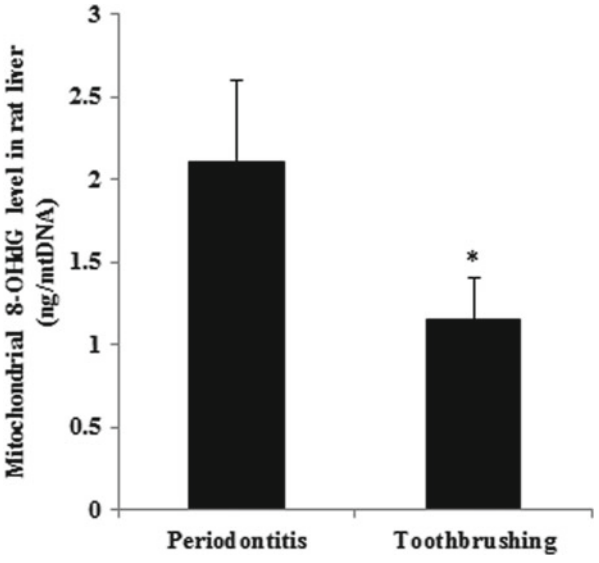

Fig. 14.3 The effects of tooth brushing on the levels of mitochondrial 8-OHdG in rat liver. Hepatic 8-OHdG level is significantly lower in the tooth brushing group than in the periodontitis group at the end of an 8-week experiment (*$p < 0.05$, using the Mann–Whitney *U*-test)

Furthermore, tooth brushing may suppress the transfer of LPS from the gingival sulcus into the blood stream. Another study also suggested that non-surgical periodontal treatment of NAFLD patients carried out for 3 months improved liver function parameters, such as serum AST and ALT [51].

Periodontal treatment decreased serum levels of ROM [80] and improved the circulating pro-oxidant/antioxidant balance [83] in chronic periodontitis patients. These observations indicated that ROS produced in periodontal lesions diffuse into the blood stream. Furthermore, clinical studies also indicated that periodontal treatment may improve periodontitis-driven impaired serum LDL cholesterol [84], increased CRP [85], increased interleukin 6 [86], and increased glycated hemoglobin levels [87]. Such responses may improve chronic liver damage. These results suggest that periodontal treatments may be useful supportive measures in the management of patients with NAFLD. Moreover, these observations support the concept that local treatment of periodontitis could be clinically effective not only for periodontal inflammation but also for prevention of systemic diseases induced by periodontal inflammation.

14.6 Conclusion

These observations support the notion that periodontitis might be an additional risk factor for the development or progression of liver disease via oxidative stress and LPS (Fig. 14.4). Maintaining and/or improving periodontal health may offer clinical

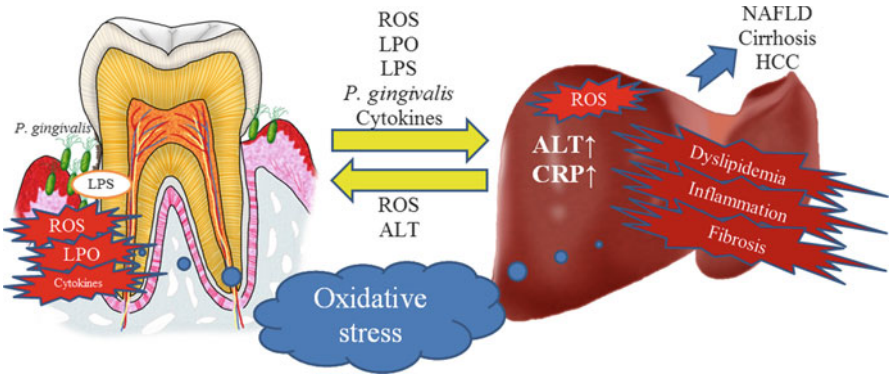

Fig. 14.4 Schema of the relationship between liver diseases and periodontitis. Periodontal inflammation induces oxidative stress and contributes to liver diseases. Increases of alanine aminotransferase (ALT) and ROS could be a potential risk factor for periodontitis. The possible mediators may include reactive oxygen species (ROS), lipid peroxide (LPO), lipopolysaccharide (LPS), *Porphyromonas gingivalis* (*P. gingivalis*), cytokines such as tumor necrosis factor alpha, and ALT. Liver injury by oxidative stress and other factors may lead to non-alcoholic fatty liver disease (NAFLD), cirrhosis, and hepatocellular carcinoma (HCC)

benefits for hepatic health. However, longitudinal clinical studies are needed to examine the causal relationships between periodontitis and hepatic conditions to clarify this issue.

References

1. Williams RC (1990) Periodontal disease. N Engl J Med 322:373–382
2. Meley BL, Oates TW (2006) American academy of periodontology: diabetes mellitus and periodontal diseases. J Periodontol 77:1289–1303
3. Cutler CW, Iacopino AM (2003) Periodontal disease: links with serum lipid/triglyceride levels? Review and new data. J Int Acad Periodontol 5:47–51
4. Morrison HI, Ellison LF, Taylor GW (1999) Periodontal disease and risk of fatal coronary heart and cerebrovascular diseases. J Cardiovasc Risk 6:7–11
5. Sculley DV, Langley-Evans SC (2002) Salivary antioxidants and periodontal disease status. Proc Nutr Soc 61:137–143
6. Valko M, Leibfritz D, Moncol J, Gronin MT, Mazur M, Telser J (2007) Free radicals and antioxidants in normal physiological functions and human diseases. Int J Biochem Cell Biol 39:44–84
7. Cahill A, Wang X, Hoek JB (2004) Increased oxidative damage to mitochondrial DNA following chronic ethanol consumption. Alcohol 235:49–58
8. Ekuni D, Tomofuji T, Sanbe T, Irie K, Azuma T, Maruyama T, Tamaki N, Murakami J, Kokeguchi S, Yamamoto T (2009) Vitamin C intake attenuates the degree of experimental atherosclerosis induced by periodontitis in the rat by decreasing oxidative stress. Arch Oral Biol 54:495–502
9. Panjamurthy K, Mannoharan S, Ramachandran CR (1999) Lipid peroxidation and antioxidant status in patients with periodontitis. Cell Mol Biol Lett 10:255–264
10. Sobaniec H, Sobaniec-Lotowska ME (2000) Morphological examinations of hard tissues of periodontium and evaluation of selected processes of lipid peroxidation in blood serum of rats in the course of experimental periodontitis. Med Sci Monit 6:875–881
11. Butterfield DA, Bader Lange ML, Sultana R (2010) Involvement of the lipid peroxidation product, HNE, in the pathogenesis and progression of Alzheimer's disease. Biochim Biophys Acta 1801:924–929
12. Singh M, Nam DT, Arseneault M, Ramassamy C (2010) Role of by-products of lipid oxidation in Alzheimer's disease brain: a focus on acrolein. J Alzheimers Dis 21:741–756
13. Davies SS, Roberts LJ 2nd (2011) F2-isoprostanes as an indicator and risk factor for coronary heart disease. Free Radic Biol Med 50:559–566
14. Wiswedel I, Hirsch D, Carluccio F, Hampl H, Siems W (2005) F2-isoprostanes as biomarkers of lipid peroxidation in patients with chronic renal failure. Biofactors 24:201–208
15. Poli G, Biasi F, Leonarduzzi G (2008) 4-Hydroxynonenal-protein adduct: a reliable biomarkers of lipid oxidation in liver diseases. Mol Aspects Med 29:67–71
16. Saito T, Shimazaki Y, Koga T, Tsuzuki M, Ohshima A (2006) Relationship between periodontitis and hepatic condition in Japanese women. J Int Acad Periodontol 8:89–95
17. Ohyama H, Nakasho K, Yamanegi K, Noiri Y, Kuhara A, Kato-Kogoe N, Yamada N, Hata M, Nishimura F, Ebisu S, Terada N (2009) An unusual autopsy case of pyogenic liver abscess caused by periodontal bacteria. Jpn J Infect Dis 62:381–383
18. Tomofuji T, Sanbe T, Ekuni D, Azuma T, Irie K, Maruyama T, Tamaki N, Yamamoto T (2008) Oxidative damage of liver induced by ligature-induced periodontitis and chronic ethanol consumption. Arch Oral Biol 53:1113–1118
19. Angulo P (2002) Nonalcoholic fatty liver disease. N Engl J Med 18:1221–1231
20. Ludwing J, Viggiano TR, McGill DB, Oh BJ (1980) Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. Mayo Clin Proc 55:434–438

21. Adams LA, Knuiman MW, Divitini ML, Olynyk JK (2008) Body mass index is a stronger predictor of alanine aminotransaminase levels than alcohol consumption. J Gastroenterol Hepatol 23:1089–1093
22. Liou I, Kowdley KV (2006) Natural history of nonalcoholic steatohepatitis. J Clin Gastroenterol 40:511–516
23. Diehl AM, Goodman Z, Ishak KG (1998) Alcohol-like disease in nonalcoholics: a clinical and histologic comparison with alcohol-induced liver injury. Gastroenterology 95:1056–1062
24. Abdelmalek MF, Diehl AM (2007) Nonalcoholic fatty liver disease as a complication of insulin resistance. Med Clin North Am 91:1125–1149
25. Marchesini G, Bugianesi E, Forlani G, Cerrelli F, Lenzi M, Manini M, Natale S, Vanni E, Villanova N, Melchionda N, Rizzetto M (2003) Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. Hepatology 37:917–923
26. Stefan N, Haring HU (2011) The metabolically benign and malignant fatty liver. Diabetes 60:2011–2017
27. Samuel VT, Shulman GI (2012) Mechanisms for insulin resistance: common threads and missing links. Cell 148:852–871
28. Wierzbicki AS, Oben J (2012) Nonalcoholic fatty liver disease and lipids. Curr Opin Lipidol 23:345–352
29. Bays HE (2011) Adiposopathy is "sick fat" a cardiovascular disease? J Am Coll Cardiol 57:2461–2473
30. Petersen KF, Oral EA, Dufour S, Befroy D, Ariyan C, Yu C, Cline GW, DePaoli AM, Taylor SI, Gorden P, Shulman GI (2002) Leptin reverses insulin resistance and hepatic steatosis in patients with severe lipodystrophy. J Clin Invest 109:1345–1350
31. Tarugi P, Averna M (2011) Hypobetalipoproteinemia: genetics, biochemistry, and clinical spectrum. Adv Clin Chem 54:81–107
32. Petersen KF, Dufour S, Hariri A, Nelson-Williams C, Foo JN, Zhang XM, Dziura J, Lifton RP, Shulman GI (2010) Apolipoprotein C3 gene variants in nonalcoholic fatty liver disease. N Engl J Med 362:1082–1089
33. Tailleux A, Wouters K, Staels B (2012) Roles of PPARs in NAFLD: potential therapeutic targets. Biochim Biophys Acta 1821:809–818
34. Romeo S, Kozlitina J, Xing C, Pertsemlidis A, Cox D, Pennacchio LA, Boerwinkle E, Cohen JC, Hobbs HH (2008) Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. Nat Genet 40:1461–1465
35. Ye R, Jung DY, Jun JY, Li J, Luo S, Ko HJ, Kim JK, Lee AS (2010) Grp78 heterozygosity promotes adaptive unfolded protein response and attenuates diet-induced obesity and insulin resistance. Diabetes 59:6–16
36. Stienstra R, Tack CJ, Kanneganti TD, Joosten LA, Netea MG (2012) The inflammasome puts obesity in the danger zone. Cell Metab 15:10–18
37. Zhang X, Xu A, Chung SK, Cresser JH, Sweeney G, Wong RL, Lin A, Lam KS (2011) Selective inactivation of c-Jun NH2-terminal kinase in adipose tissue protects against diet-induced obesity and improves insulin sensitivity in both liver and skeletal muscle in mice. Diabetes 60:486–495
38. Bataille AM, Manautou JE (2012) Nrf2: a potential target for new therapeutics in liver disease. Clin Pharmacol Ther 92:340–348
39. Rolo AP, Teodoro JS, Palmeira CM (2012) Role of oxidative stress in the pathogenesis of nonalcoholic steatohepatitis. Free Radic Biol Med 52:59–69
40. Aldaba-Muruato LR, Moreno MG, Shibayama M, Tsutsumi V, Muriel P (2012) Protective effects of allopurinol against acute liver damage and cirrhosis induced by carbon tetrachloride: modulation of NF-kappaB, cytokine production and oxidative stress. Biochim Biophys Acta 1820:65–75
41. Ivanov AV, Smirnova OA, Ivanova ON, Masalova OV, Kochetkov SN, Isaguliants MG (2011) Hepatitis C virus proteins activate NRF2/ARE pathway by distinct ROS-dependent and independent mechanisms in HUH7 cells. PLoS ONE 6:e24957

42. Hagen TM, Huang S, Curnutte J, Fowler P, Martinez V, Wehr CM, Ames BN, Chisari FV (1994) Extensive oxidative DNA damage in hepatocytes of transgenic mice with chronic active hepatitis destined to develop hepatocellular carcinoma. Proc Natl Acad Sci U S A 91: 12808–12812
43. Furuta M, Ekuni D, Yamamoto T, Irie K, Koyama R, Sanbe T, Yamanaka R, Morita M, Kuroki K, Tobe K (2010) Relationship between periodontitis and hepatic abnormalities in young adults. Acta Odontol Scand 68:27–33
44. Jaiswal G, Deo V, Bhongrade M, Jaiswal S (2011) Serum alkaline phosphatase: a potential marker in the progression of periodontal disease in cirrhosis patients. Quintessence Int 42:345–348
45. Louthan MV, Theriot JA, Zimmerman E, Stutts JT, McClain CJ (2005) Decreased prevalence of nonalcoholic fatty liver disease in black obese children. J Pediatr Gastroenterol Nutr 41:426–429
46. Tamaki N, Takaki A, Tomofuji T, Endo Y, Kasuyama K, Ekuni D, Yasunaka T, Yamamoto K, Morita M (2011) Stage of hepatocellular carcinoma is associated with periodontitis. J Clin Periodontol 38:1015–1020
47. Feingold KR, Staprans I, Memon RA, Moser AH, Shigenaga JK, Doerrler W, Dinarello CA, Grunfeld C (1992) Endotoxin rapidly induces changes in lipid metabolism that produce hypertriglyceridemia: low doses stimulate hepatic triglyceride production while high doses inhibit clearance. J Lipid Res 33:1765–1776
48. Tomofuji T, Ekuni D, Yamanaka R, Kusano H, Azuma T, Sanbe T, Tamaki N, Yamamoto T, Watanabe T, Miyauchi M, Takata T (2007) Chronic administration of lipopolysaccharide and proteases induces periodontal inflammation and hepatic steatosis in rats. J Periodontol 78:1999–2006
49. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, Neyrinck AM, Fava F, Tuohy KM, Chabo C, Waget A, Delmée E, Cousin B, Sulpice T, Chamontin B, Ferrières J, Tanti JF, Gibson GR, Casteilla L, Delzenne NM, Alessi MC, Burcelin R (2007) Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes 56:1761–1772
50. Saito T, Hayashida H, Furugen R (2007) Metabolic endotoxemia initiates obesity and insulin resistance: diabetes. Diabetes 56:1761–1772, Comment on: Cani et al. (2007) Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes 56:1761–1772
51. Yoneda M, Naka S, Nakano K, Wada K, Endo H, Mawatari H, Imajo K, Nomura R, Hokamura K, Ono M, Murata S, Tohnai I, Sumida Y, Shima T, Kuboniwa M, Umemura K, Kamisaki Y, Amano A, Okanoue T, Ooshima T, Nakajima A (2012) Involvement of a periodontal pathogen, porphyromonas gingivalis on the pathogenesis of non-alcoholic fatty liver disease. BMC Gastroenterol 12:16
52. Furusho H, Miyauchi M, Hyogo H, Inubushi T, Ao M, Ouhara K, Hisatune J, Kurihara H, Sugai M, Hayes CN, Nakahara T, Aikata H, Takahashi S, Chayama K, Takata T (2013) Dental infection of Porphyromonas gingivalis exacerbates high fat diet-induced steatohepatitis in mice. J Gastroenterol 48:1259–1270
53. Yamamoto T, Tomofuji T, Tamaki N, Ekuni D, Azuma T, Sanbe T (2010) Effects of topical application of lipopolysaccharide and proteases on hepatic injury induced by high-cholesterol diet in rats. J Periodontal Res 45:129–135
54. Schwartz J, Stinson FL, Parker RB (1972) The passage of tritiated bacterial endotoxin across intact gingival crevicular epithelium. J Periodontol 43:270–276
55. Solga SF, Diehl A (2003) Non-alcoholic fatty liver disease: limen-liver interactions and possible role for probiotics. J Hepatol 38:681–687
56. Amano A, Nakagawa I, Kataoka K, Morisaki I, Hamada S (1999) Distribution of porphyromonas gingivalis strains with fimA genotypes in periodontitis patients. J Clin Microbiol 37:1426–1430
57. Nakagawa I, Amano A, Ohara-Nemoto Y, Endoh N, Morisaki I, Kimura S, Kawabata S, Hamada S (2002) Identification of a new variant of fimA gene of porphyromonas gingivalis and its distribution in adults and disabled populations with periodontitis. J Periodontal Res 37:425–432

58. Nelson RG, Shlossman M, Budding LM, Pettitt DJ, Saad MF, Genco RJ, Knowler WC (1990) Periodontal disease and NIDDM in Pima Indians. Diabetes Care 13:836–840
59. Sconyers JR, Crawford JJ, Moriarty JD (1973) Relationship of bacteremia to tooth brushing in patients with periodontitis. J Am Dent Assoc 87:616–622
60. Carroll GC, Sebor RJ (1980) Dental flossing and its relationship to transient bacteremia. J Periodontol 51:691–692
61. Forner L, Larsen T, Kilian M, Holmstrup P (2006) Incidence of bacteremia after chewing, tooth brushing and scaling in individuals with periodontal inflammation. J Clin Periodontol 33:401–407
62. Steel DM, Whitehead AS (1994) The major acute phase reactant: C-reactive protein, serum amyloid P component and serum amyloid A protein. Immunol Today 15:81–88
63. Chitsazi MT, Pourabbas R, Shirmohammadi A, Ahmadi Zenouz G, Vatankhah AH (2008) Association of periodontal diseases with elevation of serum C-reactive protein and body mass index. J Dent Res Dent Clin Dent Prospects 2:9–14
64. Noack B, Genco RJ, Trevisan M, Grossi S, Zambon JJ, De Nardin E (2001) Periodontal infections contribute to elevated systemic C-reactive protein level. J Periodontol 72:1221–1227
65. Bain JL, Lester SR, Henry WD, Bishop CM, Turnage AA, Naftel JP, Johnson RB (2009) Comparative gender differences in local and systemic concentrations of pro-inflammatory cytokines in rats with experimental periodontitis. J Periodontal Res 44:133–140
66. Pussinen PJ, Tuomisto K, Jousilahti P, Havulinna AS, Sundvall J, Salomaa V (2007) Endotoxemia, immune response to periodontal pathogens, and systemic inflammation associate with incident cardiovascular disease events. Arterioscler Thromb Vasc Biol 27:1433–1439
67. Ebersole JL, Cappelli D, Mott G, Kesavalu L, Holt SC, Singer RE (1999) Systemic manifestations of periodontitis in the non-human primate. J Periodontal Res 34:358–362
68. Lamster IB, Novak MJ (1992) Host mediators in gingival crevicular fluid: implications for the pathogenesis of periodontal disease. Crit Rev Oral Biol Med 3:31–60
69. Ranney RR (1991) Immunologic mechanisms of pathogenesis in periodontal disease: an assessment. J Periodontal Res 26:243–254
70. Kinane DF, Adonogianaki E, Moughal N, Winstanley FP, Mooney J, Thornhill M (1991) Immunocytochemical characteristics of cellular infiltrate, related endothelial changes and determination of GCF acute-phase proteins during human experimental gingivitis. J Periodontal Res 26:286–288
71. Page RC (1991) The role of inflammatory mediators in the pathogenesis of periodontal disease. J Periodontal Res 26:230–242
72. Tomofuji T, Azuma T, Kusano H, Sanbe T, Ekuni D, Tamaki N, Yamamoto T, Watanabe T (2006) Oxidative damage of periodontal tissue in the rat periodontitis model: effects of a high-cholesterol diet. FEBS Lett 580:3601–3604
73. Maruyama T, Tomofuji T, Endo Y, Irie K, Azuma T, Ekuni D, Tamaki N, Yamamoto T, Morita M (2011) Supplementation of green tea catechins in dentifrices suppresses gingival oxidative stress and periodontal inflammation. Arch Oral Biol 56:48–53
74. Tomofuji T, Ekuni D, Irie K, Azuma T, Tamaki N, Maruyama T, Yamamoto T, Watanabe T, Morita M (2011) Relationships between periodontal inflammation, lipid peroxide and oxidative damage of multiple organs in rats. Biomed Res 32:343–349
75. Karaağaç N, Salman F, Doğru-Abbasoğlu S, Uysal M (2011) Changes in prooxidant-antioxidant balance in tissues of rats following long-term hyperglycemic status. Endocr Res 36:124–133
76. Ekuni D, Endo Y, Irie K, Azuma T, Tamaki N, Tomofuji T, Morita M (2010) Imbalance of oxidative/anti-oxidative status induced by periodontitis is involved in apoptosis of rat submandibular glands. Arch Oral Biol 55:170–176
77. Nager RM, Salameh F, Reznick AZ, Livshits V, Nahir AM (2003) Salivary gland involvement in rheumatoid arthritis and its relationship to induced oxidative stress. Rheumatology (Oxford) 42:1234–1241
78. Abdollahi M, Fooladian F, Emami B, Zafari K, Bahreini-Moghadam A (2003) Protection by sildenafil and theophylline of lead acetate-induced oxidative stress in rat submandibular gland and saliva. Hum Exp Toxicol 22:587–592

79. de la Cal C, Lomniczi A, Mohn CE, De Laurentiis A, Casal M, Chiarenza A, Paz D, McCann SM, Rettori V, Elverdín JC (2006) Decrease in salivary secretion by radiation mediated by nitric oxide and prostaglandins. Neuroimmunomodulation 13:19–27
80. Tamaki N, Tomofuji T, Ekuni D, Yamanaka R, Yamamoto T, Morita M (2009) Short-term of non-surgical periodontal treatment on plasma level of reactive oxygen metabolites in patients with chronic periodontitis. J Periodontol 80:901–906
81. Ekuni D, Tomofuji T, Tamaki N, Sanbe H, Azuma T, Yamanaka R, Yamamoto T, Watanabe T (2008) Mechanical stimulation of gingiva reduces plasma 8-OHdG level in rat periodontitis. Arch Oral Biol 53:324–329
82. Tomofuji T, Ekuni D, Sanbe T, Azuma T, Tamaki N, Irie K, Maruyama T, Yamamoto T, Watanabe T, Miyauchi M, Takata T (2009) Effects of improvement in periodontal inflammation by tooth brushing on serum lipopolysaccharide concentration and liver injury in rats. Acta Odontol Scand 67:200–205
83. Tamaki N, Tomofuji T, Ekuni D, Yamanaka R, Morita M (2011) Periodontal treatment decreases plasma oxidized LDL level and oxidative stress. Clin Oral Investig 15:953–958
84. Oz SG, Fentoglu O, Kilicarslan A, Guven GS, Tanrtover MD, Aykac Y, Sozen T (2007) Beneficial effects of periodontal treatment on metabolic control of hypercholesterolemia. South Med 100:686–691
85. Yamazaki K, Honda T, Oda T, Ueki-Maruyama K, Nakajima T, Yoshie H, Seymour GJ (2005) Effects of periodontal treatment on the C-reactive protein and proinflammatory cytokine levels in Japanese periodontitis patients. J Periodontal Res 40:53–58
86. Elter JR, Hinderliter AL, Offenbacher S, Beck JD, Caughey M, Brodala N, Madianos PN (2006) The effects of periodontal therapy on vascular endothelial function: a pilot trial. Am Heart J 151:47
87. Grossi SG, Skrepcinski FB, DeCaro T, Robertson DC, Ho AW, Dunford RG, Genco RJ (1997) Treatment of periodontal disease in diabetes reduces glycated hemoglobin. J Periodontol 68:713–719

Chapter 15
Oxidative Stress and Periodontal Disease in Down Syndrome

Tomoko Komatsu and Masaichi-Chang-Il Lee

15.1 Introduction

The various symptoms of Down syndrome (DS) were first described in the medical literature by English physician Dr. John Langdon Down in 1866. The management of clinical problems in children with DS remains a major medical challenge and depends on the understanding of the unique metabolic imbalance induced by overexpression of genes on chromosome 21. Individuals with DS undergo an accelerated process of aging, which is thought to be associated with high levels of oxidative stress throughout the lifespan [1].

The endogenous antioxidant enzyme superoxide dismutase (SOD) is responsible for the regulation of reactive oxygen species (ROS) homeostasis [2]. Increased levels or activity of SOD may contribute to neuronal death and disease progression in DS and precede the signature manifestations of the disease by decades. Various studies have indicated that SOD plays an important role in both DS and Alzheimer's disease (AD) [3].

Periodontal disease in DS patients is often severe, especially in the region of the lower anterior teeth. Rapid progression of DS-related periodontal disease is most common among younger age groups. Individuals with DS have a higher prevalence and severity of periodontal disease, which cannot be explained by poor oral hygiene alone and is related to changes in the immune response. ROS generation by activated neutrophils has been implicated in the pathogenesis of various inflammatory

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diseases, and in DS patients, it appears that the ROS-generating capacity of neutrophils is enhanced. Using electron spin resonance (ESR) and spin trapping, we demonstrated directly that ROS are generated by cultured gingival fibroblasts from DS patients [4]. It is possible that the increased generation of ROS in DS causes a variety of clinical disorders, including severe periodontal disease and early aging.

There are many advantages associated with the use of saliva as a clinical diagnostic biofluid. It has been reported that the presence of 8-hydroxy-2′-deoxyguanosine (8-OHdG) in saliva is associated with oxidative stress in aging [5], disorders such as DS [6], and periodontal disease [7], and that the salivary levels of 8-OHdG can be used to predict severe erectile dysfunction [8]. The most interesting and novel finding of the current study was that the levels of 8-OHdG in saliva, urine, and leukocytes were significantly higher in DS patients versus controls regardless of age; this phenomenon may be associated with accelerated aging in DS patients [9].

15.1.1 Down Syndrome

John Langdon Down, a nineteenth century English physician, identified the phenotypic expression of patients with circulation and coordination problems as DS, but failed to determine the underlying mechanism [10, 11]. Similarity of the distinct features of DS among patients with the disease led early researchers to suspect a chromosomal aberration as the cause. More than a century later, in the late 1950s, it was hypothesized that meiotic non-disjunction could lead to trisomy of chromosome 21 [10, 12].

Full trisomy of chromosome 21 accounts for approximately 95 % of chromosome 21 trisomy cases, and the remaining cases are attributable to other chromosomal abnormalities, including translocations to chromosome 14 or 21 (3 %), mosaicism (2 %), and partial trisomy [13]. DS is a multifactorial disease, where in abnormal expression of trisomic genes arises not only from genetic but also environmental factors. Thus, trisomy leads to deregulation that also affects disomic genes and ultimately results in largely different phenotypes. DS is associated with mental retardation and short stature, and it shared a common set of extraoral features, including epicanthal folds at the eyelids, a broad nasal bridge, frontal bossing, open mouth, and an underdeveloped midface. Intraorally, patients exhibit macroglossia, high-arch plates, pronathism, fissured tongue [14], and congenital missing teeth. Patients with DS have an increased incidence of gastrointestinal tract anomalies, congenital heart disease, acute myeloid leukemia (AML), cataractogenesis [15], nutritional difficulties during infancy, seizure disorders, asleep apnea, visual impairment, audiological dysfunction, cataracts, growth retardation, and weight gain at adulthood. Moreover, immune disorders such as respiratory infections, celiac disease [16], thyroid disorders [17], and diabetes mellitus [18, 19] are prevalent. Currently, DS is one of the most common birth defects, affecting about 1 in every 750–1,000 live births [11]. DS is associated with premature aging and Alzheimer-like dementia, as well as shortened life expectancy [20]. Worldwide, life expectancy among this

population is increasing [21]. Generally, these patients now live to age 50 and some to age 60. As the life expectancy of DS patients continues to increase, new medical and social interventions should be sought to improve quality of life [11, 22].

15.1.2 Down Syndrome and ROS

The complete DNA sequence of chromosome 21, which is the smallest human chromosome, was first reported in 2000 [16]. The successful management of clinical complications in children with DS is a major medical challenge and depends on the understanding of the unique metabolic imbalance induced by overexpression of genes on chromosome 21. Individuals with DS undergo an accelerated process of aging, which is thought to be associated with high levels of oxidative stress throughout the lifespan [1]. The evidence for a multiple prooxidant state in young DS patients supports the role of oxidative stress associated with the DS phenotype, with relevant distinctions according to patients' ages [23]. The presence of an extra copy of chromosome 21, or rather the consecutive overexpression of the genes located on it, has been regarded as the central point for the development of the DS phenotype. Overexpression of the encoded proteins leads to overconsumption of their substrates and overproduction of their metabolic end-products [15, 19].

Many proteins coded on chromosome 21 play important roles in immune and nervous system function; these proteins include amyloid precursor protein (APP) mapping in 21q21.3–22.05, the cytoplasmic enzyme Cu^{2+}/Zn^{2+} SOD-1 (mapping in 21q22.1), the DS critical/candidate region (DSCR) (q22.3), cystathionine-synthase (CBS), glycinamide ribonucleotide synthase–aminoimidazole ribonucleotide synthase–glycinamide formyl transferase (GARS–AIRS–GART) (q22.1), CD18-β chain of LFA-1, interferon receptor, and protein S-100β (Fig. 15.1). APP and SOD-1, both of which are responsible for the regulation of ROS homeostasis [2], are important factors in the development of oxidative stress. Elevated levels and/or activity of these enzymes, which occurs decades prior to the appearance of the signature pathological features of DS, are thought to contribute to neuronal death and disease progression in DS patients. DSCR is also found on the long arm of chromosome 21 (21q), including CBS. GARS–AIRS–GART is the gene coding for the trifunctional enzyme complex, which catalyzes certain display overexpression, suggesting that the DS phenotype cannot be explained simply by the "gene dosage effect" [24].

SODs are also activated by H_2O_2 and Cu(II) [25]. The presence of CuZn-SOD, Mn-SOD, or Mn(II) has also been shown to enhance the frequency of DNA damage induced by H_2O_2 and Cu(II), and to alter the site specificity of the latter: H_2O_2 induces Cu(II)-dependent DNA damage with high frequency at 5′-guanine of poly G sequences; in the presence of SODs, the frequency of cleavages at thymine and cytosine residues is increased and 8-oxo-7,8-dihydro-2′-deoxyguanosine is formed [25].

Peroxynitrite, formed by the reaction of nitric oxide (NO·) with superoxide (O_2^{-}), is a highly reactive molecule that breaks down to form the hydroxyl radical HO·. ROS can interact with cellular components such as proteins, lipids, and DNA [26],

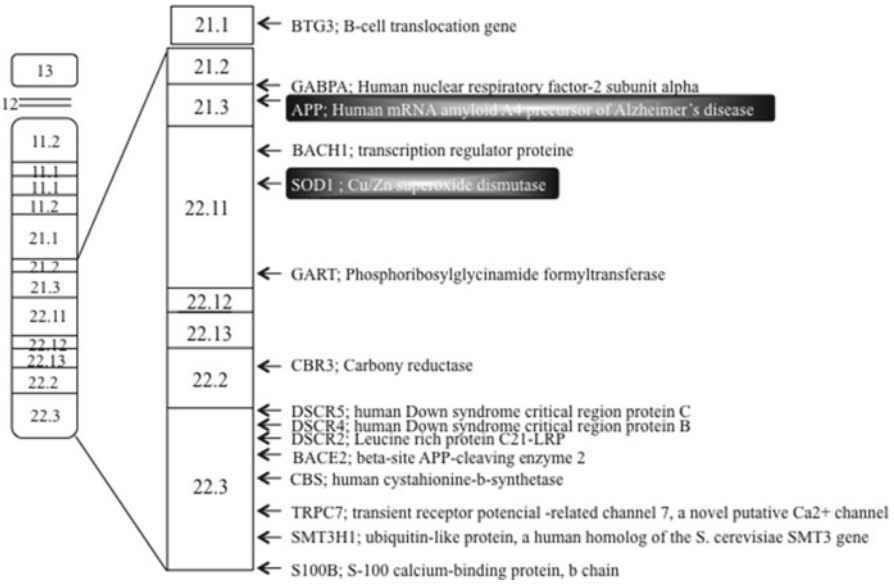

Fig. 15.1 Regional assignment of genes to chromosome 21 related to antioxidant, oxidative stress

and ultimately cause cell death. Oxidative stress is defined as an imbalance between the generation and removal of ROS, and the process may play a significant role in the pathogenesis of DS.

A considerable amount of research regarding the role of SOD in the central nervous system (CNS) has been published to data. Evidence of elevated SOD concentrations in the adult DS brain suggests the existence of a "gene dosage effect" as a response to oxidative injury [27, 28]. The increase in SOD levels results in enhanced production of H_2O_2. These results are consistent with the "gene dosage effect" hypothesis, confirming the significance of oxidative stress. In contrast, fetal DS neurons were not found to overexpress SOD, and therefore the impaired oxidative status may occur only as a consequence of the low levels of reducing agents and enzymes involved in the removal of H_2O_2 [29]. In agreement with these findings, the increased rate of apoptosis in these neurons could be inhibited by scavengers of H_2O_2, suggesting that unbalanced H_2O_2 metabolism plays a central role in the enhanced cell death that has been postulated to be associated with the progressive mental decline in DS and Alzheimer's disease (AD) [3].

In patients with DS, an altered SOD/GPX activity ratio is observed not only in the CNS but in all other tissues as well [30]. SOD levels approximately 50 % higher than normal have been reported for a variety of DS cells and tissues, including erythrocytes, B and T lymphocytes, and fibroblasts. Systemic increases in SOD, SOD/GPX, or the SOD/(GPX+CAT) activity ratio are seen in erythrocytes from children, adolescents, and adults with DS [31, 32].

Likewise, elevated SOD activity is also detected in neutrophils from DS patients, regardless of the age of the probands [31]. Consistent with these observations,

enhanced antioxidant enzyme activity ratios have also been reported in fibroblasts and lymphocytes of DS patients [33], which may affect gene expression by altering the binding and/or availability of transcription factors such as nuclear factor-κB (NF-κB) [30] Taken together, despite some conflicting results, the majority of studies have suggested that antioxidant enzymes, and particularly SOD, play an important role in both DS and AD, which is consistent with the notion that oxidative stress underlies these disorders and serves as a necessary insult to initiate (although not sufficient alone to propagate) disease pathogenesis [34, 35].

15.1.3 Down Syndrome and Periodontal Disease

Despite anatomic differences among patients with DS, the clinical features include a high prevalence of periodontal disease in association with physiological alterations. Cohen and colleagues [14] conducted an investigation of 100 young patients with DS and found that virtually all had some degree of periodontal disease, ranging from severe gingivitis in the youngest patients to periodontal disease with pocket formation and alveolar bone loss in the older patients. Both the prevalence and severity of periodontal disease was greater in the 212 individuals with DS than in their 124 unaffected siblings [36].

In those with DS, the onset of the disease process is apparent even in the deciduous dentition. Periodontal disease is often severe, especially in the region of the lower anterior teeth. Its progression is rapid and particularly so in younger age groups. Individuals with DS have a higher prevalence and severity of periodontal disease, which cannot be explained by poor oral hygiene alone and is related to changes in the immune response. In an extensive review of periodontal disease in DS, Roland-Bousma and Van Dijk [37] examined both endogenous conditions and exogenous factors that may predispose affected patients to aggressive periodontal disease. They divided the exogenous factors into local factors, related mostly to oral hygiene, and secondary factors, such as tongue thrust, malocclusion, and lack of lip seal. The bulk of the evidence suggests that although these exogenous factors clearly contribute to the development of periodontal disease they do not correlate with the severity of periodontal disease in DS patients.

Endogenous factors might contribute to the rapid progression of periodontal breakdown. The main immune defect occurs in the thymus-dependent system, which may result in a reduced amount of mature T cells together with a relatively large proportion of immature ones. This, together with the possibility of differences in collagen biosynthesis and an abnormal capillary morphology, may explain the higher susceptibility to periodontal disease observed in DS patients. Defects in neutrophils are among the endogenous factors that may exacerbate periodontal disease in DS. The first cellular anomaly linked to DS was the tendency of the nucleus of neutrophils to be consistently less segmented in DS patients than in other types of patients [38]. While circulating neutrophil counts in DS patients are not significantly different from healthy controls, some authors have reported a preponderance

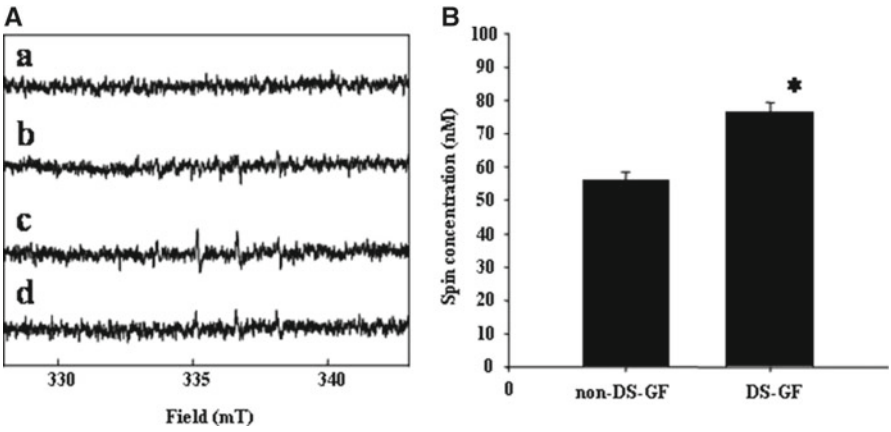

Fig. 15.2 Hydroxyl radical (HO·) generation from cultured Down syndrome-gingival fibroblasts (DS-GF) or non-DS-GF. (**A**) ESR spin trapping measurement of HO· generation from cultured DS-GF or non-DS-GF. (*a*) Culture medium, (*b*) 5,5-dimetyl-1-pyrolline-N-oxide (DMPO) and culture medium, (*c*) DMPO and cultured DS-GF, (*d*) DMPO and cultured non-DS-GF. In (**B**), are shown DMPO spin concentration of HO· generation from DS-GF or non-DS-GF. Data are presented as mean S.E. of triplicate experiments. *Significance $p<0.05$ difference from the corresponding value of non-DS-GF [4]

of younger cell forms that is independent of both the total leukocyte count and the relative number of neutrophils [39]. The abnormal function of neutrophils in DS was noted with regard to random mobility and phagocytosis. It has been reported that neutrophil chemotaxis is significantly impaired in children with DS [40, 41], and this effect would be expected to lead to a diminished ability for neutrophil phagocytosis of *Candida albicans*. Reduced bacteriocidal capacity has been reported for a number of organisms, including *Staphylococcus aureus*, *Escherichia coli*, and *C. albicans* [42, 43]. The results of tests using nitroblue tetrazolium reduction in neutrophils from DS patients have also been mixed, with lower than expected values found by Tan and colleagues and normal values noted by Seger and Barkin [38, 40, 44]. Taken together, these studies suggest that the oxidative metabolic potential of neutrophils in DS patients varies considerably. Oxidative stress due to the generation of ROS has been implicated in the pathogenesis of a variety of inflammatory diseases. ROS are generated enzymatically during inflammation as a result of the neutrophil oxidative burst [45, 46]. ROS can lead to various pathophysiological phenomena including periodontal disease [47, 48], temporomandibular disease [49], and impaired wound healing of periodontal tissue after implant surgery [50].

Questions remain regarding the extent of ROS generation from gingival fibroblasts (GFs) in DS, since the concentrations of GF-mediated ROS have not been directly monitored. Using ESR and spin trapping, we found clear and direct evidence that HO· is generated in culture media from the GFs of DS patients (Fig. 15.2) [4]; the involvement of a Fenton-type reaction in the generation of HO· was suggested by the ability of CAT to reduce the formation of DMPO-OH spin adduct (Fig. 15.3) [4].

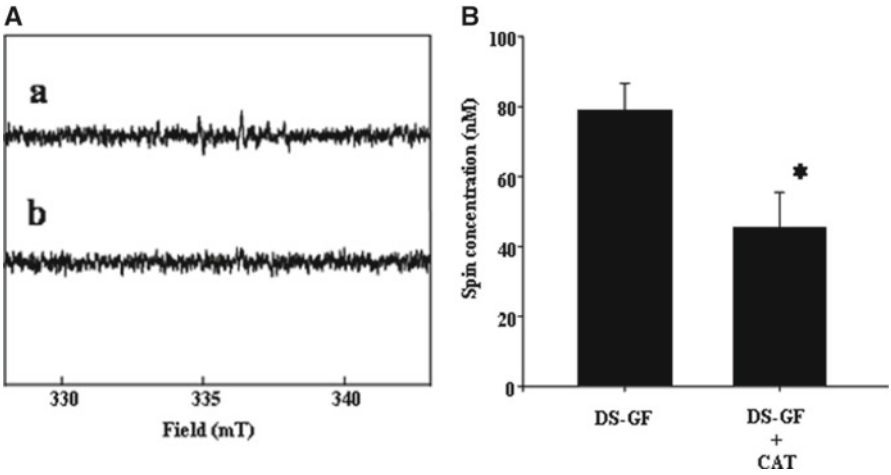

Fig. 15.3 Effects of H_2O_2 scavenger, catalase (CAT) on hydroxyl radical (HO·) generation from cultured Down syndrome-gingival fibroblasts (DS-GF). (**A**) ESR spin trapping measurement of HO· generation from cultured DS-GF or non-DS-GF. (*a*) 5,5-Dimetyl-1-pyrolline-N-oxide (DMPO) and cultured DS-GF, (*b*) DMPO with the pretreatment (1 h) of CAT (100 unit/ml) to cultured DS-GF. In (**B**), are shown the effects of CAT on DMPO spin concentration of HO· generation from DS-GF. Data are presented as mean S.E. of triplicate experiments. *Significance $p < 0.05$ difference from the corresponding value of DS-GF [4]

These results are consistent with the possibility that SOD-1 overexpression [51] in DS could result in an increase in the formation of H_2O_2. In this present study, DMPO-OH spin adduct in the culture media from GFs of DS patients was significantly decreased by desferrioxamine (DFO) (Fig. 15.4) [4]. Other clinical groups at our institution previously reported that HO· production and subsequent lipid peroxidation via the iron-catalyzed Fenton-type reaction is of critical importance in the pathophysiology of tempromadibular disease [49]. These results suggest that HO· generated via the Fenton reaction may lead to periodontal disease in individuals with DS [4].

We also showed that the concentration of DMPO-OH was enhanced in GFs from DS patients compared with non-DS subjects (Fig. 15.2B) [4]. Increased generation of HO· in DS implies an abundance of H_2O_2 due to dismutation of $O_2^{\cdot-}$ resulting from overexpression of SOD. This would arise from the ability of SOD to catalyze the formation of H_2O_2 from $O_2^{\cdot-}$, thereby increasing the availability of H_2O_2 as a substrate for the iron-dependent generation of HO· via the Fenton reaction. It is possible that the increased generation of HO· in DS causes a variety of clinical disorders, including severe periodontal disease and early aging. In addition, neutrophils activated by periodontal pathogens and/or periodontal fibroblasts induced by cytokines may reflect the periodontal destruction seen in patients with DS.

The findings to date suggest that DS patients may have inappropriate regulation of enzymes and T-cell immunodeficiency together with functional defects of PMNs

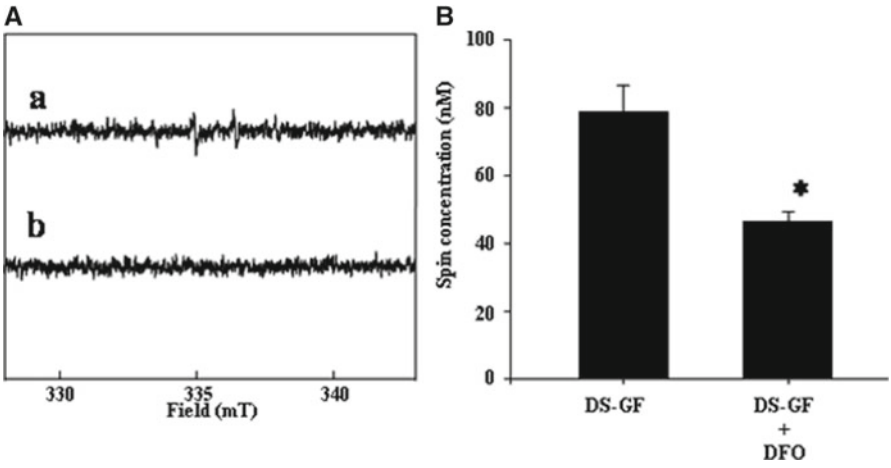

Fig. 15.4 Effects of iron chelator Desferal (DFO) on hydroxyl radical (HO·) generation from cultured Down syndrome-gingival fibroblasts (DS-GF). (**A**) Electron spin resonance (ESR) spin trapping measurement of HO· generation from cultured DS-GF or non-DS-GF. (*a*) DMPO and cultured DS-GF, (*b*) DMPO with the pretreatment (1 h) of DFO (10 mM) to cultured DS-GF. In (**B**), are shown the effects of DFO on DMPO spin concentration of HO· generation from DS-GF. Data are presented as mean S.E. of triplicate experiments. *Significance $p<0.05$ difference from the corresponding value of DS-GF [4]

and monocytes. This, together with potential differences in collagen biosynthesis as well as abnormal capillary morphology and hyperinnervation of the gingiva, may contribute to the rapid periodontal destruction observed in DS patients.

15.1.4 Measurement of Oxidative Stress in DS-Related Periodontal Disease

It is well known that proteins, lipids, and DNA are susceptible to oxidation, which results in a wide variety of chronic diseases and acute pathologic processes. ROS can attack nucleic acids in living cells. One of the byproducts of oxidative damage of DNA in the nucleus is 8-OHdG, which arises from specific enzymatic cleavage after 8-hydroxylation of guanine. Several studies have indicated that the 8-OHdG levels in body fluids are a biomarker of oxidative stress [52]. The urinary levels of 8-OHdG have been shown to be increased in association with aging and in patients with cancer, atherosclerosis, rheumatoid arthritis, Parkinson disease, diabetes, and DS [6, 53]. Recently, 8-OHdG was used as a marker for the evaluation of oxidative stress in subjects with periodontal disease [6, 54].

There are many advantages associated with the use of saliva as a clinical diagnostic biofluid. Sample collection is simple, non-invasive, and causes little anxiety on the part of patients. Saliva analysis also offers a cost-effective approach for large-scale

Table 15.1 One way ANOVA test results of comparing salivary 8-OHdG concentration in four groups and the pair-wise comparison using Scheff's test for all four groups [9]

Study group	8-OHdG concentration (ng/ml)	Pair-wise comparison among the groups	
		Study group	*p*-value
DS-1	1.88 ± 1.37	DS1 and DS2	<0.01*
DS-2	3.30 ± 1.44	DS1 and C1	<0.01*
C-1	1.04 ± 0.76	DS1 and C2	–
C-2	2.10 ± 1.41	DS2 and C1	<0.01*
F-value	16.67	DS2 and C2	<0.01*
p-value	<0.001	C1 and C2	<0.01*

The DS and C groups were subdivided according to age: group 1 consisted of DS (DS-1) and control (C-1) subjects under 12 years old, and group 2 consisted of DS (DS-2) and control (C-2) subjects over 30 years of age [9]
*Significant at 1 % level of significance ($p < 0.01$)

screening [54] and has been used for as a diagnostic alternative to blood tests [55]. A number of findings in the past decade have prompted interest in the diagnostic use of saliva biomarkers. For example, the levels of hormones (e.g., cortisol, oxytocin) and drugs (e.g., cisplatin, nicotine, methadone) in saliva reflect their concentration in serum [56]. In 2004, saliva-based HIV detection was approved by the US Food and Drug Administration (FDA) [57]. It has been reported that the presence of 8-OHdG in saliva is associated with oxidative stress in aging [5] and disorders such as DS [6] and periodontal disease [7], and that it can be used to predict severe erectile dysfunction [8].

The most interesting and novel finding of the current study was that the levels of 8-OHdG in saliva, urine, and leukocytes were significantly higher in DS patients versus controls regardless of age; this phenomenon may be associated with accelerated aging in DS patients (Table 15.1) (Elsevier) [9]. Surprisingly, the salivary 8-OHdG levels in young DS patients were similar to the levels in older control subjects (Table 15.1) (Elsevier) [9]. Furthermore, salivary 8-OHdG levels showed a statistically significant positive correlation with the gingival index in young DS patients ($r = 0.26$) but not in normal subjects (Table 15.2) (Elsevier) [9]. Thus, this evidence of the existence of a prooxidant state in young DS subjects supports the notion that gingivitis and periodontal disease are related to oxidative stress associated with the DS phenotype, and that this relationship varies according to the patient's age.

The difference in salivary 8-OHdG levels between young and old DS subjects was greater than that between young and old normal subjects, suggesting that the salivary levels of 8-OHdG were significantly higher overall in older versus younger subjects (Table 15.1) (Elsevier) [9]. This finding is again consistent with the possible involvement of oxidative stress in the DS phenotype. Our results suggest that abnormalities in redox pathways leading to accumulation of HO [4] may be a factor contributing to the early onset of severe periodontal disease in DS patients. Furthermore, we also found that the 8-OHdG level was significantly correlated with

Table 15.2 Pearson's correlation coefficient test comparing the salivary 8-OHdG to GI, and PD among the groups [9]

Study group	GI	PD
DS1	0.26*	0.05
DS2	0.40*	0.62**
C1	0.13	0.18
C2	0.73**	0.64**

The DS and C groups were subdivided according to age: group 1 consisted of DS (DS-1) and control (C-1) subjects under 12 years old, and group 2 consisted of DS (DS-2) and control (C-2) subjects over 30 years of age
*Significant at 0.05 level ($p<0.05$); **significant at 0.01 level ($p<0.01$)

probe depth (PD) in subjects greater than 30 years old but was not significantly correlated with PD in subjects less than 12 years old (Table 15.2) (Elsevier) [9]. These results indicate that the level of 8-OHdG in saliva could serve a sensitive biomarker for periodontal disease and a surrogate for PD in adults with DS. More research involving direct evaluation of oxidative stress using a biomarker such as 8-OHdG or another sensitive technique is needed to improve the diagnosis and management of periodontal disease in DS patients.

15.1.5 Conclusion

DS is considered a multifactorial disease, where abnormal expression of trisomic genes on chromosome 21 arises not only from genetic factors but also from environmental factors. Various studies have indicated that SOD plays an important role in both DS and AD, which is consistent with the notion that oxidative stress underlies these disorders and serves as a necessary insult for initiation and progression of the disease [3].

Individuals with DS have a higher prevalence and severity of periodontal disease, which cannot be explained by poor oral hygiene alone and is related to changes in the immune response. It has been reported that the 8-OHdG in saliva is a useful marker for the assessment of various oxidative stress-induced diseases [5–7] on DS, and that it may be useful for predicting a more severe disease level [8]. The salivary levels of the oxidative stress biomarker 8-OHDG were significantly higher in DS patients than in control subjects, suggesting that high oxidative stress may lead to some of the clinical features of DS, especially rapidly progressive periodontal disease associated with premature aging [9]. In the future, analysis of 8-OHdG and other biomarkers in saliva could be useful for the assessment of oxidative stress and management of periodontal disease in DS patients.

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References

1. Perluigi M, Butterfield DA (2012) Oxidative stress and Down syndrome: a route toward Alzheimer-like dementia. Curr Gerontol Geriatr Res 2012:724904
2. Schuchmann S, Heinemann U (2000) Increased mitochondrial superoxide generation in neurons from trisomy 16 mice: a model of Down's syndrome. Free Radic Biol Med 28:235–250
3. Busciglio J, Yankner BA (1995) Apoptosis and increased generation of reactive oxygen species in Down's syndrome neurons in vitro. Nature 378:776–779
4. Komatsu T, Lee MC, Miyagi A et al (2006) Reactive oxygen species generation in gingival fibroblasts of Down syndrome patients detected by electron spin resonance spectroscopy. Redox Rep 11:71–77
5. Hayakawa M, Hattori K, Sugiyama S et al (1992) Age-associated oxygen damage and mutations in mitochondrial DNA in human hearts. Biochem Biophys Res Commun 189:979–985
6. Campos C, Guzman R, Lopez-Fernandez E et al (2011) Evaluation of urinary biomarkers of oxidative/nitrosative stress in adolescents and adults with Down syndrome. Biochim Biophys Acta 1812:760–768
7. Sawamoto Y, Sugano N, Tanaka H et al (2005) Detection of periodontopathic bacteria and an oxidative stress marker in saliva from periodontal disease patients. Oral Microbiol Immunol 20:216–220
8. Yasuda M, Ide H, Furuya K et al (2008) Salivary 8-OHdG: a useful biomarker for predicting severe ED and hypogonadism. J Sex Med 5:1482–1491
9. Komatsu T, Duckyoung Y, Ito A et al (2013) Increased oxidative stress biomarkers in the saliva of Down syndrome patients. Arch Oral Biol 58:1246–1250
10. Megarbane A, Ravel A, Mircher C et al (2009) The 50th anniversary of the discovery of trisomy 21: the past, present, and future of research and treatment of Down syndrome. Genet Med 11:611–616
11. Frydman A, Nowzari H (2012) Down syndrome-associated periodontal disease: a critical review of the literature. Compend Contin Educ Dent 33:356–361
12. Hassold TJ, Jacobs PA (1984) Trisomy in man. Annu Rev Genet 18:69–97
13. Cichon P, Crawford L, Grimm WD (1998) Early-onset periodontal disease associated with Down's syndrome—clinical interventional study. Ann Periodontol 3:370–380
14. Cohen MM, Winer RA, Schwartz S et al (1961) Oral aspects of mongolism. I: periodontal disease in mongolism. Oral Surg Oral Med Oral Pathol 14:92–107
15. Roizen NJ, Patterson D (2003) Down's syndrome. Lancet 361:1281–1289
16. Sustrova M, Sarikova V (1997) Down's syndrome—effect of increased gene expression in chromosome 21 on the function of the immune and nervous system. Bratisl Lek Listy 98:221–228
17. Pueschel SM (1990) Clinical aspects of Down syndrome from infancy to adulthood. Am J Med Genet Suppl 7:52–56
18. Smith DS (2001) Health care management of adults with Down syndrome. Am Fam Phys 64:1031–1038
19. Thiel R, Fowkes SW (2005) Can cognitive deterioration associated with Down syndrome be reduced? Med Hypotheses 64:524–532
20. Zigman WB, Lott IT (2007) Alzheimer's disease in Down syndrome: neurobiology and risk. Ment Retard Dev Disabil Res Rev 13:237–246
21. Malt EA, Dahl RC, Haugsand TM et al (2013) Health and disease in adults with Down syndrome. Tidsskr Nor Laegeforen 133:290–294
22. Teksen F, Sayli BS, Aydin A et al (1998) Antioxidative metabolism in Down syndrome. Biol Trace Elem Res 63:123–127
23. Pallardo FV, Degan P, d'Ischia M et al (2006) Multiple evidence for an early age pro-oxidant state in Down syndrome patients. Biogerontology 7:211–220
24. Cheon MS, Shim KS, Kim SH et al (2003) Protein levels of genes encoded on chromosome 21 in fetal Down syndrome brain: challenging the gene dosage effect hypothesis (Part IV). Amino Acids 25:41–47

25. Midorikawa K, Kawanishi S (2001) Superoxide dismutases enhance H2O2-induced DNA damage and alter its site specificity. FEBS Lett 495:187–190
26. de Teunissen CE, Vente J, Steinbusch HW et al (2002) Biochemical markers related to Alzheimer's dementia in serum and cerebrospinal fluid. Neurobiol Aging 23:485–508
27. Brooksbank BW, Balazs R (1984) Superoxide dismutase, glutathione peroxidase and lipoperoxidation in Down's syndrome fetal brain. Brain Res 318:37–44
28. Gulesserian T, Seidl R, Hardmeier R et al (2001) Superoxide dismutase SOD1, encoded on chromosome 21, but not SOD2 is overexpressed in brains of patients with Down syndrome. J Investig Med 49:41–46
29. Gulesserian T, Engidawork E, Fountoulakis M et al (2001) Antioxidant proteins in fetal brain: superoxide dismutase-1 (SOD-1) protein is not overexpressed in fetal Down syndrome. J Neural Transm Suppl 61:71–84
30. de Haan JB, Cristiano F, Iannello RC et al (1995) Cu/Zn-superoxide dismutase and glutathione peroxidase during aging. Biochem Mol Biol Int 35:1281–1297
31. Muchova J, Sustrova M, Garaiova I et al (2001) Influence of age on activities of antioxidant enzymes and lipid peroxidation products in erythrocytes and neutrophils of Down syndrome patients. Free Radic Biol Med 31:499–508
32. Pastore A, Tozzi G, Gaeta LM et al (2003) Glutathione metabolism and antioxidant enzymes in children with Down syndrome. J Pediatr 142:583–585
33. Sikora E, Radziszewska E, Kmiec T et al (1993) The impaired transcription factor AP-1 DNA binding activity in lymphocytes derived from subjects with some symptoms of premature aging. Acta Biochim Pol 40:269–272
34. Moreira PI, Honda K, Zhu X et al (2006) Brain and brawn: parallels in oxidative strength. Neurology 66:S97–S101
35. Zhu X, Raina AK, Perry G et al (2004) Alzheimer's disease: the two-hit hypothesis. Lancet Neurol 3:219–226
36. Orner G (1976) Periodontal disease among children with Down's syndrome and their siblings. J Dent Res 55:778–782
37. van Reuland-Bosma W, Dijk J (1986) Periodontal disease in Down's syndrome: a review. J Clin Periodontol 13:64–73
38. Seger R, Buchinger G, Stroder J (1977) On the influence of age on immunity in Down's syndrome. Eur J Pediatr 124:77–87
39. Mittwoch U (1958) The leucocyte count in children with mongolism. J Ment Sci 104:457–460
40. Barkin RM, Weston WL, Humbert JR et al (1980) Phagocytic function in Down syndrome—I: chemotaxis. J Ment Defic Res 24(Pt 4):243–249
41. Khan AJ, Evans HE, Glass L et al (1975) Defective neutrophil chemotaxis in patients with Down syndrome. J Pediatr 87:87–89
42. Costello C, Webber A (1976) White cell function in Down's syndrome. Clin Genet 9:603–605
43. Gregory L, Williams R, Thompson E (1972) Leucocyte function in Down's syndrome and acute leukaemia. Lancet 1:1359–1361
44. Tan CV, Rosner F, Feldman F (1973) Nitroblue tetrazolium dye reduction in various hematologic disorders. N Y State J Med 73:952–956
45. Lee C, Miura K, Liu X et al (2000) Biphasic regulation of leukocyte superoxide generation by nitric oxide and peroxynitrite. J Biol Chem 275:38965–38972
46. Babior BM (1984) The respiratory burst of phagocytes. J Clin Invest 73:599–601
47. Chapple IL (1997) Reactive oxygen species and antioxidants in inflammatory diseases. J Clin Periodontol 24:287–296
48. Waddington RJ, Moseley R, Embery G (2000) Reactive oxygen species: a potential role in the pathogenesis of periodontal diseases. Oral Dis 6:138–151
49. Lee MC, Kawai Y, Shoji H et al (2004) Evidence of reactive oxygen species generation in synovial fluid from patients with temporomandibular disease by electron spin resonance spectroscopy. Redox Rep 9:331–336

50. Lee MC, Yoshino F, Shoji H et al (2005) Characterization by electron spin resonance spectroscopy of reactive oxygen species generated by titanium dioxide and hydrogen peroxide. J Dent Res 84:178–182
51. Sherman L, Dafni N, Lieman-Hurwitz J et al (1983) Nucleotide sequence and expression of human chromosome 21-encoded superoxide dismutase mRNA. Proc Natl Acad Sci U S A 80:5465–5469
52. Chen HI, Liou SH, Ho SF et al (2007) Oxidative DNA damage estimated by plasma 8-hydroxydeoxyguanosine (8-OHdG): influence of 4,4′-methylenebis (2-chloroaniline) exposure and smoking. J Occup Health 49:389–398
53. Wu LL, Chiou CC, Chang PY et al (2004) Urinary 8-OHdG: a marker of oxidative stress to DNA and a risk factor for cancer, atherosclerosis and diabetics. Clin Chim Acta 339:1–9
54. Takane M, Sugano N, Ezawa T et al (2005) A marker of oxidative stress in saliva: association with periodontally-involved teeth of a hopeless prognosis. J Oral Sci 47:53–57
55. Kaufman E, Lamster IB (2002) The diagnostic applications of saliva—a review. Crit Rev Oral Biol Med 13:197–212
56. Hofman LF (2001) Human saliva as a diagnostic specimen. J Nutr 131:1621S–1625S
57. Branson BM (2004) FDA approves OraQuick for use in saliva: on March 25, the FDA approved the first rapid test for HIV in oral fluids. AIDS Clin Care 16:39

Part IV
Future Strategies (Control of ROS, Roles of Nutrition/Antioxidants and Application to Dentistry)

Chapter 16
New Theories and Their Clinical Relevance to the Onset and Development of Periodontal Diseases

Pedro Bullon

16.1 Introduction

Every form of life needs a source of energy to survive. In humans it is supplied by the oxygen combustion of different foods. These foods need to be degraded to simple chemical compounds to be assimilated by the cells. This process starts in the mouth with the chewing process mediated by teeth. To do this job the teeth should be supported by the periodontium.

We know as periodontium all the structures that support the teeth resist the chewing rubbing and isolate the external septic portion from the internal one that should be aseptic. For an adequate chewing and good nutrition it should be essential the health of the periodontal tissues.

The term periodontal disease usually refers to the common inflammatory disorders of gingivitis and periodontitis that take place in the periodontium. Gingivitis, the mildest form of periodontal disease, is highly prevalent and readily reversible by simple, effective oral hygiene. Gingivitis affects 50–90 % of adults worldwide, depending on its precise definition [1]. Inflammation that extends deep into the tissues and causes loss of supporting connective tissue and alveolar bone is known as periodontitis. Periodontitis results in the formation of soft tissue pockets or deepened crevices between the gingiva and tooth root. Severe periodontitis can result in loosening of teeth, occasional pain and discomfort, impaired mastication, and eventual tooth loss. In Europe 30–60 % of the population above 35 years is affected [2].

Chronic health diseases, such as cardiovascular disease and diabetes, are considered a social problem due to high incidence rate and the risk for life. Periodontal disease is one of the most prevalent diseases suffered by humans. The tooth loss damage does not produce mortality but the pathological mechanisms involved have

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common conditions with cardiovascular disease and diabetes, main causes of death in the developing countries.

The cause of periodontal diseases is attributed to bacteria. The mouth, like all external surfaces of the body and the gut, has a substantial microflora living in symbiosis with a healthy host. These organisms grow on tooth surfaces as complex, mixed, interdependent colonies in biofilms and are attached and densely packed against the tooth in the deeper layers. Cultural studies indicate that more than 500 distinct microbial species can be found in dental plaque [3]. However, molecular methods of 16S rDNA amplification reveal an even more diverse view of the subgingival bacterial flora and suggest that a large proportion of even this well-studied and familiar microbial environment remains uncharacterized [4]. Tooth cleanings every 48 h can maintain the biofilm mass at an amount compatible with gingival health. Unfortunately, few individuals achieve this, and the host response with an inflammatory reaction. An enormous research effort has been devoted to the study of periodontal-disease-associated microflora, certain clusters of bacterial species commonly cohabit subgingival sites and are reproducibly associated with periodontitis. The main putative pathogens include *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, and *Actinobacillus actinomycetemcomitans* [5]. The increasing amount of bacteria or some specific pathogenic types produce the inflammation as host response. Inflammation is the response of living tissues to injury or infection. It is part of the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. Inflammation is a protective attempt by the organism to remove the injurious stimuli and to heal the damaged tissues. However, sometimes inflammation can lead to progressive destruction of the tissue, starting a disease. In gingivitis the inflammatory reaction takes place in connective and epithelial tissues from the gingiva and all the lesions can be solved with the recovery of the morphological and physiological functions. Periodontitis has been shown to result from an imbalance among the natural microbial biofilm on the teeth, dental plaque, and the host inflammatory/immune response. Produce an irreversible alveolar bone resorption that leads to teeth loss.

Nowadays the key question is why in some patients the inflammatory response to bacterial infections is limited to gingivitis and in others produces alveolar bone loss periodontitis. We know that aggressive localized periodontitis presents an alveolar bone loss limited to molar mesial surface and incisors with few inflammatory clinical symptoms. In chronic periodontitis a small proportion of subjects exhibit severe and extensive periodontitis in any given age-group, but the proportion affected is greater in older age-groups. A specific group of population has to be considered as high risk to suffer from periodontitis [6]. Therefore our efforts should be to identify what are the characteristics of these patients to prevent and start the treatment as soon as possible. One of the possible mechanisms is an altered inflammatory response. In recent years it has been demonstrated an epidemiological relationship between periodontitis and systemic diseases mainly atheromatous disorders (especially coronary heart disease, peripheral vascular arteriosclerosis, and stroke) and diabetes. All these diseases present in its pathogenic process an altered inflammation. We have to highlight that host inflammation response mechanisms are

shared by tissues and systems throughout the body. It is a reaction against a specific stress situation for any organism that could affect different organs or tissues. Therefore, we can conclude that inflammation could be the key process to improve our understanding of the periodontal disease pathogenia.

This chapter reviews new theories about inflammation and their clinical relevance to the onset and development of periodontal diseases.

16.2 Tissues Inflammatory Mechanism

Classically inflammation has been classified as either acute or chronic. Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes (especially granulocytes) from the blood into the injured tissues. A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue. Prolonged inflammation, known as chronic inflammation, leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and not healing of the tissue. In the acute inflammation the causative agents are mainly pathogens and irritants, have an immediate onset, last for few days and finalized with a resolution, abscess formation, or transform into a chronic inflammation. The chronic inflammation is caused by nondegradable pathogens and persistent foreign bodies, it has a delayed onset, late months or years and finalized with tissue destruction, necrosis, or fibrosis.

Inflammation is characterized by five cardinal signs (PRISH): pain, redness, immobility (loss of function), swelling, and heat. These clinical signs have been produced by the following biological events:

16.2.1 Vascular Changes

The inflammation first step includes vasodilation and the increased capillary permeability that produces the extravasation of plasma, big molecules, and biochemical inflammatory mediators to the perivascular connective tissue. The number of vessels increased with a blood ectasia.

16.2.2 Inflammatory Cells

The cellular component involves leukocytes, which normally reside in blood and must move into the inflamed tissue via extravasation. It has to involve in a margination and adhesion to the endothelial cells, a migration across the endothelial cells,

via the process of diapedesis and the movement across the tissue. Some act as phagocytes, ingesting bacteria, viruses, and cellular debris. Others release enzymatic granules which damage pathogenic invaders. Leukocytes also release inflammatory mediators which develop and maintain the inflammatory response. Acute inflammation is mediated by granulocytes, while chronic inflammation is mediated by mononuclear cells such as monocytes and lymphocytes.

16.2.3 Biochemical Mediators and Its Membrane Receptors

All the inflammatory steps have been conducted by different chemical components produced by different cells. There are linking tools between the inflammation components with specific effects. The biochemical mediators can be grouped in four different systems:

(a) The complement system, when activated, results in the increased removal of pathogens via opsonization and phagocytosis.
(b) The kinin system generates proteins capable of sustaining vasodilation and other physical inflammatory effects.
(c) The coagulation system or clotting cascade which forms a protective protein mesh over sites of injury.
(d) The fibrinolysis system, which acts in opposition to the coagulation system, to counterbalance clotting and generate several other inflammatory mediators.

All of them produce its effect through the activation of target cell membrane receptors. These are specialized integral membrane proteins put across the phospholipid bilayer cell membrane that take part in communication between the cell and the outside world. The biochemical inflammatory mediators attach to the extracellular domain receptor and trigger changes in the function of the cell. Usually this produces a modification of gene expression through the activation of inflammatory transcription factors, such as NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), MAP-kinases (mitogen-activated protein kinases), and PPAR (peroxisome proliferator-activated receptor) [7].

16.2.4 Tissue Destruction and Systemic Effects

An important step in the inflammatory process is the destruction of the affected tissues to isolate it for a better control. The tissue destruction is mediated by metalloproteinases [8]. These are proteolytic enzymes whose catalytic mechanism involves a metal. Most of them are zinc-dependent, some use cobalt. The metal ion is coordinated with the protein via three ligands. The ligands co-coordinating the metal ion can vary with histidine, glutamate, aspartate, lysine, and arginine, all possible ligands. The fourth coordination position is taken up by a labile water molecule.

But some infectious organisms can escape the confines of the infected tissue spread to other parts of the body and also the inflammatory mediators can produce some systemic effects. These and other inflammatory molecules induced a systemic inflammatory response characterized by increasing body temperature, higher heart and respiratory rate, and white blood cell count alterations.

16.2.5 Aggression Control with Pathogens Destroys

The major mechanism to remove pathogens is the phagocytosis [9]. It is the cellular process of engulfing bacteria to form an internal phagosome by white blood cells and macrophages. It is activated by attachment to pathogen-associated molecular patterns (PAMPS), which leads to NF-κB activation. Opsonins such as C3b and antibodies can act as attachment sites and aid in the phagocytosis of pathogens. Engulfment of material is facilitated by the actin–myosin contractile system. The phagosome of ingested material is then fused with the lysosome, leading to degradation. Degradation can be oxygen-dependent or oxygen-independent:

(a) Oxygen-dependent degradation depends on a coenzyme nicotinamide adenine dinucleotide phosphate (NADPH) and the production of reactive oxygen species (ROS). Hydrogen peroxide and myeloperoxidase activate a halogenating system, which leads to the destruction of bacteria.
(b) Oxygen-independent degradation depends on the release of granules, containing proteolytic enzymes such as defensins, lysozyme, and cationic proteins. Other antimicrobial peptides are present in these granules, including lactoferrin, which sequesters iron to provide unfavorable growth conditions for bacteria.

16.2.6 Healing/Recovery

The outcomes of the inflammatory process could be [10]:

(a) *Resolution*. It is the complete restoration of the inflamed tissue back to a normal status. Inflammatory items such as vasodilation, chemical production, and leukocyte infiltration cease, and damaged parenchymal cells regenerate. It is mediated through lipoxins, resolvins, and protectins. In some situations where limited or short-lived inflammation has occurred; this is usually the outcome produced.
(b) *Fibrosis*. Large amounts of tissue destruction, or damage in tissues unable to regenerate, cannot be recovered completely by the body. Fibrous scarring occurs in these areas of damage, forming a scar composed primarily of collagen. The scar will not contain any specialized structures, such as parenchymal cells, hence functional impairment may occur.
(c) *Abscess Formation*. A cavity is formed containing pus, an opaque liquid containing dead white blood cells and bacteria with general debris from destroyed cells.

(d) *Chronic Inflammation*. In acute inflammation, if the injurious agent persists, then chronic inflammation will ensue. This process marked by inflammation lasting many days, months, or even years may lead to the formation of a chronic wound. Chronic inflammation is characterized by the dominating presence of macrophages in the injured tissue. These cells are powerful defensive agents of the body, but the toxins they release (including ROS) are injurious to the organism's own tissues as well as invading agents. Consequently, chronic inflammation is almost always accompanied by tissue destruction.

16.2.7 Cytokines

All these inflammatory mechanisms involved different tissues and a large number of cells. These all need to be interconnected through messengers called cytokines. Cytokines are small cell-signaling protein molecules that are secreted by numerous cells and are a category of signaling molecules used extensively in intercellular communication [11]. Each cytokine has a matching cell-surface receptor. Subsequent cascades of intracellular signaling then stimulate specific cell functions. This may include the upregulation and/or downregulation of several genes and their transcription factors, resulting in the production of other cytokines, an increase in the number of surface receptors for other molecules, or the suppression of their own effect by feedback inhibition. Cytokines have been classed as lymphokines, interleukins, and chemokines, based on their presumed function, cell of secretion, or target of action.

Lymphokines are a subset of cytokines that are produced by lymphocyte. They are protein mediators typically produced by T cells to direct the immune system response by signaling between its cells. Lymphokines have many roles, including the attraction of other immune cells, macrophages, and other lymphocytes to an infected site and their subsequent activation to prepare them to mount an immune response.

Interleukins are produced by a wide variety of body cells to modulate the immune system. The majority of interleukins are synthesized by helper CD4+ T lymphocytes, as well as through monocytes, macrophages, and endothelial cells. They promote the development and differentiation of T, B, and hematopoietic cells. Nowadays at least 32 different interleukines have been described.

Chemokines induce directed chemotaxis in nearby responsive cells; they are chemotactic cytokines. Some chemokines are considered proinflammatory and can be induced during an immune response to recruit cells of the immune system to a site of infection, while others are considered homeostatic and are involved in controlling the migration of cells during normal processes of tissue maintenance or development. These proteins exert their biological effects by interacting with G protein-linked transmembrane receptors called chemokine receptors that are selectively found on the surfaces of their target cells.

16.3 Cellular Inflammatory Mechanisms

All this inflammatory mechanism describes all the tissue aspects. We should think inflammation can take place in every organ or tissue in our body and involved cells. The cell is the basic structural and functional unit of all known living organisms. It is the smallest unit of life that is classified as a living thing and is often called the building block of life. To improve our understanding of the inflammation we need to know the intracellular physiology and how it can be related to the inflammatory process. Inflammation is a mechanism response to an aggression, a protective attempt by the organism to remove the injurious stimuli and to heal the damaged tissues. It is a specific stress situation for any cell; in such a condition, the cell should function perfectly with all necessary biochemical processes operating properly. In periodontitis inflammation takes place in the gingival connective tissue, mediated by cells and fibroblasts as main connective tissue cells and white blood cells as main participants' inflammatory cells. But any cell of the body can participate in the inflammation; therefore, host inflammation response mechanisms are shared by tissues and systems throughout the body. The main systemic diseases related to periodontitis are atheromatous disorders (especially coronary heart disease, peripheral vascular arteriosclerosis, and stroke), obesity, hypertension, and diabetes. As in periodontitis in all of them inflammation is an essential part of the pathogenesis taken place in different tissues. We will highlight the mounting evidence that the basis for the interrelationships lies at a fundamental intracellular level, as a meeting background among such chronic diseases and periodontitis. We will describe the intracellular physiological metabolic process related to inflammation.

16.3.1 Isolation/Individualization: Biological/Cell Membrane

All the cells need to maintain its individuality to be isolated from the exterior with a membrane. The biological membrane is a major structure in all living systems from one-celled prokaryotic microorganisms to complex many-celled eukaryotic organisms. It is a biological membrane that separates the interior of all cells from the outside environment. This membrane allows the cell to optimize its internal medium and protect sensitive components from the vagaries of a potentially harsh external environment. But functional compartmentalization may also occur within the cell in membrane-bound organelles such as the mitochondria, lysosomes, and Golgi apparatus. Biological membranes, however, are not simply inert impermeable barriers but play a crucial role in almost all cellular events. They are very highly selective filters and devices for active transport and are involved in many complex processes of living cells such as endocytosis, exocytosis, cell adhesion, cell movement, cell–cell recognition, cell–cell communication, and signal transduction. In addition, they permit the specialization of cellular functions within a cell by defining various compartments with different enzymatic activities which are a

prerequisite for many complex biochemical processes. As described by Singer and Nicholson in 1972 [12], this consists of a fluid lipid bilayer in which intrinsic proteins are either partly inserted or which they completely traverse. Both lipids and proteins are free to diffuse within the plane of the membrane unless constrained, for example, by extrinsic proteins associated with the cytoskeleton. It should not come as too much of a surprise that biological membranes are considerably more complex than lipid bilayers. Membranes are dynamic and fluid structures and their components are able to move freely within the plane of the membrane. A combination of glycosphingolipids and protein receptors is organized in glycolipoprotein microdomains termed lipid rafts. These specialized membrane microdomains compartmentalize cellular processes by serving as organizing centers for the assembly of signaling molecules, influencing membrane fluidity and membrane protein trafficking, and regulating neurotransmission and receptor trafficking. Lipid rafts are more ordered and tightly packed than the surrounding bilayer, but float freely in the membrane bilayer [13].

Nevertheless, all membranes are structurally organized in a similar manner. Lipid molecules are able to form sheet-like structures which provide the basic structure for the so-called lipid bilayer. This lipid bilayer is a relatively impermeable barrier and forms closed boundaries between compartments. The membrane proteins take over most of the membrane functions such as transport and transmembrane signaling or serve as a structural link to the extracellular matrix or to cytoskeletal elements. Although all biological membranes are thought to be constructed on a common pattern, there exist noticeable diversities among different types of membranes, i.e., the composition and behavior of membranes from one cell type to another, and from one organelle to another, can vary remarkably. These variations give each kind of biological membrane its distinctive identity and specialized function. The diversity is primarily the result of the different functions of the proteins present in each membrane and the way in which they interact with lipids, with each other, or with cytoplasmic components. Although proteins clearly mediate the specific membrane functions, lipids are increasingly being recognized as active participants in membrane-associated processes.

In the composition of biological membranes the ratio of lipid to protein varies significantly from membrane to membrane, ranging from approximately 1:4 to 4:1 and is dependent on the specific function of the membrane. For example, myelin membranes which serve mainly to insulate nerve cell axons contain less than 25 % of proteins, whereas in membranes which are involved in energy transduction (e.g., internal membranes of mitochondria and chloroplasts) the protein content is approximately 75 %. The most striking feature of lipids found in biological membranes is their enormous diversity. Any single membrane can contain well over 100 unique lipid species. The biological significance of this lipid heterogeneity is not known, but may be related to the recently recognized fact that lipids are active participants in many membrane-associated processes. Although the major role of lipids is to form the bilayer matrix, they are also involved in the proper organization of particular protein molecules within membranes.

Lipids are organic biomolecules which consist primarily of carbon and hydrogen and oxygen also generally, but in much lower percentages. Furthermore, they may

also contain phosphorus, nitrogen, and sulfur. It is a very heterogeneous group of substances that have only these two characteristics in common: they are insoluble in water and they are soluble in organic solvents such as ether, chloroform, and benzene. Lipids as biological molecules are responsible for: (1) a unique class of cellular structures, particles, and organizations; (2) providing life's most dynamic and efficient fuelling and energetic schemes; and (3) complex signaling systems within and between cells [14]. Lipids constitute a broad group of naturally occurring molecules that include fats, waxes, sterols, fat-soluble vitamins (such as vitamins A, D, E, and K), monoglycerides, diglycerides, triglycerides, phospholipids, and others. Lipids may be divided into eight categories: fatty acids, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, and polyketides (derived from condensation of ketoacyl subunits); and sterol lipids and prenol lipids (derived from condensation of isoprene subunits). There are two groups of fatty acids: saturated (SFA) that have only single bonds between carbon atoms and unsaturated that have one (monounsaturated fatty acid: MUFA) or more double bonds (polyunsaturated fatty acid: PUFA) in their chain and their molecules have kinks, with changes of direction in the places where the double bond. A critical property of unsaturated fatty acids is their configuration (*cis* or *trans*) around double-bonded carbon atoms.

Fatty acids are important sources of fuel because, when metabolized, they yield large quantities of ATP. Many cell types can use either glucose or fatty acids for this purpose. In particular, heart and skeletal muscle prefer fatty acids. The brain cannot use fatty acids as a source of fuel; it relies on glucose or ketone bodies.

The three major types of lipids in biological membranes are phospholipids, glycolipids, and cholesterol. All three are amphiphilic molecules: they have a hydrophilic ("water-loving" or polar) end and a hydrophobic ("water-hating" or nonpolar) end. The amphiphilic nature of some lipids allows them to form structures such as vesicles, liposomes, or membranes in an aqueous environment. The main biological functions of lipids include energy storage, as structural components of cell membranes, and as important signaling molecules. The specific lipid composition of a membrane can change, according to physiological conditions and diet. Most of the fat found in food is in the form of triglycerides, cholesterol, and phospholipids. Some dietary fat is necessary to facilitate absorption of fat-soluble vitamins (A, D, E, and K) and carotenoids. Humans and other mammals have a dietary requirement for certain essential fatty acids, such as linoleic acid (an omega-6 fatty acid) and alpha-linolenic acid (an omega-3 fatty acid) because they cannot be synthesized from simple precursors in the diet. Both of these fatty acids are 18-carbon PUFA differing in the number and position of the double bonds. Most vegetable oils are rich in linoleic acid (safflower, sunflower, and corn oils). Alpha-linolenic acid is found in the green leaves of plants, and in selected seeds, nuts, and legumes (in particular flax, rapeseed, walnut, and soy). Fish oils are particularly rich in the longer-chain omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

One of the possible fates of fatty acids is to be used for the synthesis of polar lipids, such as phospholipids and sphingolipids, which are essential components of the cell membranes. They form the permeability barrier and modulate the structural properties and functions of the several membrane proteins, responsible for all the

membrane-associated activities. The differences between fatty acids in its length and degree of unsaturation have deep influence on dynamics and structural properties of the lipid bilayers and can affect membrane plasticity. For example, SFA, such as palmitate, significantly decrease membrane fluidity [15].

It is now widely accepted that cell membrane composition and structure play a central role in controlling metabolic and cardiovascular disorders. The more saturated tails there are in the lipid bilayer, the less fluid (and active) a membrane becomes. Evidence has been demonstrated that SFA and *trans*-fat decrease membrane fluidity and represent prominent risk factors for different metabolic and cardiovascular pathologies. Long-chain PUFA are also involved in modulating the function of mitochondria. Of central importance is their effect on the efficiency of ligand recognition by receptors which control second messenger systems and signal transduction inside the cell. PUFA can improve cell sensitivity to hormones, and in particular to insulin [16]. There is evidence for potentially detrimental metabolic effects of low-fat, high-carbohydrate diets in some population groups, such as type 2 diabetics [17], and it is argued that n-6 PUFA and MUFA substitution is preferable since both would achieve further reductions in LDL cholesterol that cannot be achieved with the removal of SFA alone. A large number of studies have shown positive health benefits associated with consumption of omega-3 fatty acids on infant development, cancer, cardiovascular diseases, and various mental illnesses, such as depression, attention-deficit hyperactivity disorder, and dementia [18]. In contrast, it is now well established that consumption of *trans*-fats, such as those present in partially hydrogenated vegetable oils, is a risk factor for cardiovascular disease [19]. Long-chain fatty acids influence inflammation through a variety of mechanisms; many of these are mediated by, or at least associated with, changes in fatty acid composition of cell membranes. Cells involved in the inflammatory response are typically rich in the n-6 fatty acid arachidonic acid, but the contents of arachidonic acid and of the n-3 fatty acids EPA and DHA can be altered through oral administration of EPA and DHA. Increased membrane content of EPA and DHA and decreased arachidonic acid content result in a changed pattern of production of eicosanoids and resolvins. Changing the fatty acid composition of cells involved in the inflammatory response also affects production of peptide mediators of inflammation (adhesion molecules, cytokines, etc.). Thus, the fatty acid composition of cells involved in the inflammatory response influences their function; the contents of arachidonic acid, EPA, and DHA appear to be especially important [20]. Lipid rafts are potentially modifiable by diet, particularly (but not exclusively) by dietary fatty acids. In vitro and animal studies show that n-3 PUFAs, cholesterol, and gangliosides modulate the structure and composition of lipid rafts, potentially influencing a wide range of biological processes, including immune function, neuronal signaling, cancer cell growth, entry of pathogens through the gut barrier, and insulin resistance in metabolic disorders [21].

Since the 1980s, a large body of evidence has evolved that suggests that fatty acids are capable of modulating immune function. The suggestion that unsaturated fatty acids modulate immune function arose primarily from animal studies, which demonstrated potent anti-inflammatory effects of the n-3 PUFAs. In vitro studies

suggest that PUFAs cause substantial reorganization of membrane microdomains, which has an impact on signaling and function in a number of cell types. Lipid rafts are potentially modifiable by diet, particularly by dietary fatty acids, gangliosides, and cholesterol [22].

In periodontitis it has been found that some perturbation exists in lipid biomarkers, such as increased serum total cholesterol and low-density lipoprotein cholesterol. Also total plasma fatty acids, saturated, n-6 polyunsaturated, and monounsaturated fatty acids were significantly higher in the periodontitis patients [23].

16.3.2 Energy Production Process: Mitochondria

All the inflammation processes need energy to take place. It is provided by the oxygen combustion of foods previously degraded in the digestive tube to simple chemical compounds. This one is known as aerobic respiration, they used oxygen as a common oxidizing agent (electron acceptor). The energy released in respiration is used to synthesize ATP, which stores this energy. It is the most profitable way of energy production. In aerobic respiration a molecule of glucose produces a net worth of 34 ATP molecules and in anaerobic respiration just 2. The energy stored in ATP can then be used to drive processes requiring energy, including biosynthesis, locomotion, or transportation of molecules across cell membranes. Glucose, fatty acids and amino acids are the main power source used by the cell respiration. The main intracellular organelle responsible for energy production is the mitochondria. A mitochondrion is a double membrane-enclosed organelle and a constituent of most eukaryotic cells. Mitochondria are descended from α-proteobacteria, and became part of the present-day eukaryotic cell through an endosymbiotic event approximately two billion years ago [24]. The two membranes each with a phospholipid bilayer separate four distinct compartments: the outer membrane; the intermembrane space; the inner membrane; and the matrix. The inner membrane forms multiple invaginations (known as cristae) into the matrix compartment. ATP is generated by the mitochondria in the process of oxidative phosphorylation. The inner membrane houses the megadalton complexes of the electron transport chain and ATP synthase that control the basic rates of cell metabolism necessary for oxidative phosphorylation. The energy is generated through a process in which reducing equivalents, derived from the oxidation of acetyl coenzyme A in the tricarboxylic acid cycle and from other oxidative processes (i.e., fatty-acid oxidation, the urea cycle, and amino-acid degradation), are transferred from nicotinamide adenine dinucleotide and flavin adenine dinucleotide to the electron transport chain and ultimately to oxygen, a process which produces an electrochemical gradient that is used to synthesize ATP. The mitochondrial respiratory chain, located in the inner mitochondrial membrane, is composed of enzymes, protein (i.e., cytochrome *c*), and low-molecular-weight redox intermediates (i.e., coenzymes such as ubiquinone or coenzyme Q) that transport reducing equivalents, in the form of hydrogen atoms or just their electrons, down the redox potential from respiratory substrates to

oxygen: an oxidative pathway composed of four multiple-subunit complexes. In this pathway, electrons are transferred from the reduced form of nicotinamide adenine dinucleotide to oxygen through the electron transport chain consisting of complex I (nicotinamide adenine dinucleotide dehydrogenase or nicotinamide adenine dinucleotide: ubiquinone oxidoreductase), complex II (succinate dehydrogenase or succinate: ubiquinone oxidoreductase), complex III (ubiquinol: cytochrome *c* oxidoreductase), and complex IV (cytochrome *c* oxidase) [25]. Although oxidative phosphorylation is more efficient than glycolysis in generating ATP, it carries the inherent risk of generating ROS as a result of the premature reaction of electrons with oxygen at respiratory complex I or complex III. ROS are chemically reactive molecules containing oxygen. Examples include oxygen ions and peroxides. ROS are highly reactive due to the presence of unpaired valence shell electrons. They can start chain reactions and cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions. Plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, and vitamin E as well as enzymes such as catalase, superoxide dismutase, and various peroxidases. Insufficient levels of antioxidants, or inhibition of the antioxidant enzymes, cause oxidative stress and may damage or kill cells. Oxidative stress is an imbalance between the systemic manifestation of ROS and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA. Further, some reactive oxidative species act as cellular messengers in redox signaling [26]. Thus, oxidative stress can cause disruptions in normal mechanisms of cellular signaling.

It is widely accepted that inflammation is a process essential for the maintenance of the integrity of living structures. Its mechanisms need energy, which is produced in the mitochondria by means of oxidative processes. Therefore, it can be assumed that changes in the oxidation processes of mitochondria can affect the inflammatory process. Cardiovascular disease, diabetes, and metabolic syndrome are the major cause of death in western countries [27]. It is generally accepted that the origin of all those metabolic disorders is a "proinflammatory" state derived from excessive caloric intake and over nutrition, and, perhaps, other chronic inflammatory conditions. This proinflammatory state also leads to an increase in oxidative stress, with the potential to impair several crucial biological mechanisms.

One example of how mitochondrial oxidative stress is produced is the influence of diet lipids in diabetes cardiomyopathy. High-fat diet-induced hyperlipidemia will lead to an increased facilitated diffusion of fatty acids (FA) over the plasma membrane into the cytoplasm. The higher intracellular concentration of FA will lead to an activation of PPAR signaling pathways. This enhances mitochondrial b-oxidation and CD36 translocation, which will speed up FA import and further boost PPAR stimulation. The resulting vicious circle of increased uptake of FA and FA-induced uptake stimulation eventually leads to mitochondrial FA overload. FA becomes the preferred substrate for mitochondrial b-oxidation at the expense of glucose.

Excessive b-oxidation results in massive ROS production. ROS production causes mitochondrial dysfunction, which leads to cardiomyocyte apoptosis and affects the complex signaling patterns of insulin action and glucose utilization [28].

Oxidative stress has been proposed as a common mechanism in the development of several features related to cardiovascular disease, diabetes and metabolic syndrome, and periodontitis, and perhaps an interaction between these conditions may result in a worse evolution of all those [29]. It has been demonstrated in peripheral blood mononuclear cells (PBMCs) from periodontitis patients a lower CoQ10 levels and citrate synthase activity, together with high levels of ROS production. Also human gingival fibroblasts treated with *P. gingivalis* lipopolysaccharide (a potent periodontal destruction mediator) provoked increased oxidative stress and mitochondrial dysfunction by a decrease in mitochondrial protein expression, mitochondrial mass, and mitochondrial membrane potential. Therefore mitochondrial dysfunction could represent a pathogenic mechanism in periodontitis and could be a possible link to understanding the interrelationships between two prominent inflammatory diseases: periodontitis and cardiovascular disease [30].

As we have mentioned previously oxidative process is used by the cells not only to produce energy but also to destroy bacteria and external molecules; therefore, it has been used as defense mechanism by the cells. With phagocytosis the bacteria is engulfed by white blood cells and macrophages, formed a phagosome, and fused with the lysosome. This one has an oxygen-dependent degradation that depends on the production of ROS. Therefore, it is an essential step in the inflammatory process.

16.3.3 Energy Production Control: AMPK System

We can consider mitochondria as the cell "rechargeable battery," with a key chemicals process that involves a balance between ATP and ADP. ATP is generated by the mitochondrial ATP synthase, thus "charging the battery" then is used by every cell function that requires energy by the hydrolysis of ATP back to ADP and phosphate, thus "discharging the battery." The reaction ATP ↔ ADP + phosphate is maintained by catabolism many orders of magnitude away from equilibrium, yielding a high ratio of ATP to ADP that is used to drive energy-requiring processes. ATP generation needs to remain in balance with ATP consumption, and regulatory proteins that sense ATP and ADP levels would be a logical way to achieve this. This recognition system that identifies the cellular energy status is mediated by the adenosine monophosphate P-activated protein kinase (AMPK). In general, AMPK switches on catabolic processes that provide alternative pathways to generate ATP, while switching off anabolic pathways and other processes consuming ATP, thus acting to restore cellular energy homeostasis. It is an enzyme that consists of three proteins (α, β, and γ subunits), conserved from yeast to humans, genes encoding the three subunits of the kinase are found in essentially all eukaryotic genomes. AMPK is activated not only by increases in ADP/ATP ratio but also by increases in Ca^{2+}, ROS, drugs such

as metformin, and xenobiotics such as resveratrol [31]. The net effect of AMPK activation is stimulation of hepatic fatty acid oxidation and ketogenesis, inhibition of cholesterol synthesis, triglyceride synthesis, inhibition of adipocyte lipolysis and lipogenesis, stimulation of skeletal muscle fatty acid oxidation and muscle glucose uptake, and modulation of insulin secretion by pancreatic beta-cells. Because the energy status of the cell is a crucial factor in all aspects of cell function, it is not surprising that AMPK has many downstream targets whose phosphorylation mediates dramatic changes in cell metabolism, cell growth, and other functions. The kinase evolved in single-celled eukaryotes and is still involved in multicellular organisms in regulating energy balance in a cell-autonomous manner. However, it is now clear that new functions were acquired during the development of metazoans so that AMPK is also regulated by hormones and adipokines that regulate energy balance at the whole body level. The maintaining of body energy homeostasis by AMPK includes: regulation of glucose uptake through glucose transporter type 4 in muscle contraction; promotion of fatty acid uptake in cardiac myocytes; regulation of mitochondrial biogenesis and mitophagy; regulation of biosynthesis of lipids, carbohydrates, proteins, and ribosomal RNA; mediation of the effects of hormones and other agents acting on neurons in different hypothalamic regions, which regulate intake of food (and hence energy) and energy expenditure; regulation of diurnal rhythms of feeding and metabolism. By switching off biosynthetic pathways required for cell growth, AMPK activation exerts a cytostatic effect, helping to explain why its upstream activator, LKB1, is a tumor suppressor. Commensurate with its role in preserving cellular energy homeostasis, AMPK also downregulates ATP-requiring processes outside metabolism, including progress through the cell cycle (another potential tumor suppressor effect) and firing of action potentials in neurons [32]. These exciting data might lead to a new pathogenic explanation of some diseases, such as periodontitis as inflammatory disease, and to elaborate new treatment approaching with new drugs.

16.3.4 Aggression Recognition: Inflammasome

As we have seen cell to survive needs to be well isolated through a membrane, and produce energy. But also the cell needs to defense against biological aggression, other organisms try to destroy it and use all the cellular components as nutrients. We have shown that inflammation is the body reaction to this aggression, but to start this process it is essential for a multicellular organism to recognize the harmful microbes. This is mediated through the pattern recognition receptors (PRRs). They are proteins expressed by hematopoietic and nonhematopoietic cells such as macrophages, dendritic cells, and epithelial cells. These may either be on the membrane surface, e.g., Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) or inside the cytoplasm, e.g., Nod-like receptors (NLRs) and RIG-I-like receptors (RLRs). PRRs enable innate immune cells to instantly detect and respond to the presence of danger- and pathogen-associated molecular patterns (DAMPs and

PAMPs, respectively) [33]. PAMPs are conserved microbial molecules that are not produced by mammalian host cells, such as nucleic acid structures that are unique to microorganisms, bacterial secretion systems and their effector proteins, and microbial cell wall components such as lipoproteins and lipopolysaccharides (LPSs). In contrast, DAMPs are a set of host-derived molecules that signal cellular stress, damage, or nonphysiological cell death. Orchestration of an appropriate immune response against these microbial threats is accomplished in part through the production of potent inflammatory cytokines. In particular, the related cytokines interleukin-1β (IL-1β) and IL-18 were recognized early on for their ability to cause a wide variety of biological effects associated with infection, inflammation, and autoimmune processes. In addition to belonging to the same cytokine family, IL-1β and IL-18 have in common a unique maturation and secretory mechanism. Although most cytokines traffic through the Golgi complex prior to exocytosis, biologically active IL-1β and IL-18 are liberated from their cytosolic precursors by the cysteine protease caspase-1 and -11 complexes termed inflammasomes. Caspase-1 is the founding member of a family of conserved metazoan aspartate-specific cysteine proteases, with 11 human (caspases 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 14) representatives [34]. The term "inflammasome" describes a large (700 kDa), multiprotein complex considered as a key regulator of innative, adaptative, and host responses that survey the cytosol and other intracellular compartments for the presence of PAMPs and DAMPs. These multiprotein complexes have been characterized in a variety of cells, although the focus has been mainly on epithelial cells in tissues with mucosal surfaces and immune cells of the myeloid lineage. Genetic studies using mice deficient for different nucleotide-binding and NLRs suggest that at least four inflammasomes of distinct composition are formed in vivo in a stimulus-dependent manner. They are the NLR proteins Nlrp1b, Nlrp3, Nlrc4, and Nlrp6 as well as the HIN200 protein AIM2 that assemble inflammasomes in a stimulus-specific manner [35]. Once secreted, IL-1β and IL-18 mediate a variety of local and systemic responses to infection. IL-1β induces fever; promotes T cell survival, B cell proliferation, and antibody production; contributes to polarization of T helper 1 (TH1), TH2, and TH17 responses; and mediates transmigration of leukocytes. A second inflammasome effector mechanism that may contribute to host response is pyroptosis, a proinflammatory cell death. It is a critical mechanism by which inflammasome contributes to host responses against Gram-negative bacterial pathogens in vivo. This genetically programmed cell death mode differs morphologically from apoptosis in that it features cytoplasmic swelling and early plasma membrane rupture. The consequent release of the cytoplasmic content into the extracellular space is thought to render pyroptosis proinflammatory, whereas apoptosis is generally considered an immunologically silent cell death mechanism. It prevents intracellular replication of infectious agents, therefore is an intriguing inflammasome-mediated host defense mechanism against intracellular pathogens. A third emerging mechanism by which inflammasomes may contribute to immune signaling is the secretion of leaderless cytokines and growth factors. Recent studies have extended the list of unconventionally secreted cytokines and growth factors to more than 20 proteins. The biochemical mechanisms by which leaderless proteins are secreted into the

extracellular space largely remains to be characterized, but inflammasomes might play a central role in this process. Apart from these effector mechanisms, inflammasomes have been implicated in inactivation of glycolysis enzymes, activation of sterol-regulatory element binding protein-1 and -2. These mechanisms illustrate that inflammasomes can contribute to a diverse set of responses that collectively may help the host to effectively fight microbial pathogens and other threats [36].

Inflammasome activation contributes significantly to host and inflammatory responses, but the association of gain-of-function mutations in NLRP3, NLRP1, and other inflammasome components with autoimmune and autoinflammatory disorders illustrates that excessive inflammasome activity can be harmful. Vitiligo, Addison's disease, Crohn's disease, and multiple sclerosis have been related to altered inflammasome signaling. Also the inflammasome mechanism illustrates the importance of preventing unwarranted and disproportional activation of inflammasome effector pathways. It is thus not surprising that pathogens evolved different virulence mechanisms to modulate inflammasome activation to their benefit. Certain viruses and bacterial pathogens express proteins that inhibit inflammasome assembly and activity (e.g., *Rabbitpox virus*, *Myxoma virus*, *Mycobacterium tuberculosis*, *Pseudomona aeruginosa*). The metabolic inflammasome may act as a link between ER stress and more global stress responses, including inflammation and metabolic dysfunction (as observed in insulin resistance and obesity).

Inflammation is one of the key events that underlies the development of obesity-induced insulin resistance. Although different roads may lead to its activation, the contribution of IL-1β to the development of insulin resistance at the level of the β cell, as well as peripherally, in obese individuals is now well established. Active IL-1β is produced by cleavage of pro-IL-1β by caspase-1, which is part of the inflammasome protein complex. Although most studies performed to date provide indirect and associative evidence, growing evidence indicates that the inflammasome can be activated by fatty acids, high glucose levels, uric acid, and IAPP, linking metabolic danger signals to activation of IL-1β synthesis. The described correlations and associations, however, do not necessarily prove causative relationships [37]. Although most components of the metabolic inflammasome promote autophagy, the induction of autophagy by this signaling complex would be expected to serve as a negative-feedback mechanism that limits ER stress and disease progression. Consistent with this postulated protective effect of autophagy, hepatic suppression of the autophagy gene *Atg7* in mice results in increased ER stress and insulin resistance, and mice deficient in the autophagy adaptor protein p62 develop mature-onset obesity and insulin resistance [38].

16.3.5 Debris Elimination: Apoptosis/Autophagy/Hormesis

Nature is frugal; therefore, every form of life should spend the lowest possible energy and eliminate every structure, cell, or intracellular organelle not needed. Every organism should be adapted for physiological development, new environment

conditions, and aggression to survive. One way it is done is with apoptosis and autophagy mechanisms.

Apoptosis is defined as the process of programmed cell death (PCD) that may occur in multicellular organisms. In contrast to necrosis, which is a form of traumatic cell death that results from acute cellular injury, apoptosis, in general, confers advantages during an organism's life cycle. For example, the differentiation of fingers and toes in a developing human embryo occurs because cells between the fingers apoptosis; the result is that the digits are separate. Between 50 and 70 billion cells die each day due to apoptosis in the average human adult. Failure of apoptosis is one of the main contributions to tumor development and autoimmune diseases; this, coupled with the unwanted apoptosis that occurs with ischemia or Alzheimer's disease, has stimulated interest in caspases as potential therapeutic targets since they were discovered in the mid-1990s. The process of apoptosis is controlled by a diverse range of cell signals, which may originate either extracellularly (extrinsic inducers) or intracellularly (intrinsic inducers). Extracellular signals may include toxins, hormones, growth factors, nitric oxide, or cytokines that must either cross the plasma membrane or transduce to induce a response. These signals may positively (i.e., trigger) or negatively (i.e., repress, inhibit, or dampen) affect apoptosis. A cell initiates intracellular apoptotic signaling in response to a stress, which may bring about cell suicide. The release of intracellular apoptotic signals by a damaged cell can be started by: the binding of nuclear receptors by glucocorticoids, heat, radiation, nutrient deprivation, viral infection, hypoxia, and increased intracellular calcium concentration. Intrinsic apoptosis, which requires the permeabilization of the mitochondrial membrane, is closely regulated by members of the Bcl-2 protein family. The pro-apoptotic Bcl-2 family members Bax and Bak serve to permeabilize the mitochondrial membrane, leading to the release of cytochrome *c*, caspase activation, and cell death. This pro-death function is opposed by the anti-apoptotic Bcl-2 family members Bcl-2, Bcl-x, and Mcl-1. A third group of Bcl-2 proteins, referred to as BH3-only proteins, serves a pro-apoptotic function. Extrinsic apoptosis is initiated by the binding of a death receptor, such as Fas, TNFR1, or TRAIL, to its ligand, which results in the formation of the death-inducing signaling complex (DISC). Adapter proteins (FADD and/or TRADD) bind to the cytoplasmic tail of the death receptor through DD-DD interactions and subsequently recruit pro-caspase 8 to the DISC through DED–DED interactions. Recruitment of pro-caspase 8 to the DISC allows for its homodimerization and cleavage, resulting in the release of active caspase 8, which cleaves downstream substrates, including effector caspases, leading to DNA fragmentation and cell death [39]. After a cell receives stimulus, it undergoes organized degradation of cellular organelles by activated proteolytic caspases. Caspases are a family of enzymes that degrade polypeptides called cysteine proteases that act in concert in a cascade. Members of the caspase family of cysteine proteases coordinate the morphological and biochemical events that typify apoptosis. However, neutralization of caspase activity in mammals fails to block death in response to most proapoptotic stimuli. This is because many cell death triggers provoke mitochondrial dysfunction upstream of caspase activation as a consequence of BAX/BAK channel opening. Although genetic or pharmacological

inactivation of caspases fails to block cell death in most instances, it does convert the phenotype from apoptosis to necrosis. This has important implications for how the immune system responds to such cells, as necrotic cells provoke inflammation, whereas apoptotic cells typically do not. It has been proposed an alternative perspective on apoptosis-associated caspase function by suggesting that these proteases are activated, not to kill, but to extinguish the proinflammatory properties of dying cells. This perspective unifies the mammalian caspase family as either positive or negative regulators of inflammation [40].

Altered molecular mechanisms involved in apoptotic signaling have been related to human diseases. Indeed both reduced and increased apoptosis can result in pathology. Cancer, neurological disorders (Alzheimer's disease, Parkinson's disease, stroke), heart diseases (ischemia reperfusion, chronic heart failure), and autoimmune diseases (rheumatoid arthritis, thyroiditis) are the main diseases with altered apoptosis. Also more recently these findings have led to the development of therapeutic approaches based on the regulation of apoptosis, some of which are in clinical trials and need to solve many problems [41].

Autophagy is an essential, homeostatic process by which cells break down their own components. The autophagy machinery is thought to have evolved as a stress response that allows unicellular eukaryotic organisms to survive during harsh conditions, probably by regulating energy homeostasis and/or by protein and organelle quality control. The same machinery might therefore be expected to diversify functionally in complex metazoan organisms, so as to regulate new layers of defenses used by multicellular organisms to confront different forms of stress. Autophagy is a lysosomal degradation pathway in which portions of the cytoplasm (organelles or cytosol) are enwrapped in double-membraned vesicles (called autophagosomes) that fuse with lysosomes and get degraded by lysosomal hydrolases. Basal levels of autophagy contribute to the maintenance of intracellular homoeostasis by ensuring the turnover of supernumerary, aged, and/or damaged components. Under conditions of starvation, the autophagic pathway operates to supply cells with metabolic substrates, and hence represents an important pro-survival mechanism. Autophagy has never been shown to be the cause of death in mammalian cells under physiologically relevant conditions, accumulating evidence points to have a pro-survival function [39]. The pro-survival function of autophagy has been demonstrated at the cellular and organismal level in different contexts, including during nutrient and growth factor deprivation, endoplasmic reticulum stress, development, microbial infection, and diseases characterized by the accumulation of protein aggregates. This pro-survival function is generally believed to be adaptive, but, in the context of cancer, is potentially maladaptive. An apparent conundrum is that autophagy acts both in cytoprotection and in cell death. In response to most forms of cellular stress, autophagy plays a cytoprotective role, because ATG gene knockdown/knockout accelerates rather than delays cell death. However, in certain settings where there is uncontrolled upregulation of autophagy (e.g., overexpression of the autophagy protein Beclin 1 in mammalian cells14, and Atg1 overexpression in Drosophila), autophagy can lead to cell death, possibly through activating apoptosis or possibly as a result of the inability of cells to survive the nonspecific degradation of large

amounts of cytoplasmic contents. Notably, many examples of ATG-gene-dependent cell death occur in cells deficient in apoptosis, suggesting that autophagy, as a route to cell death, maybe a choice of last resort [42]. Moreover, autophagy is required for normal development and for the protective response to intracellular pathogens. Perhaps the most primordial function of this lysosomal degradation pathway is adaptation to nutrient deprivation. However, in complex multicellular organisms, the core molecular machinery of autophagy—the "autophagy proteins"—orchestrates diverse aspects of cellular and organismal responses to other dangerous stimuli such as infection. Recent developments reveal a crucial role for the autophagy pathway and proteins in immunity and inflammation. They balance the beneficial and detrimental effects of immunity and inflammation, and thereby may protect against infectious, autoimmune, and inflammatory diseases. Autophagy was originally considered to be a nonselective bulk degradation process, but it is now clear that autophagosomes can degrade substrates in a selective manner. In addition to endogenous substrates, autophagy degrades intracellular pathogens in a selective form of autophagy, termed xenophagy. Perturbations in autophagy-protein-dependent functions in immunity may contribute not only to increased susceptibility to infection but also to chronic inflammatory diseases and autoimmune diseases. Defects in autophagy may contribute to inflammation-associated metabolic diseases such as diabetes and obesity, which are both linked to insulin resistance. Obesity is associated with the accumulation and activation of macrophages and subsets of T cells in adipose tissue and the production of cytokines such as TNF-α and IL-6. Thus, the failure of autophagy-dependent control of ER stress, immune cell homeostasis, immune cell activation, and/or proinflammatory cytokine secretion may contribute to inflammation-associated responses that underlie the pathogenesis of metabolic diseases [43]. Autophagy can lead to the removal of damaged, potentially dangerous mitochondria and is called mitophagy. Both mitochondrion-specific autophagy (mitophagy) and general autophagy can reduce the propensity of cells to undergo apoptosis. Importantly, autophagy and apoptosis exhibit a consistent degree of crosstalk, at multiple levels. Some molecular mechanisms that sense cellular stress can induce both autophagy and apoptosis. Caspase-dependent apoptosis is associated with the degradation of Beclin 1 by caspases. As Beclin 1 is essential for the initial steps of autophagy, caspase activation most often results into the inhibition of the autophagic pathway. This reflects a general pattern according to which pro-apoptotic signals result in the inhibition of pro-survival systems.

Recent data have been demonstrated that autophagy is involved in periodontitis [44]. PBMCs from periodontitis patients show an increased level of autophagy gene expression and high levels of mitochondrial ROS production, positively correlated. In human gingival fibroblasts treated with lipopolysaccharide from *P. gingivalis* there was an increase of protein and transcript of autophagy-related protein 12 (ATG12) and microtubule-associated protein 1 light chain 3 alpha LC3. A reduction of mitochondrial ROS induced a decrease in autophagy, whereas inhibition of autophagy in infected cells increased apoptosis, showing the protective role of autophagy.

Finally a new concept has been stated in this issue, "hormesis." Frequently, low doses of toxins and other stressors not only are harmless but also activate an

adaptive stress response that raises the resistance of the organism against high doses of the same agent. This phenomenon is known as “hormesis” and describes a favorable biological response to harmless doses of toxins and other stressors. Hormesis-stimulating compounds initiate an adaptive stress response that renders cells/organisms resistant against high (and normally harmful) doses of the same agent. On the theoretical level, hormesis may constitute one of the mechanisms that allows stressed cells to avoid senescence and death, and hence might have some impact on the physiology of aging. Thus, measures that reportedly prolong the healthy life-span of multiple species, such as caloric restriction and the administration of resveratrol, may do so by inducing a hormetic response [45]. As we have exposed nowadays one important issue in the host response in periodontitis is the altered inflammation mechanism. It is therefore essential to determine the factors, genetic and environmental, that induce a chronic inflammation and the way we can improve the inflammatory response. The decline in hormetic stimuli in our daily life may be leading to increased systemic subclinical inflammatory tone, decreased metabolic flexibility, and suppression of exercise salience. All of which translate into a significant increase in chronic diseases and maybe in periodontitis. Whether we like it or not, a long and healthy life needs to include regular exposure to occasional doses of environmental stressors, including fasting, natural temperature changes, polyphenols, and exercise. Although human intelligence has enabled us to remove most stressors from the environment, common sense may be required to reintroduce some of them.

16.4 Clinical Relevance

Now our diagnostic and treatment approach to periodontal disease is based on the concept that it is an infectious disease. But the statement of susceptibility (biofilm can produce different presentation of the disease) and the concept of progression (burst hypothesis) lead to an increasing importance of the host response. Inflammation is the systemic defense mechanism triggered by a biological, chemical, or physical aggression. It has been known that the tissue involvement is mainly done with a vascular response. But inflammation implicates different cellular events. New technical tools implemented in the laboratory allow us to study different biochemical intracellular processes. It is necessary to understand all this process to improve our knowledge regarding the pathological inflammation.

This one has been related with some diseases, not only autoimmune disease but also in diabetes, atherosclerosis, obesity, metabolic syndrome, some neurological disorder and cancer has been described some new pathogenic data. Every tissues or organs can response to an aggression in a similar way, according to the physiological conditions. Therefore pathological inflammation could be the meeting point for all those diseases. Periodontal disease as an inflammatory disease could highlight some of these pathological inflammatory conditions.

In the future we probably should change our way of diagnosing and treating our periodontal patients. In diagnostic we might introduce laboratory techniques to analyze intracellular statement and analyze lifestyle, nutrition, and other concomitant diseases with more details. Maybe we should treat our patients, analyzing systemic diseases, especially chronic inflammatory, change lifestyle and diet and improve metabolic conditions and the response to aggression.

Also due to the fact that periodontal disease is the most frequent chronic infection in humans it could be a good way to check the statement of the inflammatory response in our patients and a potential target for prevention strategies for systemic diseases with important clinical systemic implications.

In this chapter we try to highlight the new theories and their clinical relevance to the onset and development of periodontal diseases that will change our way of managing our periodontal patients and should change the periodontal teaching in the future.

References

1. Albandar JM, Rams TE (2002) Global epidemiology of periodontal diseases. Periodontol 2000 29:7–10
2. König J, Holtfreter B, Kocher T (2010) Periodontal health in Europe: future trends based on treatment needs and the provision of periodontal services—position paper 1. Eur J Dent Educ 14(Suppl 1):4–24
3. Moore WE, Moore LV (1994) The bacteria of periodontal diseases. Periodontol 2000 5:66–77
4. Lepp PW, Brinig MM, Ouverney CC et al (2004) Methanogenic Archaea and human periodontal disease. Proc Natl Acad Sci U S A 101:6176–6181
5. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr (1998) Microbial complexes in subgingival plaque. J Clin Periodontol 25:134–144
6. Thomson WM, Sheiham A, Spencer AJ (2012) Sociobehavioral aspects of periodontal disease. Periodontol 2000 60:54–63
7. Sertznig P, Seifert M, Tilgen W, Reichrath J (2008) Peroxisome proliferator-activated receptors (PPARs) and the human skin: importance of PPARs in skin physiology and dermatologic diseases. Am J Clin Dermatol 9:15–31
8. Hadler-Olsen E, Fadnes B, Sylte I, Uhlin-Hansen L, Winberg JO (2011) Regulation of matrix metalloproteinase activity in health and disease. FEBS J 278:28–45
9. Stuart LM, Ezekowitz RA (2005) Phagocytosis: elegant complexity. Immunity 22:539–550
10. Eming SA, Krieg T, Davidson JM (2007) Inflammation in wound repair: molecular and cellular mechanisms. J Invest Dermatol 127:514–525
11. Liu YC, Lerner UH, Teng YT (2010) Cytokine responses against periodontal infection: protective and destructive roles. Periodontol 2000 52:163–206
12. Singer SJ, Nicolson GL (1972) The fluid mosaic model of the structure of cell membranes. Science 175:720–731
13. Pike LJ (2008) The challenge of lipid rafts. J Lipid Res 50:S323–S328
14. German JB (2011) Dietary lipids from an evolutionary perspective: sources, structures and functions. Matern Child Nutr 7(2):2–16
15. Kien CL (2009) Dietary interventions for metabolic syndrome: role of modifying dietary fats. Curr Diab Rep 9:43–50

16. Cascio G, Schiera G, Di Liegro I (2012) Dietary fatty acids in metabolic syndrome, diabetes and cardiovascular diseases. Curr Diabetes Rev 8:2–17
17. Kodama S, Saito K, Tanaka S, Maki M, Yachi Y, Sato M, Sugawara A, Totsuka K, Shimano H, Ohashi Y, Yamada N, Sone H (2009) Influence of fat and carbohydrate proportions on the metabolic profile in patients with type 2 diabetes: a meta-analysis. Diabetes Care 32:959–965
18. Riediger ND, Othman RA, Suh M, Moghadasian MH (2009) A systemic review of the roles of n-3 fatty acids in health and disease. J Am Diet Assoc 109:668–679
19. Micha R, Mozaffarian D (2008) Trans fatty acids: effects on cardiometabolic health and implications for policy. Prostaglandins Leukot Essent Fatty Acids 79:147–152
20. Calder PC (2010) Omega-3 fatty acids and inflammatory processes. Nutrients 2:355–374
21. Yaqoob P, Shaikh SR (2010) The nutritional and clinical significance of lipid rafts. Curr Opin Clin Nutr Metab Care 13:156–166
22. Yaqoob P (2009) The nutritional significance of lipid rafts. Annu Rev Nutr 29:257–282
23. Ramirez-Tortosa MC, Quiles JL, Battino M, Granados S, Morillo JM, Bompadre S, Newman HN, Bullon P (2010) Periodontitis is associated with altered plasma fatty acids and cardiovascular risk markers. Nutr Metab Cardiovasc Dis 20:133–139
24. Zimmer C (2009) On the origin of eukaryotes. Science 325:666–668
25. Wallace DC, Fan W, Procaccio V (2010) Mitochondrial energetics and therapeutics. Annu Rev Pathol 5:297–348
26. Battino M, Bullon P, Wilson M, Newman H (1999) Oxidative injury and inflammatory periodontal diseases: the challenge of anti-oxidants to free radicals and reactive oxygen species. Crit Rev Oral Biol Med 10:458–476
27. World Health Organization (2005) The World Health Organization warns of the rising threat of heart disease and stroke as overweight and obesity rapidly increase. http://www.who.int/mediacentre/news/releases/2005/pr44/en/index.html
28. Dirkx E, Schwenk RW, Glatz JF, Luiken JJ, van Eys GJ (2011) High fat diet induced diabetic cardiomyopathy. Prostaglandins Leukot Essent Fatty Acids 85:219–225
29. Bullon P, Morillo JM, Ramirez-Tortosa MC, Quiles JL, Newman HN, Battino M (2009) Metabolic syndrome and periodontitis: is oxidative stress a common link? J Dent Res 88:503–518
30. Bullon P, Cordero MD, Quiles JL, Morillo JM, del Carmen Ramirez-Tortosa M, Battino M (2011) Mitochondrial dysfunction promoted by Porphyromonas gingivalis lipopolysaccharide as a possible link between cardiovascular disease and periodontitis. Free Radic Biol Med 50:1336–1343
31. Hardie DG, Ross FA, Hawley SA (2012) AMP-activated protein kinase: a target for drugs both ancient and modern. Chem Biol 19:1222–1236
32. Hardie DG, Ross FA, Hawley SA (2012) AMPK: a nutrient and energy sensor that maintains energy homeostasis. Nat Rev Mol Cell Biol 13:251–262
33. Kanneganti TD, Lamkanfi M, Nunez G (2007) Intracellular NOD-like receptors in host defense and disease. Immunity 27:549–559
34. Fischer U, Janicke RU, Schulze-Osthoff K (2003) Many cuts to ruin: a comprehensive update of caspase substrates. Cell Death Differ 10:76–100
35. Lamkanfi M, Dixit VM (2009) Inflammasomes: guardians of cytosolic sanctity. Immunol Rev 227:95–105
36. Lamkanfi M, Dixit VM (2012) Inflammasomes and their roles in health and disease. Annu Rev Cell Dev Biol 28:137–161
37. Stienstra R, Tack CJ, Kanneganti TD, Joosten LA, Netea MG (2012) The inflammasome puts obesity in the danger zone. Cell Metab 15:10–18
38. Nakamura T, Furuhashi M, Li P, Cao H, Tuncman G, Sonenberg N, Gorgun CZ, Hotamisligil GS (2010) Double-stranded RNA-dependent protein kinase links pathogen sensing with stress and metabolic homeostasis. Cell 140:338–348
39. Gordy C, He YW (2012) The crosstalk between autophagy and apoptosis: where does this lead? Protein Cell 3:17–27

40. Martin SJ, Henry CM, Cullen SP (2012) A perspective on mammalian caspases as positive and negative regulators of inflammation. Mol Cell 46:387–397
41. Favaloro B, Allocati N, Graziano V, Di Ilio C, De Laurenzi V (2012) Role of apoptosis in disease. Aging (Albany, NY) 4:330–349
42. Mizushima N, Levine B, Cuervo AM, Klionsky DJ (2008) Autophagy fights disease through cellular self-digestion. Nature 451:1069–1075
43. Levine B, Mizushima N, Virgin HW (2011) Autophagy in immunity and inflammation. Nature 469:323–335
44. Bullon P, Cordero MD, Quiles JL, Ramirez-Tortosa MC, Gonzalez-Alonso A, Alfonsi S, Garcia-Marin R, de Miguel M, Battino M (2012) Autophagy in periodontitis patients and gingival fibroblasts: unraveling the link between chronic diseases and inflammation. BMC Med 10:122
45. Martins I, Galluzzi L, Kroemer G (2011) Hormesis, cell death and aging. Aging (Albany, NY) 3:821–828

Chapter 17
The Role of Nutrition in Periodontal Diseases

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Abbreviations

$1,25(OH)_2D$	1,25-Dihydroxyvitamin D
25(OH)D	25-Hydroxyvitamin D concentrations
8OHdG	8-Hydroxy-deoxyguanosine
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
GLA	γ-Linolenic acid
GSH/GSSG	Reduced glutathione reduced/oxidized ratio
IgG	Immunoglobulin G
IL-1β	Interleukin-1β
iNOS	Nitric oxide synthase inducible
LNA	Linolenic acid
MUFA	Monounsaturated fatty acids
NHANES	National health and nutrition examination survey
P. gingivalis	*Porphyromonas gingivalis*
PGE2	Prostaglandin E2
PLS	Papillon–Lefèvre syndrome
PUFA	Polyunsaturated fatty acids
RANKL	Receptor activator of nuclear factor kappa-B ligand
ROS	Reactive oxygen species
Th	T-helper

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TNF	Tumor necrosis factor-α
VD	Vitamin D
VDR	Vitamin D receptor

17.1 Introduction

The impact of nutrition on oral disease has traditionally focused on the local effects of the diet on caries risk [1]. However, the role of diet in the development and progression of periodontal diseases is less well understood. Periodontium consists of hard and soft tissues that surround the dentition and includes the gingiva, periodontal ligament, connective tissue, alveolar bone, and cementum [2]. Periodontal disease can lead to increased risk of other oral problems (root caries, tooth mobility, and tooth loss) and has been associated with an increasing list of chronic systemic diseases, including cardiovascular diseases, diabetes, obesity, metabolic syndrome, and impaired cognition [3–16]. There is agreement about that nutritional status may be a modifying factor in the progression and healing of the periodontal tissues. In fact, despite the primary etiology of periodontal disease is bacterial, a susceptible host is also necessary for disease initiation. Nutrition is one of the modifiable factors that impact the host's immune response and the integrity of the hard and soft tissues of the oral cavity [2].

Diet can exert a topical or a systemic effect on the body and its tissues. Before tooth eruption, foods provide a nutritional or systemic effect during tooth development and in the maturation of dentine and enamel. After the tooth erupts, foods play a topical or dietary role in the maintenance of tooth structure. As an example, during growth and development, nutritional fluoride provides a systemic effect, making the tooth more resistant to decalcification by incorporation into the structure of the tooth. After the tooth has formed and erupted into the oral cavity, dietary fluoride provides a topical effect by modification of the surface layer of exposed enamel, cementum, and dentin [17].

Nutrients can be considered major or minor as determined by the amounts consumed in our diets. Major nutrients are consumed in gram quantities. Minor nutrients are required in milligram to microgram quantities and include vitamins and minerals. Apart from nutrients, many other molecules provided by diet might be important from the point of view of periodontium health, among these, antioxidants, prebiotics, probiotics, herbal extracts, and some other may be included. In the present review, we will discuss on major and minor nutrients, since antioxidants are treated into another chapter of this book and because the study of other molecules might avoid a proper focus of the matter. For each nutrient, a brief introduction considering main functions of this nutrient in health will be exposed. Then, main studies performed on each nutrient in relation to periodontitis will be analyzed, distinguishing human (cross-sectional, follow-up, case–control, intervention studies) from experimental (animals and cell cultures, when available) studies.

17.2 Macronutrients

17.2.1 Proteins

Protein is the most common substance in the body after water, making up about 50 % of the body's dry weight. They can provide structure in the body, as occur with collagen, or support different bodily functions, like in the enzymes case. Amino acids from protein in the diet can be utilized for protein synthesis and repair. Twenty-two amino acids are needed for protein synthesis, nine of which are considered to be essential amino acids: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Excess amino acids are not used for protein synthesis or repair; instead they are utilized for energy [18].

Proteins are components of defensive molecules and barriers that help to control the disease process. The periodontal defenses include cell-mediated immunity, antibody or humoral immunity, the complement system and innate immunity. Also, the crevicular and junctional epithelia provide an epithelial barrier function that provides a major defensive barrier to invasion by antigens, noxious products and bacteria, and undergoes a rapid turnover [18].

Protein energy malnutrition appears related to periodontal diseases susceptibility. It has been found that periodontal disease progresses more rapidly in undernourished populations [19], presumably due to overgrowth of periodontopathic organisms [20, 21]. In African populations, it has been commonly found acute necrotizing gingivitis in children, and periodontal pockets in adolescent and adults, although tooth loss seems infrequent [22]. The severity of protein energy malnutrition has shown important effects on saliva that has a major role in the maintenance of oral health [23]. Children's saliva presented decreases of volume and secretion rate, protein content [24, 25], and a reduction in immunologic and agglutinating defense factors [26, 27]. It seems more important when individuals are young if these results are compared with studies in adults [28] accompanied by changes in salivary gland function and structure if observations in rats are considered [29–31]. However, most of the epidemiological studies on protein deficiencies have added complications. Protein deficiencies have usually observed on malnourished populations, so it is not strange that they are accompanied by deficiencies in other nutrients. Furthermore it used to be accompanied by many risk factors like poor oral hygiene habits [32]. Altogether makes difficult to consider malnutrition and protein energy malnutrition as the main cause of the observations from epidemiological studies.

17.2.2 Glucids

The main role of glucids or carbohydrates is to provide the body with energy. Carbohydrates are primarily used as a source of energy but they also aid in fat metabolism. Carbohydrates are found within the body as glycoprotein and

glycosaminoglycans. They are essential for synthesis of the ground substance of the connective tissues, such as chondroitin, keratin, and dermatan sulfates. Glucose is also essential for erythrocyte and brain function. The body stores carbohydrates as glycogen (polysaccharides composed of α-linked glucose molecules). Carbohydrates are protein sparing, in that when inadequate amounts of dietary carbohydrates are ingested, the body breaks down protein to provide glucose. Major sources of carbohydrates are sugars and starches [17]. The groups of glucids that have shown relevant roles for development and progression of periodontitis or periodontal diseases are described below.

17.2.2.1 Simple Sugars: Monosaccharides and Dissacharides

Monosaccharides and disaccharides, also known as simple sugars or sugars, provide substrate for oral bacteria to synthesize extracellular polysaccharides that enhance plaque mass [33], which may contain cariogenic and periodontal pathogens, and these could affect the periodontal tissue negatively [34].

Studies in humans that have examined the effect of sugars intake on periodontitis or gingivitis, are few [35–37] and no clear trend has emerged [38]. The most severe effect was noted in a follow-up study in older subjects. In this research, sugar consumption at baseline was positively associated with number of periodontal disease events, determined by the attachment level changes, during 6 years [39].

The effect of sugar-rich diets on periodontal disease has been studied experimentally in humans too. Most of them have shown higher plaque volumes in subjects taking high sugar diets compared with those taking low sugar diets [38, 40–42]. According to periodontal diseases, it has been observed that these diets combined with oral hygiene absence lead to gingival inflammation [38, 42], but not to other severe form of periodontal disease which could be due to experimental period too short. At least in one case there were no differences on plaque amount between dietary groups, both without oral hygiene [38]. These results would suggest that oral hygiene is a major factor for plaque development, but the organisms present in it, when subject with high intake of sugars, seems to have more irritant effects on gum.

Experiments in animals have provided diverse results that could help to understand this association. Many studies have shown that dietary sugar has possible role in progressive periodontal disease in animal models, including rice rats (Oryzomyspalustris) [43, 44], hamsters [45, 46], and rats [47]. In rice rats a high sucrose diet, maintained for 12 and 18 weeks, increased alveolar bone loss in mandibles and maxillae [44]. However sucrose intake might be irrelevant when other factors are present as occur in certain experimental models. In rats receiving ligatures to induce periodontitis, sucrose-rich diets had no effect over periodontal destruction, although in this case, they were fed diets for 30 days only [48].

17.2.2.2 Dietary Fiber

Carbohydrates non-digestible by humans are considered dietary fiber. They are present in soluble and insoluble forms in nature, which manifest different properties.

Insoluble fiber can retain fluid and provides bulk in the gastrointestinal system. Soluble fiber helps bind cholesterol molecules and might decrease cholesterol from the diet [49]. Diets high in fiber have been shown beneficial effects on diabetes mellitus, cardiovascular disease, obesity, intestinal disorders, and several forms of colon cancer [50–58].

A diet rich in fiber could protect against periodontal disease through several mechanical actions, including cleansing the tooth surfaces of plaque [59] and forcing more chewing, which stimulates the parotid salivary gland to increase salivary flow [60] and remove potentially harmful bacteria [61]. Additional interest in dietary fiber effect on periodontal health stems from fiber's effect on systemic condition and body's weight. Briefly, fiber controls serum glucose levels and lowers lipids, blood pressure, body mass index and even inflammatory agents excreted by adipose tissue, which have been associated with periodontal disease [50, 51, 53, 57, 58].

There are several evidences that support the beneficial role of dietary fiber, although the reason behind this is not entirely clear. In cross-sectional studies, a low dietary fiber intake increases periodontal inflammation in middle-age women [62], and gingivitis risk in female adolescent [63]. Dietary habits related to cardiovascular diseases risk including low intakes of fiber had been noted higher in edentulous women compared with women with 25–32 teeth [64].

If it is taken into account the intake of dietary fiber-rich foods, there are follow-up studies whose results suggest the same effect. It was found that high whole-grain cereal intake had lower risk to get periodontitis, but it was not associated with refined-grain intake. In addition, cereal fiber was inversely related to periodontitis risk, but the association was not significant after adjustment for whole-grain intake [65]. In a survey in dwelling men, foods that provided 2.5 g or more of fiber per serving, were considered good and excellent fiber sources. It showed that each serving of good to excellent sources of total fiber was associated with lower risk of alveolar bone loss progression and tooth loss, but only when they are older than 65 years. Also, intake of fruits which were good to excellent sources of fiber was associated with lower risk of progression of alveolar bone loss, probing pocket depth and tooth loss [61]. In other study in women, severity of periodontitis was inversely correlated to frequency of intakes of green vegetables and fruits [62].

17.2.2.3 Ethanol and Alcoholic Beverages

Alcoholism or alcohol abuse can influence host defenses causing toxic damage as fatty liver, cirrhosis, cerebral atrophy, cardiomyopathy, gastrointestinal bleeding, and pancreatitis [66]. Alcoholism is a chronic illness with a slow evolution, taking on average 15–20 years to present clinical evidence [67]. Apart from the reliable evidence that alcohol intake cause serious damage to general health, studies have suggested that alcohol consumption and alcohol dependence may cause harmful effects in the oral cavity such as caries, loss of teeth, oropharyngeal cancers, and periodontal disease [68–70].

According to periodontal disease, there are many epidemiological studies that have taken into account the alcohol consumption role. Most of the cross-sectional

and follow-up studies in adults have shown that high intake of ethanol was associated with different parameters or indices using to assess periodontal disease severity or progression. They included community periodontal index [71], gingival bleeding [72], pocket depth [73, 74], clinical attachment level [72, 75], and alveolar bone loss [76], although the last two did not show any relationship with ethanol intake in some studies [72, 73]. Likewise, periodontitis prevalence was higher in those groups that consumed more alcohol, when periodontitis presence was determined through pocket depth and clinical attachment level [76], or by means of alveolar bone loss [77].

The influence of ethanol consumption seems to be reduced when certain risk factors, as tobacco use, are present. That fact could explain the reason because it has been found no relationships in a few researches [78, 79]. However in these cases, periodontal condition was assessed by only one parameter which may not be sufficient if the disease severity was low. Results observed in other could support this idea. In univariate models, it has observed more moderate and severe periodontitis cases in alcohol drinkers. Meanwhile in multivariate models ethanol consumption had no effect [80]. In a case–control survey, it was noted more horizontal bone loss and periodontal destruction in alcoholic compared with non-alcoholic but only when they were non-smokers [70]. Additionally, in certain occasions the associations only have been present in men if gender was considered [81].

Research in rats has confirmed the harmful role of alcohol but only under certain conditions. High alcohol consumption generally exacerbated alveolar bone loss in rats triggered by ligatures placement, in experimentally induced periodontitis models [82, 83]. Nevertheless, it had no effect when ligatures were not present [82, 83]. The effect of ethanol on periodontitis could be due at least in part to their energetic role. It has been found no difference between alcohol containing-diet and sucrose-rich diet with the same calories [82]. However a high sucrose diet has shown increased alveolar bone loss in mandibles in rice rats [44], so its use as a control diets can be discussed. These observations could be due to changes in oxidative stress and inflammatory state. Rats fed diet containing ethanol presented elevated markers [reduced glutathione reduced/oxidized (GSH/GSSG) ratio and 8-hydroxy-deoxyguanosine (8OHdG) and tumor necrosis factor (TNF)-α levels increased] related to inflammation and oxidative stress in gingival tissues. Ethanol administration enhanced oxidative stress and inflammatory marker levels associated to periodontitis induced by ligatures, on periodontal tissues. They included nitric oxide synthase inducible (iNOS) mRNA expression and activity, interleukin (IL)-1β mRNA expression [84] reduced GSH/GSSG ratio and TNF-α levels [85]. At histological level, ethanol consumption has also shown an additive effect on polymorphonuclear leukocyte infiltration [85]. Moreover, stress oxidative markers showed the same trend in liver and blood [86]. The intake of ethanol without additional treatments seems to produce some of these effects at systemic levels too, which explain the additive effect mentioned. In rats drinking ethanol, it has been found increased gingival levels of 8OHdG and TNF-α and reduced GSH/GSSG ratio, and blood hydroperoxides. Curiously, there is a study in that ethanol intake prevented alveolar bone loss in teeth without ligature, but it has no interaction with

ligature effects [87]. Additionally, it has avoided PGE2 increase produced by ligatures placement in other experimental model, but in this case other inflammatory markers are elevated [84].

17.2.3 Lipids

Lipids are a more concentrated source of energy than carbohydrates or proteins. Properties that confer them have two functions in the body, they help provide energy and store energy. Also lipids are important for thermal insulation. Two essential fatty acids are required in our diet: linoleic acid and linolenic acid. Obesity is a risk factor for periodontal disease [18], probably through the secretion of pro-inflammatory cytokines by adipocytes as being described above [53, 57]. Recent research has started to include fatty acids that attenuate the inflammatory process or have anti-inflammatory properties, like n-3 polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and linolenic acid (LNA), as well as the n-6 fatty acid, γ-linolenic acid (GLA) [15]. However, no research has been conducted on the putative role of other well-documented healthy fatty acids such as monounsaturated fatty acids (MUFA).

Several studies have investigated the potential effects of different fatty acids (main attention has been paid to PUFA, particularly to n-3 PUFA) derived from diet or as supplements, on periodontal disease, both in humans [88–93] and in experimental models [94–99]. In humans, almost irrelevant has been the study of MUFA, with one study using olive oil (not virgin olive oil) as placebo [88]. Two studies focused on gingivitis [88, 89] and results did not show positive effects of the n-3 PUFA treatment. However, in general, and despite the varying experimental conditions (patient size, periodontal measures, and others), a protective association of n-3 PUFA on periodontitis has been found after the treatment with or higher intake of these fatty acids [90–93]. In animals, all revised studies focused on n-3 PUFA. Results were different depending on the dietary treatment schedule (in a preventive way or as treatment after infection) and duration; and on the way to induce periodontitis (by the injection of lipopolysaccharide or by the infection with bacteria). Overall, when periodontitis was induced by the injection of bacterial lipopolysaccharide, n-3 PUFA did not lead to reductions in alveolar bone loss [94–96]. However, when bacterial inoculation was used, n-3 PUFA reduced alveolar bone loss in a significant manner [97–99].

17.3 Vitamins

17.3.1 Vitamin C

Vitamin C is also known as ascorbic acid. It was named for its ability to cure scurvy. Vitamin C is involved in many cellular functions. It is needed for the hydroxylation of proline and lysine during collagen production and functions as an antioxidant.

The classic vitamin C deficiency disease is scurvy, a hemorrhagic disease, which presents with muscle weakness, lethargy, diffuse tissue bleeding, painful and swollen joints, ecchymoses, increased fractures, poor wound healing, gingivitis, and loss of integrity of the periodontal ligament [17].

The research into the relationship between vitamin C (ascorbic/ascorbate acid) and periodontal-related diseases comes from as far as the eighteenth century when it was observed that scurvy was fully recovered after a treatment with oranges and lemons [100]. Since then, many experimental and epidemiological studies tried to address this question. However, differences concerning methodological approaches (like the assessment of vitamin C levels by inferring from dietary intake, by analyzing saliva, plasma or urine, and so on) avoid in some situation a comparative analysis of the available studies. Also, differences in the study design (cross-sectional, follow-up, case–control, etc.) or population characteristics (healthy subjects, smoker, diabetic patients, children, old persons, etc.) should be considered. According to that, finding from cross-sectional studies with healthy voluntaries suggest that when plasma or serum vitamin C is evaluated, concentrations of the vitamin are inversely correlated with the number of seropositive subjects in *Porphyromonas gingivalis* [101], with clinical attachment loss [102, 103] or with the risk to suffer for periodontitis [104]. Also, an inverse correlation between vitamin C and periodontitis risk has been shown in cross-sectional studies involving a great number of individuals and based on the intake of vitamin C analysis [105, 106]. From follow-up studies, it can be concluded that low serum levels of vitamin C led to a higher adjusted relative risk for periodontal disease [104]; to a significant effect on edentulism [107] or to the number of teeth with periodontal disease progression [108]. Results from case–control studies are contradictory and depends on the biological matrix used to test vitamin C levels. Studies that used saliva as biological sample did not show differences between case and controls [109–111]. However, studies in which vitamin C was assessed in plasma, serum, or white blood cells [112, 113] reported lower vitamin C levels in periodontitis patients than in healthy controls. Despite the evident relationship between vitamin C levels and periodontitis, only a few number of intervention studies reported positive effects on the supplementation of periodontitis patients or healthy volunteers under experimental periodontitis or gingivitis with vitamin C [114, 115], with most of them showing no effects [116–121]. According to that, it might be possible to conclude that a correct vitamin C intake is necessary to avoid periodontal problems but that when the pathological state has been instituted, a supplementation with vitamin C is not enough to revert to the healthy estate.

Ascorbic acid is a cofactor for lysyl and prolyl hydroxylase, two iron essential enzymes in the collagen biosynthesis pathway. Interestingly, lysyl hydroxylase is downregulated in oral epithelial cells when exposed to the challenge of periodontal pathogens, providing some evidence for collagen dysmetabolism as a feature of frustrated healing. The role of vitamin C is to promote the synthesis of a normal mature collagen network by preventing iron-dependent oxidation of lysyl and prolylhydroxylase and protecting these enzymes against auto-inactivation [122]. This has been confirmed by several investigations conducted on human cell lines [123, 124].

Oxidative stress and other aspects associated with free radicals, like inflammation or apoptosis have been shown to be involved in the relationship between vitamin C and periodontitis [125–128]. Finally, vitamin C has also been investigated in relation to changes in osteoclastic cells differentiation in rats under experimental periodontitis induced by teeth ligature [129]. These authors found that vitamin C inhibited bone resorption via a lower osteoclast activation and RANKL expression.

17.3.2 Vitamin-B Complex

Thiamin, niacin, riboflavin, pantothenic acid, pyridoxine, folic acid, cyanocobalamin, and biotin together form the water-soluble vitamin-B complex. Thiamin, also known as vitamin B1, is required for the normal function of muscles and nerves because it converts glucose to energy. Niacin, known as vitamin B3, plays a role mainly in helping enzymes to function properly. Riboflavin, also known as vitamin B2, has been proven to be essential to normal growth, muscle development, and hair coat. Pantothenic acid enables the body to create usable energy from carbohydrates, fats, and proteins. Vitamin B6 is used by the body in the utilization of amino acids. Folic acid and vitamin B12 (also called cyanocobalamin) are two closely related B complex vitamins that are necessary for the bone marrow to produce red blood cells, and a deficiency of either can lead to advanced anemia. Biotin is one of the most discussed of all vitamins, primarily because of its role in collagen synthesis. It is generally necessary for growth, digestion, and muscle function [130]. The vitamin-B complex may be important for periodontal wound healing [131]. Cross-sectional studies have found that low serum folate level was independently associated with periodontal disease [132], as well as a negative correlation between dietary folate level and bleeding on probing [133], although no significant association was found among periodontal index scores and folate intake [133]. Another cross-sectional study found positive effects of the intake of several components of the vitamin-B complex (B1, B6, niacin, and pantothenic acid) on the number of teeth preserved in old adults [134]. In 2005 Hung et al. reported a follow-up study based on 83,104 women [64]. After adjusting for age, total calorie intake, smoking, and physical activity, edentulous women appeared to have dietary intake associated with increased risk for cardiovascular diseases, including significantly higher intake of vitamin B12, and lower intake of vitamin B6 and folate.

The role of smoking as a risk factor for periodontal disease is well documented. Cigarette smoking is a strong predictor of deeper probing depths, greater attachment loss, more bone loss, and fewer teeth [135]. Cigarette smoking also affects vitamin B12 and folic acid mechanisms. A case–control study based on smokers (45 individual) and non-smokers (43 individual) aged 31–68 years and affected by chronic periodontal disease demonstrated that serum folic acid was lower in smokers, who showed higher plaque index, gingival index, pocket depth, and white blood cells [135]. These same authors, extending the study in a longitudinal way and including a non-surgical intervention for chronic periodontitis, observed that the clinical

response was impaired by smoking, and smoking negatively influenced serum levels of folic acid following non-surgical intervention [136]. According to some studies, folic acid supplementation produces significant reduction of gingival inflammation as determined by decreased redness, bleeding, tenderness, and exudates [137–142]. More recently, Neiva et al. [130] in a placebo-controlled trial, found that vitamin-B complex supplement in combination with access flap surgery resulted in statistically significant superior clinical attachment level gains when compared to placebo.

17.3.3 Vitamin K

Vitamin K exists naturally in multiple dietary forms. Phylloquinone (vitamin K1) is a 2-methyl-1,4-napthoquinone ring with aphytyl group at the 3-position. Menaquinones (vitamin K2) are endogenously synthesized and differ in structure from phylloquinone in their 3′-substituted [143]. Vitamin K is required for blood clotting. The "K" is derived from the Danish word "koagulation." Vitamin K is needed for the carboxylation of glutamic acid residues found in the clotting factors produced by the liver. The drug warfarin (coumadin), a vitamin K antagonist, functions by inhibiting this carboxylation and preventing the function of these factors [18]. It has been proposed that vitamin K has multiple roles beyond coagulation, both dependent and independent of its known biochemical function as an enzyme cofactor. This expanded scope of potential functions of vitamin K in the maintenance of human health has been accompanied by a substantial number of observational studies and, to a lesser extent, randomized controlled trials designed to isolate the role(s) of vitamin K in the prevention of specific chronic diseases, including osteoporosis and cardiovascular disease [143].

Up to date, there is paucity in studies evaluating vitamin K in periodontitis. In 1998, Rawlinson et al. performed a cross-sectional study investigating healthy and diseased sites from eighteen subjects with adult periodontitis aged 27–64 years [144]. Findings from this study suggest that the levels of phylloquinone in gingival crevicular liquid are lower in periodontal health than in disease sites. Since phylloquinone is an absolute growth requirement for black-pigmented anaerobes, many of which are implicated in the etiology of periodontal diseases, authors suggested that total phylloquinone at diseased sites may provide the nutritional requirements favoring the growth of black-pigmented anaerobes. In 2007, Hojo et al. assessed the possibility that bifidobacteria compete with *P. gingivalis* for their mutual growth factor vitamin K [145]. This study also examined whether salivary Bifidobacterium species decrease vitamin K concentration in the growth medium. Authors concluded that *Bifidobacterium adolescentis* S2-1 decreased vitamin K concentration and inhibited the growth of *P. gingivalis* by possibly competing for the growth factor and that according to these results, salivary bifidobacteria may possess the potential to suppress the growth of *P. gingivalis* by reducing the growth factors in the environment.

17.3.4 Vitamin A

Vitamin A, a fat-soluble vitamin, is needed for the maturation of epithelial tissues and is required for vision, being a component of visual purple (essential for night vision). Preformed vitamin A is found primarily in animal fats and fish oils as retinoids, which can be toxic when taken in high doses. Carotenoids (a provitamin) are present mainly in vegetables and fruits and are precursors of vitamin A. β-Carotene, the main carotenoid found in foods, is nontoxic in high doses and functions as an antioxidant [18]. Retinoids are required for maintaining many essential physiological processes in the body, including normal growth and development, normal vision, a healthy immune system, normal reproduction, and healthy skin and barrier functions [146]. Concerning vitamin A and periodontitis, in 1976, Freeland et al. in a cross-sectional study with 80 dental patients, reported that dietary vitamin A was inversely related to periodontal index [147]. In 2009 Linden et al. performed a cross-sectional study with 1,358 men, founding that the levels of α- and β-carotene, β-cryptoxanthin, and zeaxanthin were significantly lower in men with periodontitis [148]. Participants in the third national health and nutrition examination survey (NHANES III) were used in a study [149] to investigate the relationship between monthly tomato consumption and serum lycopene levels, and a self-reported history of congestive heart failure in individuals with periodontitis. Conclusions of the study were that a relationship exists between periodontitis and congestive heart failure risk, and high monthly tomato consumption appeared to affect this relationship in a positive direction in periodontitis subjects. In 2007, Chandra et al. reported results from a treatment-placebo trial with 20 systemically healthy patients showing clinical signs of gingivitis [150]. Treatment with lycopene resulted in a statistically significant decrease in gingivitis when compared with placebo.

17.3.5 Vitamin E

Vitamin E is a fat-soluble vitamin whose primary role is to function as an antioxidant. It is composed of eight related compounds called tocopherols or tocotrienols. The most active form is α-tocopherol, which is incorporated into the lipid membrane of cells helping to quench free radicals, thus protecting the fatty acids in the lipid bilayer [17].

In man, greater dietary intake of vitamin E has been associated with fewer reported oral symptoms [151]. Linden et al. [148], investigating 1,258 men aged 60–70 years found no significant differences in the levels of α- and γ-tocopherol in relation to periodontitis. On the other hand, Battino et al. [152] working with a group of patients belonging to three generations of a family with different degrees of severity of Papillon–Lefèvre syndrome (PLS), an uncommon disease in which palmoplanar ectodermal dysplasia is accompanied by a particularly aggressive periodontal disease [153], found that serum vitamin E levels were very low in the child of the third

generation (phenotypically affected) and his mother. These results have been contrasted with more patients [154]. A follow-up study with 83,104 women in USA [64] found that edentulous women showed lower intake of vitamin E. Another follow-up study performed on 224 Japanese aged 71 years found that low serum levels of vitamin E showed higher adjusted relative risk for periodontal disease events. On the other hand, old studies showed that circulating concentrations of the vitamin were the same in patients with and without periodontitis [155]. Concerning supplementation studies, it was early showed that patients with periodontal disease who were given vitamin E daily for 21 days to swish in their mouths and swallow exhibited a significant decrease in fluid flow from the gingival sulcus than in controls with disease but no vitamin E supplementation [156]. Also, subjects given vitamin E supplementation for 12 weeks exhibited a reduction in Russell's Periodontal Index [157]. However, topical vitamin E did not reduce gingivitis over a 4-week period relative to a placebo [158], but the method appeared insensitive in that chlorhexidine similarly had non-significant effects on gingivitis. The implication of vitamin E in periodontal diseases is related to its role on reactive oxygen species, inflammation, and immunomodulation biology. All these aspects have been assessed by investigating with cell cultures [127, 159] and rats [160, 161].

17.3.6 Vitamin D

It is well known that the active vitamin D (VD) hormone, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), is a major component in the regulation of bone metabolism and bone-related biomarkers by playing a significant role in promoting calcium and phosphate absorption [162]. If plasma calcium concentrations decrease, calcium reabsorption from bone increases leading to decreased bone mineralization [163]. VD also regulates the expression of a number of bone-related genes (e.g., osteocalcin and alkaline phosphatase) via VD receptor (VDR) [164–167]. More recently, $1,25(OH)_2D$ has been shown to increase transcription of antimicrobial peptides [168, 169] which fight foreign invaders (e.g., pathogenic oral bacteria). VD may also protect against periodontitis because of anti-inflammatory properties of $1,25(OH)_2D$ [170–172]. For example, the VDR is expressed on a number of human immune cells [170–172] and $1,25(OH)_2D$ has been shown to decrease proliferation of T and B lymphocytes [173, 174] and inhibit the T-helper (Th)1 and Th17 (pro-inflammatory) cell response [175] while promoting a Th2 (anti-inflammatory) cell response [176, 177].

Studies on vitamin D might be divided between those focused on VD itself and those aboarding aspects related to VDR. Concerning studies on VD, two cross-sectional studies conducted using the NHANES III (1988–1994) observed an inverse association between serum 25-hydroxyvitamin D concentrations [25(OH)D] and gingival inflammation and periodontal clinical attachment level [178].

Few studies have investigated the association between vitamin D status, assessed with a blood biomarker, and periodontal disease. Of those conducted, the majority were small case–control studies [179–181].

17.4 Minerals

17.4.1 Calcium

Almost all body's calcium content is in the skeletal system, where most of the mineral portion being present as hydroxyapatite. Due to this structural role, calcium is needed for normal bone metabolism, where there is an interplay among the osteoblasts, osteocytes, and osteoclasts. Therefore it plays a major role in nerve conduction and blood clotting [18].

Calcium is in equilibrium among bone, extracellular water and soft tissue, with about 0.7 g being absorbed and redeposited daily, and its deficiency can lead to a decrease in serum calcium, resulting in mobilization from host tissues [18]. Under these conditions, alveolar bone may be affected in the same way as other parts of skeleton. In this sense, Amarasena et al. [182] observed that serum calcium was associated with the progression of periodontal disease in elderly Japanese. However, in other study in a wide population from NHAMES III participants, it was observed that low serum calcium levels were related to periodontal disease only in younger females [183]. According to this relationship, it had been found a negative correlation among loss of clinical attachment and bone mineral density of some bones assessed [184], but when old women with or without periodontal disease have been compared, there were no differences in bone mass index or in absolute percentage or percentage change in bone mass index of hip and its subregions [185].

Attending to the impact of calcium consumption, several studies have shown that low calcium intakes were associated with low number of teeth [186], periodontal disease [183], or periodontal index [147]. If dietary recommendations were taken into account, calcium intake below recommendations was associated with increased risk of subsequent tooth loss only in men [186]. Calcium provided by dairy products seems more important at least in relation to periodontitis. Adegboye et al. [187] have shown a similar relationship only with calcium from dairy products in older adults, and only with milk and fermented foods when previous intake was subdivided. In the same sense, these authors and other have found that dairy products intake was related to periodontitis prevalence [188] or risk [187, 188], particularly in milk and fermented foods case [187]. Interventional trials including dietary calcium always have been made in conjunction with vitamin D. Periodontitis patients receiving periodontal maintenance therapy [189] or extraction of several teeth and immediate placement of dentures [190] who have taken vitamin D and calcium combined showed lower values several on clinical measures related to periodontal diseases, probing depth, bleeding on probing, gingival index, furcation involvement, clinical attachment loss, height alveolar crest [189], or alveolar bone loss than placebo-treated individuals [190].

Studies developed in animals, mainly rats, have supported the importance of calcium for these pathologies. In a model of experimental periodontitis induced by elastic ring insertion, female rats were fed (before and during the experimental procedure period) on different amounts of calcium [11, 192]. In this study, it was found that calcium intake was positively related with a higher bone mineral density and

with a lower alveolar bone height [191, 192]. These effects were more evident for lactating rats [192], but there was no differences when dietary calcium were high enough [191]. Bone mineral density in their pups, when they were present, was affected in the same manner [191]. In female mice, it has been observed decreases in bone in femur and alveolar crest, being affected trabecular bone but not alveolar crest height, when they received a calcium-deficient diet. This effect could be reverted when calcium were replacement for the same period [193].

Parotid saliva and gland features have been affected by dietary calcium too. In rats fed low calcium diets, salivary flow amylase activity and content, and acinar cells AMPc from parotid gland, firstly increased, but decreased at 4 weeks, just when the gland weight started to decrease [194].

17.4.2 Magnesium

The average person contains about 25 g of magnesium, with the majority stored in bone and about 25 % present in soft tissues [17]. However it is present in all tissues playing a crucial role in many physiological functions. Magnesium is the physiologic calcium antagonist [122]. Intracellular magnesium is concentrated in mitochondria and involved in energy transfer [17]. Magnesium deficiency has been suggested to be involved in the etiology of cardiovascular diseases, diabetes, pre-eclampsia, eclampsia, sickle cell disease, and chronic alcoholism [195].

A cross-sectional study has not found any association of magnesium serum concentration with periodontal index [147]. Attending to its relationship with calcium, serum calcium/magnesium ratio has been studied. It has been observed an inverse dose–response relationship between calcium/magnesium ratio and periodontal disease events in elderly persons, but only when they were smokers [196]. If it considered people aged between 20 and 80 years old, a high serum calcium/magnesium ratio was associated with reduced probing depth, less attachment loss, and a higher number of remaining teeth [197]. According to possible magnesium consumption effect, the only evidence found has been that people taking magnesium-containing drugs showed less attachment loss and more remaining teeth compared with their matched counterparts, in subjects aged 40 years old and older [197].

Furthermore, it has been found that salivary levels of magnesium and clinical attachment level were correlated in smokers [198]. Certain in vitro studies could explain a relevant role of magnesium on this fluid, magnesium cation enhanced lactoferrin killing activity of *A. actinomycetemcomitans*, while other ion, like potassium and calcium, had no effect. Furthermore it seems to participate in opsonization and subsequent phagocytosis of at least *P. gingivalis* [199]. On the other hand, in *Streptococcus gordoni* cultures, peroxidogenesis was stimulated with magnesium cation presence [200].

17.4.3 Phosphorus

Phosphorus is found in all plant and animal cells. A primary dietary deficiency of phosphorous is not known. About 600–900 g of phosphorus is present in bone in hydroxyapatite. In the past, it was thought that phosphate intake could influence calcium absorption, but it is now known that phosphate intake has little consequence for calcium absorption at normal levels of intake [18]. In hamster, a diet with $Ca_3(PO_4)_2$ or a mixture of dibasic sodium phosphate and monobasic potassium phosphate showed inhibition of alveolar bone loss, but in the second case it was not significant [201]. An in vitro study investigated the role of phosphorus alone or combinated with ascorbate [124]. Results shown a synergistinc action between phosphate and L-ascorbate, leading together to an improved intracellular vitamin C, collagen synthesis and decrease ROS production and IL-8 expression in a more effective manner than L-ascorbate sodium [124]. We found only a study in which phosphorus effect on periodontitis was studied in humas [202]. In this study, subgingival local irrigation with inorganic polyphosphate was studied in a randomised double-blind study of 33 patients with periodontitis. Scaling and root planing were performed 1 week after the initial examination. No significant differences between the inorganic polyphosphate group and control were detected in each item except IL-1b. Patients in whom both the bleeding on probing and gingival index at 1 week had improved were significantly older in the inorganic polyphosphate group than in the control group ($p < 0.05$). Bone regeneration was seen in one case of the inorganic polyphosphate group. Author concluded that inorganic polyphosphate was useful in the treatment of periodontitis in the elderly, indicating a probable effect of anti-ageing, with similar bone regenerations occurring in both groups.

17.4.4 Iron

Iron is important as a functional component of hemoglobin and it aids immune function. The typical person has about 4 g of iron: 2.5 g in hemoglobin, 0.3 g in myoglobin and cytochromes, and about 1 g in iron stores (ferritin). Most iron is used to make red blood cells in the bone marrow. Iron deficiency leads to anemia, which is seen more in women during their reproductive years and in children due to their rapid growth [17]. Some epidemiological studies have found a negative relationship between periodontal diseases related parameters, like periodontal index or IgG against *P. gingivalis*, and iron levels in blood or serum [203, 204], but it was not always present [147]. According to iron intake, no association with periodontal index has been noted in a cross-sectional study [147]. However, in a vitamin B12 and iron severe anemia case associated to generalize alveolar bone loss, treatment for one year maintained the values stable during this period [205].

Associations with clinical parameters related to anemia also have been searched. Community periodontal index has been related to glycosylated hemoglobin A1 and A1c [203]. In some case–control studies, periodontitis patients have shown lower hematocrit, lower number of erythrocytes and hemoglobin compared with healthy subjects [206, 207], and higher erythrocytes sedimentation [206], but in other it was not found any associations [208]. Some authors [209, 210] have suggested that anemia is one of the causes of destructive periodontitis, but others [211] have proposed that periodontitis cause an anemia type termed as anemia of chronic disease. This condition is defined as the anemia occurring in chronic infections, and occurring despite the presence of adequate iron stores and vitamins [212]. The possible etiology cited for decreased blood counts was the downregulation of erythropoiesis in the bone marrow by pro-inflammatory cytokines due to periodontal disease [206, 207]. Nevertheless if the data provided above are taken into account it seems that anemia of chronic disease is not always present.

In rats, it was noted that iron deficiency can influence saliva characteristics too. They exhibited salivary peroxidase decrease and also secretion rate, but only in growing rats [213].

17.4.5 Copper

Copper is stored bound to ceruloplasmin, a copper-dependent ferroxidase that help so oxidize iron. Ceruloplasmin is required for optimal use of ferritin. Copper is found in two members of the superoxide dismutase family, which help quench superoxide free radicals. Furthermore copper is required for the formation of hemoglobin [17]. It has been observed a direct and linear relationship between serum copper level and periodontal index [147]. Similarly, two Wilson diseased female patients aged 28 and 53 years presented multiple oral manifestation including gingival enlargement and early onset periodontitis [214]. On the other hand, it has noted possible antimicrobial effects against periodontopathogenic bacteria, at least against *P. gingivalis*, for copper ions or copper-containing compounds, in vitro experiences, although this role remains unclear [215–217]. Moreover, in vitro studies have revealed matrix metalloproteinases 2 and 9 activity in the presence of $CuSO_4$ [218].

17.4.6 Zinc

Zinc is a cofactor for over 50 enzymes (e.g., carbonic anhydrase, alkaline phosphatase, alcohol dehydrogenase, and superoxide dismutase). About 2 g of Zn is stored in the body, with most present in bone. A Zn deficiency can lead to small stature, mild anemia, and impaired wound healing [17]. Freeland et al. [147] have searched for associations among periodontal index and dietary intake or serum levels of zinc, but they did not found it. It seemed to exert beneficial effects improving plaque

index in children taken Zn supplement, but it has not been related to gingival index changes because it also improved in placebo-treated children [219]. The effect of dietary zinc has been tested in rats too, animals fed Zn-deficient diet showed changes related to saliva among others. Reduction in acidic proline-rich protein and secretory activity of parotid gland accompanied by differential secretory granules in the acini were observed in this group [220]. However, in other rat studies, zinc-deficient diet was related to lower plaque and gingival indices scores, but probing depth was unaffected. On the other side, other oral histological abnormalities were more frequent on rats fed zinc-deficient diet like ulcers and hyperkeratinosis, mainly in tongue [221].

17.4.7 Manganese

Manganese is a cofactor for enzymes involved in the synthesis of proteoglycans and other enzymes are present in mitochondria [17] like superoxide dismutase. Magnesium should be of interest due to the important superoxides dismutases in antioxidant systems. Also there is one experiment that has shown the importance of this enzyme in periodontitis. In rats with periodontitis experimentally induced by ligatures, extravasation in gingivomucosal tissue and alveolar bone destruction were decreased by a synthetic form of superoxide dismutase intraperitoneally administered [222].

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References

1. Scardina GA, Messina P (2012) Good oral health and diet. J Biomed Biotechnol. doi:10.1155/2012/720692
2. Boyd LD, Lampi KJ (2001) Importance of nutrition for optimum health of the periodontium. J Contemp Dent Pract 2:1–14
3. Battino M, Ferreiro MS, Fattorini D, Bullon P (2002) In vitro antioxidant activities of mouthrinses and their components. J Clin Periodontol 29:462–467
4. Battino M, Ferreiro MS, Gallardo I, Newman HN, Bullon P (2002) The antioxidant capacity of saliva. J Clin Periodontol 29:189–194
5. Battino M, Bompadre S, Leone L, Pugnaloni A, Rubini C, Ferreiro MS, Gallardo I, Bullon P (2003) The effect of cyclosporine A chronic administration on the antioxidant pattern of rat liver mitochondria: structural and functional consequences. Biofactors 18:271–275
6. Battino M, Bompadre S, Politi A, Fioroni M, Rubini C, Bullon P (2005) Antioxidant status (CoQ10 and Vit. E levels) and immunohistochemical analysis of soft tissues in periodontal diseases. Biofactors 25:213–217
7. Battino M, Ferreiro MS, Armeni T, Politi A, Bompadre S, Massoli A, Bullon P (2005) In vitro antioxidant activities of antioxidant-enriched toothpastes. Free Radic Res 39:343–350

8. Bullon P, Pugnaloni A, Gallardo I, Machuca G, Hevia A, Battino M (2003) Ultrastructure of the gingiva in cardiac patients treated with or without calcium channel blockers. J Clin Periodontol 30:682–690
9. Bullon P, Fioroni M, Goteri G, Rubini C, Battino M (2004) Immunohistochemical analysis of soft tissues in implants with healthy and peri-implantitis condition, and aggressive periodontitis. Clin Oral Implants Res 15:553–559
10. Bullon P, Gallardo I, Goteri G, Rubini C, Battino M, Ribas J, Newman HN (2007) Nifedipine and cyclosporin affect fibroblast calcium and gingiva. J Dent Res 86:357–362
11. Bullon P, Morillo JM, Ramirez-Tortosa MC, Quiles JL, Newman HN, Battino M (2009) Metabolic syndrome and periodontitis: is oxidative stress a common link? J Dent Res 88:503–518
12. Bullon P, Quiles JL, Morillo JM, Rubini C, Goteri G, Granados-Principal S, Battino M, Ramirez-Tortosa M (2009) Gingival vascular damage in atherosclerotic rabbits: hydroxytyrosol and squalene benefits. Food Chem Toxicol 47:2327–2331. doi:10.1016/j.fct.2009.06.026
13. Bullon P, Cordero MD, Quiles JL, Morillo JM, del Carmen Ramirez-Tortosa M, Battino M (2011) Mitochondrial dysfunction promoted by *Porphyromonas gingivalis* lipopolysaccharide as a possible link between cardiovascular disease and periodontitis. Free Radic Biol Med 50:1336–1343. doi:10.1016/j.freeradbiomed.2011.02.018
14. Bullon P, Cordero MD, Quiles JL, del Carmen Ramirez-Tortosa M, Gonzalez-Alonso A, Alfonsi S, García-Marín R, de Miguel M, Battino M (2012) Autophagy in periodontitis patients and gingival fibroblasts: unraveling the link between chronic diseases and inflammation. BMC Med 10:122. doi:10.1186/1741-7015-10-122
15. Kaye EK (2010) n-3 fatty acid intake and periodontal disease. J Am Diet Assoc 110:1650–1652
16. Ramirez-Tortosa MC, Quiles JL, Battino M, Granados S, Morillo JM, Bompadre S, Newman HN, Bullon P (2010) Periodontitis is associated with altered plasma fatty acids and cardiovascular risk markers. Nutr Metab Cardiovasc Dis 20:133–139. doi:10.1016/j.numecd.2009.03.003
17. Schifferle RE (2005) Nutrition and periodontal disease. Dent Clin N Am 49:595–610. doi:10.1016/j.cden.2005.03.008
18. Schifferle RE (2009) Periodontal disease and nutrition: separating the evidence from current fads. Periodontol 2000 50:78–89. doi:10.1111/j.1600-0757.2008.00297.x
19. Enwonwu CO (1995) Interface of malnutrition and periodontal diseases. Am J Clin Nutr 61:430S–436S
20. Enwonwu CO (1994) Cellular and molecular effects of malnutrition and their relevance to periodontal disease. J Clin Periodontol 21:643–657. doi:10.1111/j.1600-051X.1994.tb00782.x
21. Enwonwu CO, Meeks VI (1996) Oral candidiasis, HIV, and saliva glucocorticoids. Am J Pathol 148:1313–1318
22. Baelum V, Scheutz F (2002) Periodontal diseases in Africa. Periodontol 2000 29:79–103. doi:10.1034/j.1600-0757.2002.290105.x
23. Navia JM (1996) Nutrition and dental caries: ten findings to be remembered. Int Dent J 46:381–387
24. Johansson I, Ericson T (1987) Saliva composition and caries development during protein deficiency and β-receptor stimulation or inhibition. J Oral Pathol 16:145
25. Agarwal PK, Agarwal KN, Agarwal DK (1984) Biochemical changes in saliva of malnourished children. Am J Clin Nutr 39:181–184
26. Reddy V, Raghuramulu N, Bhaskaram C (1976) Secretory IgA in protein-calorie malnutrition. Arch Dis Child 51:871–874. doi:10.1136/adc.51.11.871
27. McMurray DN, Rey H, Casazza LJ, Watson RR (1977) Effect of moderate malnutrition on concentrations of immunoglobulins and enzymes in tears and saliva of young Colombian children. Am J Clin Nutr 30:1944–1948
28. Johansson I, Ericson T (1986) Effect of chewing on the secretion of salivary components during fasting. Caries Res 20:141–147. doi:10.1159/000260933
29. Menaker L, Navia JM (1974) Effect of undernutrition during the perinatal period on caries development in the rat: V. Changes in whole saliva volume and protein content. J Dent Res 53:592–597. doi:10.1177/00220345740530031201

30. Johansson I, Alm P (1989) Effect of moderate protein-deficiency on ultrastructure in parotid and submandibuar acinar cells in the adult rat. Scand J Dent Res 97:505–510. doi:10.1111/j.1600-0722.1989.tb00924.x
31. Johansson I, Ryberg M (1991) The effects of moderate protein deficiency on β-adrenoceptor density in rat parotid and submandibular salivary glands. Arch Oral Biol 36:591–594. doi:10.1016/0003-9969(91)90109-8
32. Enwonwu CO, Phillips RS, Ibrahim CD, Danfillo IS (2004) Nutrition and oral health in Africa. Int Dent J 54:344–351. doi:10.1111/j.1875-595X.2004.tb00010.x
33. Morhart RE, Fitzgerald RJ (1976) Nutritional determinants of the ecology of the oral flora. Dent Clin North Am 20:473–489
34. Hujoel P (2009) Dietary carbohydrates and dental-systemic diseases. J Dent Res 88:490–502. doi:10.1177/0022034509337700
35. Chung CS, Hankin JH, Miyamoto W, Kau MK (1977) Dental plaque and dietary intakes in school children in Hawaii. J Dent Res 56:11–16. doi:10.1177/00220345770560010101
36. Harjola U, Liesma H (1978) Effect of polyol and sucrose candies on plaque, gingivitis and lactobacillus index scores. Acta Odontol Scand 36:237–242. doi:10.3109/00016357809004674
37. Roberts IF, Roberts GJ (1979) Relation between medicines sweetened with sucrose and dental disease. Br Med J 2:14–16
38. Sidi AD, Ashley FP (1984) Influence of frequent sugar intakes on experimental gingivitis. J Periodontol 55:419–423
39. Yoshihara A, Watanabe R, Hanada N, Miyazaki H (2009) Longitudinal study of the relationship between diet intake and dental caries and periodontal disease in elderly Japanese subjects. Gerodontology 26:130–136. doi:10.1111/j.1741-2358.2008.00244.x
40. Carlsson J, Sundström B (1968) Variations in composition of early dental plaque following ingestion of sucrose and glucose. Odontol Revy 19:161–169
41. Fry AJ, Grenby TH (1972) The effects of reduced sucrose intake on the formation and composition of dental plaque in a group of men in the Antartic. Arch Oral Biol 17:873–882. doi:10.1016/0003-9969(72)90030-1
42. Scheinin A, Makinen KK, Ylitalo K (1976) Turku sugar studies. V. Final report on the effect of sucrose, fructose and xylitol diets on the caries incidence in man. Acta Odontol Scand 34:179–198
43. Auskaps AM, Gupta OP, Shaw JH (1957) Periodontal disease in the rice rat. III. Survey of dietary influences. J Nutr 63:325–343
44. Aguirre JI, Akhter MP, Kimmel DB, Pingel J, Xia X, Williams A, Jorgensen M, Edmonds K, Lee JY, Reinhard MK, Battles AH, Kesavalu L, Wronski TJ (2012) Enhanced alveolar bone loss in a model of non-invasive periodontitis in rice rats. Oral Dis 18:459–468. doi:10.1111/j.1601-0825.2011.01893.x
45. Keyes PH, Jordan HV (1964) Periodontal lesions in the Syrian hamster. III. Findings related to an infectious and transmissible component. Arch Oral Biol 9:377–400. doi:10.1016/0003-9969(64)90024-X
46. Jordan HV, Keyes PH, Lim S (1969) Plaque formation and implantation of Odontomyces viscosus in hamsters fed different carbohydrates. J Dent Res 48:824–831. doi:10.1177/00220345690480053601
47. Socransky SS, Huberas kC, Propas D (1970) Induction of periodontal destruction in gnotobiotic rats by a human oral strain of Actinomyces naeslundii. Arch Oral Biol 15:993–995
48. Galvão MP, Chapper A, Rösing CK, Ferreira MB, de Souza MA (2003) Methodological considerations on descriptive studies of induced periodontal diseases in rats. Pesqui Odontol Bras 17:56–62. doi:10.1590/S1517-74912003000100011
49. Liu S (2002) Intake of refined carbohydrates and whole grain foods in relation to risk of type 2 diabetes mellitus and coronary heart disease. J Am Coll Nutr 21:298–306
50. Ripsin CM, Keenan JM, Jacobs DR Jr, Elmer PJ, Welch RR, Van Horn L, Liu K, Turnbull WH, Thye FW, Kestin M, Hegsted M, Davidson DM, Davidson MH MD, Dugan LD, Demark-Wahnefried W, Beling S (1992) Oat products and lipid lowering. A meta-analysis. JAMA 267:3317–3325. doi:10.1001/jama.1992.03480240079039

51. Alfieri MA, Pomerleau J, Grace DM, Anderson L (1995) Fiber intake of normal weight, moderately obese and severely obese subjects. Obes Res 3:541–547. doi:10.1002/j.1550-8528.1995.tb00188.x
52. Beck B, Burlet A, Nicolas JP, Burlet C (1996) Opposite influence of carbohydrates and fat on hypothalamic neurotensin in Long-Evans rats. Life Sci 59:349–356
53. Montague CT, O'Rahilly S (2000) The perils of portliness: causes and consequences of visceral adiposity. Diabetes 49:883–888. doi:10.2337/diabetes.49.6.883
54. Esposito K, Marfella R, Ciotola M, Di Palo C, Giugliano F, Giugliano G, D'Armiento M, D'Andrea F, Giugliano D (2004) Effect of a Mediterranean-style diet on endothelial dysfunction on markers of vascular inflammation in the metabolic syndrome: a randomized trial. JAMA 292:1440–1446. doi:10.1001/jama.292.12.1440
55. Slattery ML, Curtin KP, Edwards SL, Schaffer DM (2004) Plant foods, fiber, and rectal cancer. Am J Clin Nutr 79:274–281
56. Mann J (2007) Dietary carbohydrate: relationship to cardiovascular disease and disorders of carbohydrate metabolism. Eur J Clin Nutr 61:S100–S111. doi:10.1038/sj.ejcn.1602940
57. Pischon N, Heng N, Bernimoulin J-P, Kleber B-M, Willich SN, Pischon T (2007) Obesity, inflammation, and periodontal disease. J Dent Res 86:400–409. doi:10.1177/154405910708600503
58. Schulze MB, Schulz M, Heidemann C, Schienkiewitz A, Hoffmann K, Boeing H (2007) Fiber and magnesium intake and incidence of type 2 diabetes: a prospective study and meta-analysis. Arch Intern Med 167:956–965. doi:10.1001/archinte.167.9.956
59. König KG, Navia JM (1995) Nutritional role of sugars in oral health. Am J Clin Nutr 62:275S–283S
60. Proctor GB, Carpenter GH (2001) Chewing stimulates secretion of human salivary secretory immunoglobulin A. J Dent Res 80:909–913. doi:10.1111/j.1532-5415.2011.03866.x
61. Schwartz N, Kaye EK, Nunn ME, Spiro A 3rd, Garcia RI (2012) High-fiber foods reduce periodontal disease progression in men aged 65 and older: the Veterans Affairs normative aging study/Dental Longitudinal Study. J Am Geriatr Soc 60:676–683. doi:10.1111/j.1532-5415.2011.03866.x
62. Yamori M, Njelekela M, Mtabaji J, Yamori Y, Bessho K (2011) Hypertension, periodontal disease, and potassium intake in nonsmoking, nondrinker African women on no medication. Int J Hypertens 2011:695719. doi:10.4061/2011/695719
63. Petti S, Cairella G, Tarsitani G (2000) Nutritional variables related to gingival health in adolescent girls. Community Dent Oral Epidemiol 28:407–413. doi:10.1034/j.1600-0528.2000.028006407.x
64. Hung HC, Colditz G, Joshipura KJ (2005) The association between tooth loss and the self-reported intake of selected CVD-related nutrients and foods among US women. Community Dent Oral Epidemiol 33:167–173. doi:10.1111/j.1600-0528.2005.00200.x
65. Merchant AT, Pitiphat W, Franz M, Joshipura KJ (2006) Whole-grain and fiber intakes and periodontitis risk in men. Am J Clin Nutr 83:1395–1400
66. Hornecker E, Muuß T, Ehrenreich MD, Mausberg RF (2003) A pilot study on the oral conditions of severely alcohol addicted persons. J Contemp Dent Pract 4:51–59
67. WHO (1992) The classification of mental and behavioral disorders. Clinical descriptions and diagnostic guidelines. World Health Organization, Geneva
68. Larato DC (1972) Oral tissue changes in the chronic alcoholic. J Periodontol 43:772–773
69. Harris CK, Warnakularsuriya KA, Johnson NW, Gelbier S, Peters TJ (1996) Oral health in alcohol misusers. Community Dent Health 13:199–203
70. Enberg N, Wolf J, Ainamo A, Alho H, Heinälä P, Lenander-Lumikari M (2001) Dental diseases and loss of teeth in a group of Finnish alcoholics: a radiological study. Acta Odontol Scand 59:341–347. doi:10.1080/000163501317153176
71. Shizukuishi S, Hayashi N, Tamagawa H, Hanioka T, Maruyama S, Takeshita T, Morimoto K (1998) Lifestyle and periodontal health status of Japanese factory workers. Ann Periodontol 3:303–311
72. Tezal M, Grossi SG, Ho AW, Genco RJ (2001) The effect of alcohol consumption on periodontal disease. J Periodontol 72:183–189. doi:10.1902/jop.2001.72.2.183

73. Shimazaki Y, Saito T, Kiyohara Y, Kato I, Kubo M, Iida M, Yamashita Y (2005) Relationship between drinking and periodontitis: the Hisayama Study. J Periodontol 76:1534–1541. doi:10.1902/jop.2005.76.9.1534
74. Nishida N, Tanaka M, Sekine S, Takeshita T, Nakayama K, Morimoto K, Shizukuishi S (2010) Association of ALDH2 genotypes with periodontitis progression. J Dent Res 89:138–142. doi:10.1177/0022034509356045
75. Tezal M, Grossi SG, Ho AW, Genco RJ (2004) Alcohol consumption and periodontal disease. The Third National Health and Nutrition Examination Survey. J Clin Periodontol 31:484–488. doi:10.1111/j.1600-051X.2004.00503.x
76. Lages EJ, Costa FO, Lages EM, Cota LO, Cortelli SC, Nobre-Franco GC, Cyrino RM, Cortelli JR (2012) Risk variables in the association between frequency of alcohol consumption and periodontitis. J Clin Periodontol 39:115–122. doi:10.1111/j.1600-051X.2011.01809.x
77. Pitiphat W, Merchant AT, Rimm EB, Joshipura KJ (2003) Alcohol consumption increases periodontitis risk. J Dent Res 82:509–513. doi:10.1177/154405910308200704
78. Okamoto Y, Tsuboi S, Suzuki S, Nakagaki H, Ogura Y, Maeda K, Tokudome S (2006) Effects of smoking and drinking habits on the incidence of periodontal disease and tooth loss among Japanese males: a 4-yr longitudinal study. J Periodontal Res 41:560–566. doi:10.1111/j.1600-0765.2006.00907.x
79. Jansson L (2008) Association between alcohol consumption and dental health. J Clin Periodontol 35:379–384. doi:10.1111/j.1600-051X.2008.01210.x
80. Torrungruang K, Tamsailom S, Rojanasomsith K, Sutdhibhisal S, Nisapakultorn K, Vanichjakvong O, Prapakamol S, Premsirinirund T, Pusiri T, Jaratkulangkoon O, Unkurapinun N, Sritara P (2005) Risk indicators of periodontal disease in older Thai adults. J Periodontol 76:558–565. doi:10.1902/jop.2005.76.4.558
81. Kongstad J, Hvidtfeldt UA, Grønbaek M, Jontell M, Stoltze K, Holmstrup P (2008) Amount and type of alcohol and periodontitis in the Copenhagen City Heart Study. J Clin Periodontol 35:1032–1039. doi:10.1111/j.1600-051X.2008.01325.x
82. de Souza DM, Ricardo LH, Prado Mde A, Prado Fde A, da Rocha RF (2006) The effect of alcohol consumption on periodontal bone support in experimental periodontitis in rats. J Appl Oral Sci 14:443–447. doi:10.1590/S1678-77572006000600010
83. Souza DM, Ricardo LH, Kantoski KZ, Rocha RF (2009) Influence of alcohol consumption on alveolar bone level associated with ligature-induced periodontitis in rats. Braz Oral Res 23:326–332. doi:10.1590/S1806-83242009000300017
84. Dantas AM, Mohn CE, Burdet B, Zorrilla Zubilete M, Mandalunis PM, Elverdin JC, Fernández-Solari J (2012) Ethanol consumption enhances periodontal inflammatory markers in rats. Arch Oral Biol 57:1211–1217. doi:10.1016/j.archoralbio.2012.02.008
85. Irie K, Tomofuji T, Tamaki N, Sanbe T, Ekuni D, Azuma T, Maruyama T, Yamamoto T (2008) Effects of ethanol consumption on periodontal inflammation in rats. J Dent Res 87:456–460. doi:10.1177/154405910808700511
86. Tomofuji T, Sanbe T, Ekuni D, Azuma T, Irie K, Maruyama T, Tamaki N, Yamamoto T (2008) Oxidative damage of rat liver induced by ligature-induced periodontitis and chronic ethanol consumption. Arch Oral Biol 53:1113–1118. doi:10.1016/j.archoralbio.2008.05.015
87. Liberman DN, Pilau RM, Gaio EJ, Orlandini LF, Rösing CK (2011) Low concentration alcohol intake may inhibit spontaneous alveolar bone loss in Wistar rats. Arch Oral Biol 56:109–113. doi:10.1016/j.archoralbio.2010.09.012
88. Campan P, Planchand P, Duran D (1997) Pilot study on n-3 polyunsaturated fatty acids in the treatment of human experimental gingivitis. J Clin Periodontol 24:907–913. doi:10.1111/j.1600-051X.1997.tb01210.x
89. Eberhard J, Heilmann F, Açil Y, Albers KH, Jepsen S (2002) Local application of n-3 or n-6 polyunsaturated fatty acids in the treatment of human experimental gingivitis. J Clin Periodontol 29:364–369. doi:10.1034/j.1600-051X.2002.290413.x
90. Rosenstein ED, Kushnera LJ, Kramera N, Kazandjian G (2003) Pilot study of dietary fatty acid supplementation in the treatment of adult periodontitis. Prostaglandins Leukot Essent Fatty Acids 68:213–218. doi:10.1016/S0952-3278(02)00272-7

91. El-Sharkawy H, Aboelsaad N, Eliwa M, Darweesh M, Alshahat M, Kantarci A, Hasturk H, Van Dyke TE (2010) Adjunctive treatment of chronic periodontitis with daily dietary supplementation with omega-3 fatty acids and low-dose aspirin. J Periodontol 81:1635–1643. doi:10.1902/jop.2010.090628
92. Iwasaki M, Yoshihara A, Moynihan P, Watanabe R, Taylor GW, Miyazaki H (2010) Longitudinal relationship between dietary ω-3 fatty acids and periodontal disease. Nutrition 26:1105–1109. doi:10.1016/j.nut.2009.09.010
93. Naqvi AZ, Buettner C, Phillips RS, Davis RB, Mukamal KJ (2010) n-3 fatty acids and periodontitis in US adults. J Am Diet Assoc 110:1669–1675. doi:10.1016/j.jada.2010.08.009
94. Vardar S, Buduneli E, Türkoğlu O, Berdeli AH, Baylas H, Başkesen A, Atilla G (2004) Therapeutic versus prophylactic plus therapeutic administration of omega-3 fatty acid on endotoxin-induced periodontitis in rats. J Periodontol 75:1640–1646. doi:10.1902/jop.2004.75.12.1640
95. Vardar S, Buduneli E, Baylas H, Berdeli AH, Buduneli N, Atilla G (2005) Individual and combined effects of selective cyclooxygenase-2 inhibitor and omega-3 fatty acid on endotoxin-induced periodontitis in rats. J Periodontol 76:99–106. doi:10.1902/jop.2005.76.1.99
96. Vardar S, Buduneli N, Buduneli E, Kardesxler L, Baylas H, Atilla G, Lappin D, Kinane DF (2006) Dietary supplementation of omega-3 fatty acid and circulating levels of interleukin-1β, osteocalcin, and c-reactive protein in rats. J Periodontol 77:814–820. doi:10.1902/jop.2006.050214
97. Kesavalu L, Vasudevan B, Raghu B, Browning E, Dawson D, Novak JM, Correll MC, Steffen MJ, Bhattacharya A, Fernandes G, Ebersole JL (2006) Omega-3 fatty acid effect on alveolar bone loss in rats. J Dent Res 85:648–652. doi:10.1177/154405910608500713
98. Kesavalu L, Bakthavatchalu V, Rahman MM, Su J, Raghu B, Dawson D, Fernandes G, Ebersole JL (2007) Omega-3 fatty acid regulates inflammatory cytokine/mediator messenger RNA expression in Porphyromonas gingivalis-induced experimental periodontal disease. Oral Microbiol Immunol 22:232–239. doi:10.1111/j.1399-302X.2007.00346.x
99. Bendyk A, Marino V, Zilm PS, Howe P, Bartold PM (2009) Effect of dietary ω-3 polyunsaturated fatty acids on experimental periodontitis in the mouse. J Periodontal Res 44:211–216. doi:10.1111/j.1600-0765.2008.01108.x
100. Rubinoff AB, Latner PA, Pasut CAL (1989) Vitamin C and oral health. J Can Dent Assoc 55:705–707
101. Pussinen PJ, Laatikainen T, Alfthan G, Asikainen S, Jousilahti P (2003) Periodontitis is associated with a low concentration of vitamin C in plasma. Clin Diagn Lab Immunol 10:897–902. doi:10.1128/CDLI.10.5.897-902.2003
102. Amarasena N, Ogawa H, Yoshihara A, Hanada N, Miyazaki H (2005) Serum vitamin C periodontal relationship in community-dwelling elderly Japanese. J Clin Periodontol 32:93–97. doi:10.1111/j.1600-051X.2004.00643.x
103. Amaliya, Timmerman MF, Abbas F, Loos BG, Van der Weijden GA, Van Winkelhoff AJ, Winkel EG, Van der Velden U (2007) Java project on periodontal diseases: the relationship between vitamin C and the severity of periodontitis. J Clin Periodontol 34:299–304. doi:10.1111/j.1600-051X.2007.01053.x
104. Iwasaki M, Manz MC, Taylor GW, Yoshihara A, Miyazaki H (2012) Relations of serum ascorbic acid and α-tocopherol to periodontal disease. J Dent Res 91:167–172. doi:10.1177/0022034511431702
105. Chapple ILC, Milward MR, Dietrich T (2007) The prevalence of inflammatory periodontitis is negatively associated with serum antioxidant concentrations. J Nutr 137:657–664
106. Nishida M, Grossi SG, Dunford RG, Ho AW, Trevisan M, Genko RJ (2000) Dietary vitamin C and the risk for periodontal disease. J Periodontol 71:1215–1223
107. Eklund SA, Burt BA (1994) Risk factors for total tooth loss in the United States; longitudinal analysis of National data. J Public Health Dent 54:5–14
108. Iwasaki M, Moynihan P, Manz MC, Taylor GW, Yoshihara A, Muramatsu K, Watanabe R, Miyazaki H (2013) Dietary antioxidants and periodontal disease in community based older

Japanese: a 2 year follow up study. Public Health Nutr 16:330–338. doi:10.1017/S1368980012002637
109. Diab-Ladki R, Pellat B, Chahine R (2003) Decrease in the total antioxidant activity of saliva in patients with periodontal diseases. Clin Oral Investig 7:103–107. doi:10.1007/s00784-003-0208-5
110. Buduneli N, Kardeşler L, Işik H, Willis CS, Hawkins SI, Kinane DF, Scott DA (2006) Effects of smoking and gingival inflammation on salivary antioxidant capacity. J Clin Periodontol 33:159–164. doi:10.1111/j.1600-051X.2006.00892.x
111. Gümüş P, Buduneli N, Cxetinkalp S, Hawkins SI, Renaud D, Kinane DF, Scott DA (2009) Salivary antioxidants in patients with type 1 or 2 diabetes and inflammatory periodontal disease: a case-control study. J Periodontol 80:1440–1446. doi:10.1902/jop.2009.090159
112. Thomas B, Kumari S, Ramitha K, Kumari MBA (2010) Comparative evaluation of micronutrient status in the serum of diabetes mellitus patients and healthy individuals with periodontitis. J Indian Soc Periodontol 14:46–49. doi:10.4103/0972-124X.65439
113. Kuzmanova D, Jansen IDC, Schoenmaker T, Nazmi K, Teeuw WJ, Bizzarro S, Loos BG, van der Velden U (2012) Vitamin C in plasma and leucocytes in relation to periodontitis. J Clin Periodontol 39:905–912. doi:10.1111/j.1600-051X.2012.01927.x
114. Campbell HG, Cook RP (1941) Treatment of gingivitis with ascorbic acid. Br Med J 1:360–361. doi:10.1136/bmj.1.4183.360
115. Leggott PJ, Robertson PB, Rothman DL, Murray PA, Jacobf RA (1986) The effect of controlled ascorbic acid depletion and supplementation on periodontal health. J Periodontol 57:480–485
116. Thomas AE, Busby MC Jr, Ringsdorf WM Jr, Cheraskin E (1962) Ascorbic acid and alveolar bone loss. Oral Surg Oral Med Oral Pathol 15:555–565. doi:10.1016/0030-4220(62)90173-1
117. Glickman I, Dines MM (1963) Effect of increased ascorbic acid blood levels on the ascorbic acid level in treated and non-treated. J Dent Res 42:1152–1158
118. Coven EM (1965) Effect of prophylasis and vitamin supplementation upon periodontal index on children 60-66. J Periodontol 36:494–500
119. Prentice AM, Lamb WH, Bates CJ (1983) A trial of ascorbic acid and of multivitamin supplementation on the oral health of West African children. Trans R Soc Trop Med Hyg 77:792–795
120. Vogel RI, Lamster IB, Wechsler SA, Macedo B, Hartley LJ, Macedo JA (1986) The effects of megadoses of ascorbic acid on PMN chemotaxis and experimental gingivitis. J Periodontol 57:472–479
121. Abou Sulaiman AEA, Shehadeh RMH (2010) Assessment of total antioxidant capacity and the use of vitamin C in the treatment of non-smokers with chronic periodontitis. J Periodontol 81:1547–1554. doi:10.1902/jop.2010.100173
122. Van der Velden U, Kuzmanova D, Chapple ILC (2011) Micronutritional approaches to periodontal therapy. J Clin Periodontol 38:142–158. doi:10.1111/j.1600-051X.2010.01663.x
123. Shiga M, Kapila YL, Zhang Q, Hayami T, Kapila S (2003) Ascorbic acid induces collagenase-1 in human periodontal ligament cells but not in MC3T3-E1 osteoblast-like cells: potential association between collagenase expression and changes in alkaline phosphatase phenotype. J Bone Miner Res 18:67–77
124. Tsutsumi K, Fujikawa H, Kajikawa T, Takedachi M, Yamamoto T, Murakami S (2012) Effects of L-ascorbic acid 2-phosphate magnesium salt on the properties of human gingival fibroblasts. J Periodontal Res 47:263–271. doi:10.1111/j.1600-0765.2011.01430.x
125. Sanbe T, Tomofuji T, Ekuni D, Azuma T, Tamaki N, Yamamoto T (2007) Oral administration of vitamin C prevents alveolar bone resorption induced by high dietary cholesterol in rats. J Periodontol 78:2165–2170
126. Staudte H, Güntsch A, Völpel A, Sigusch BW (2010) Vitamin C attenuates the cytotoxic effects of Porphyromonas gingivalis on human gingival fibroblasts. Arch Oral Biol 55:40–45
127. Chapple ILC, Matthews JB, Wright HJ, Scott AE, Griffiths HR, Grant MM (2012) Ascorbate and α-tocopherol differentially modulate reactive oxygen species generation by

neutrophils in response to FcγR and TLR agonists. Innate Immun 0:1–8. doi:10.1177/1753425912455207
128. Tomofuji T, Ekuni D, Sanbe T, Irie K, Azuma T, Maruyama T, Tamaki N, Murakami J, Kokeguchi S, Yamamoto T (2009) Effects of vitamin C intake on gingival oxidative stress in rat periodontitis. Free Radic Biol Med 46:163–168
129. Akman S, Canakci V, Kara A, Tozoglu U, Arabaci T (2012) Therapeutic effects of alpha-lipoic acid and vitamin C on alveolar bone resorption after experimental periodontitis in rats. J Periodontol. doi:10.1902/jop.2012.120252
130. Neiva RF, Al-Shammari K, Nociti FH Jr, Soehren S, Wang H (2005) Effects of vitamin-B complex supplementation on periodontal wound healing. J Periodontol 76:1084–1091
131. Albina JE (1994) Nutrition and wound healing. J Parenter Enteral Nutr 18:367–376. doi:10.1177/014860719401800417
132. Yu YH, Kuo HK, Lai YL (2007) The association between serum folate levels and periodontal disease in older adults: data from the National Health and Nutrition Examination Survey 2001/02. J Am Geriatr Soc 55:108–113. doi:10.1111/j.1532-5415.2006.01020.x
133. Esaki M, Morita M, Akhter R, Akino K, Honda O (2010) Relationship between folic acid intake and gingival health in non-smoking adults in Japan. Oral Dis 16:96–101. doi:10.1111/j.1601-0825.2009.01619.x
134. Yoshihara A, Watanabe R, Nishimuta M, Hanada N, Miyazaki H (2005) The relationship between dietary intake and the number of teeth in elderly Japanese subjects. Gerodontology 22:211–218. doi:10.1111/j.1741-2358.2005.00083.x
135. Erdemir EO, Bergstrom J (2006) Relationship between smoking and folic acid, vitamin B12 and some haematological variables in patients with chronic periodontal disease. J Clin Periodontol 33:878–884. doi:10.1111/j.1600-051X.2006.01007.x
136. Erdemir EO, Bergstrom J (2007) Effect of smoking on folic acid and vitamin B12 after nonsurgical periodontal intervention. J Clin Periodontol 34:1074–1081. doi:10.1111/j.1600-051X.2007.01154.x
137. da Costa M, Rothenberg S (1974) Appearance of folate binder in leukocytes and serum of women who are pregnant or taking oral contraceptives. J Lab Clin Med 83:207–214
138. Vogel RI, Fink RA, Schneider LC, Frank O, Baker H (1976) The effect of folic acid on gingival health. J Periodontol 47:667–668
139. Vogel RI, Fink RA, Frank O, Baker H (1978) The effect of topical application of folic acid on gingival health. J Oral Med 33:20–22
140. Pack AR, Thomson ME (1980) Effects of topical and systemic folic acid supplementation on gingivitis in pregnancy. J Clin Periodontol 7:402–414
141. Thomson ME, Pack AR (1982) Effects of extended systemic and topical folate supplementation on gingivitis of pregnancy. J Clin Periodontol 9:275–280
142. Pack AR (1984) Folate mouthwash: effects on established gingivitis in periodontal patients. J Clin Periodontol 11:619–628
143. Booth SL (2009) Roles for vitamin K beyond coagulation. Annu Rev Nutr 29:89–110. doi:10.1146/annurev-nutr-080508-141217
144. Rawlinson A, Walsh TF, Lee A, Hodges SJ (1998) Phyltoquinone in gingival crevicular fluid in adult periodontitis. J Clin Periodontol 25:662–665
145. Hojo K, Nagaoka S, Murata S, Taketomo N, Ohshima T, Maeda N (2007) Reduction of vitamin K concentration by salivary Bifidobacterium strains and their possible nutritional competition with Porphyromonas gingivalis. J Appl Microbiol 103:1969–1974. doi:10.1111/j.1365-2672.2007.03436.x
146. D'Ambrosio DN, Clugston RD, Blaner WS (2011) Vitamin A metabolism: an update. Nutrients 3:63–103. doi:10.3390/nu3010063
147. Freeland JH, Cousins RJ, Schwartz R (1976) Relationship of mineral status and intake to periodontal disease. Am J Clin Nutr 29:745–749
148. Linden GJ, McClean KM, Woodside JV, Patterson CC, Evans A, Young IS, Kee F (2009) Antioxidants and periodontitis in 60–70-year-old men. J Clin Periodontol 36:843–849. doi:10.1111/j.1600-051X.2009.01468.x

149. Wood N, Johnson RB (2004) The relationship between tomato intake and congestive heart failure riskinperiodontitissubjects.JClinPeriodontol31:574–580.doi:10.1111/j.1600-051X.2004.00531.x
150. Chandra RV, Prabhuji MLV, Roopa DA, Ravirajan S, Kishore HC (2007) Efficacy of lycopene in the treatment of gingivitis: a randomised placebo-controlled clinical Trial. Oral Health Prev Dent 5:327–336
151. Cheraskin E, Ringsdorf WM (1970) Relationship of reported oral symptoms and signs versus daily vitamin E consumption. Oral Surg Oral Med Oral Pathol 29:361–364. doi:10.1016/0030-4220(70)90134-9
152. Battino M, Ferreiro M-S, Bompadre S, Leone L, Mosca F, Bullon P (2001) Elevated hydroperoxide levels and antioxidants patterns in Papillon-Lefèvre syndrome. J Periodontol 72:1760–1766. doi:10.1902/jop.2001.72.12.1760
153. Hewitt C, McCormick D, Linden G, Turk D, Stern I, Wallace I, Southern L, Zhang L, Howard R, Bullon P, Wong M, Widmer R, Gaffar KA, Awawdeh L, Briggs J, Yaghmai R, Jabs EW, Hoeger P, Bleck O, Rüdiger SG, Petersilka G, Battino M, Brett P, Hattab F, Al-Hamed M, Sloan P, Toomes C, Dixon M, James J, Read AP, Thakker N (2004) The role of cathepsin C in Papillon-Lefèvre syndrome, prepubertal periodontitis, and aggressive periodontitis. Hum Mutat 23:222–228
154. Battino M, Ferreiro MS, Quiles JL, Bompadre S, Leone L, Bullon P (2003) Alterations in the oxidation products, antioxidant markers, antioxidant capacity and lipid patterns in plasma of patients affected by Papillon-Lefèvre syndrome. Free Radic Res 37:603–609
155. Slade EW Jr, Bartuska D, Rose LF, Cohen DW (1976) Vitamin E and periodontal disease. J Periodontol 32:1072–1077
156. Goodson JM, Bowles D (1973) The effect of α-tocopherol on sulcus fluid flow in periodontal disease. J Dent Res 52:217 (abstract)
157. Cerna H, Fiala B, Fingerov H, Pohanka J, Szwarcova E (1984) Contribution to indication of total therapy with vitamin E in chronic periodontal disease. Acta Univ Palacki Olomuc Fac Med 107:167–170
158. Cohen RE, Ciancio SG, Mather ML, Curro FA (1991) Effect of vitamin E gel, placebo gel, and chlorhexidine on periodontal diseases. Clin Prev Dent 13:20–24
159. Sheikhi M, Bouhafs RKL, Hammarström KJ, Jarstrand C (2001) Lipid peroxidation caused by oxygen radicals from Fusobacterium-stimulated neutrophils as a possible model for the emergence of periodontitis. Oral Dis 7:41–46
160. Cohen ME, Meyer DM (1993) Effect of dietary vitamin E supplementation and rotational stress on alveolar bone loss in rice rats. Arch Oral Biol 38:601–606. doi:10.1016/0003-9969(93)90126-7
161. Carvalho RS, de Souza CM, Neves JCS, Holanda-Pinto SA, Pinto LMS, Brito GAC, de Andrade GM (2013) Vitamin E does not prevent bone loss and induced anxiety in rats with ligature-induced periodontitis. Arch Oral Biol 58:50–58. doi:10.1016/jarchoralbio.2012.04.020
162. Liu K, Meng H, Tang X, Xu L, Zhang L, Chen Z, Shi D, Feng X, Lu R (2009) Elevated plasma calcifediol is associated with aggressive periodontitis. J Periodontol 80:1114–1120. doi:10.1902/jop.2009.080675
163. Millen AE, Hovey KM, LaMonte MJ, Swanson M, Andrews CA, Kluczynski MA, Genco RJ, Wactawski-Wende J (2012) Plasma 25-hydroxyvitamin D concentrations and periodontal disease in postmenopausal women. J Periodontol. doi:10.1902/jop.2012.120445
164. Kyeyune-Nyombi E, Lau KH, Baylink DJ, Strong DD (1991) 1,25-Dihydroxyvitamin D3 stimulates both alkaline phosphatase gene transcription and mRNA stability in human bone cells. Arch Biochem Biophys 291:316–325
165. Ryhänen S, Mahonen A, Jääskeläinen T, Mäenpää PH (1996) Synthetic 20-epi analogs of calcitriol are potent inducers of target-gene activation in osteoblastic cells. Eur J Biochem 238:97–103
166. Christakos S, Dhawan P, Liu Y, Peng X, Porta A (2003) New insights into the mechanisms of vitamin D action. J Cell Biochem 88:695–705. doi:10.1002/jcb.10423
167. Takahashi E, Nakagawa K, Suhara Y et al (2006) Biological activities of 2alpha-substituted analogues of 1alpha, 25-dihydroxyvitamin D3 in transcriptional regulation and human pro-

myelocytic leukemia (HL-60) cell proliferation and differentiation. Biol Pharm Bull 29:2246–2250
168. Wang TT, Nestel FP, Bourdeau V, Nagai Y, Wang Q, Liao J, Tavera-Mendoza L, Lin R, Hanrahan JW, Mader S, White JH (2004) Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. J Immunol 173:2909–2912
169. Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR, Ochoa MT, Schauber J, Wu K, Meinken C, Kamen DL, Wagner M, Bals R, Steinmeyer A, Zügel U, Gallo RL, Eisenberg D, Hewison M, Hollis BW, Adams JS, Bloom BR, Modlin RL (2006) Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. Science 311:1770–1773
170. Mora JR, Iwata M, von Andrian UH (2008) Vitamin effects on the immune system: vitamins A and D take centre stage. Nat Rev Immunol 8:685–698. doi:10.1038/nri2378
171. Bikle DD (2009) Vitamin D and immune function: understanding common pathways. Curr Osteoporos Rep 7:58–63. doi:10.1007/s11914-009-0011-6
172. Kamen DL, Tangpricha V (2010) Vitamin D and molecular actions on the immune system: modulation of innate and autoimmunity. J Mol Med 88:441–450
173. Rigby WF, Stacy T, Fanger MW (1984) Inhibition of T lymphocyte mitogenesis by 1,25-dihydroxyvitamin D3 (calcitriol). J Clin Invest 74:1451–1455
174. Chen S, Sims GP, Chen XX, Gu YY, Chen S, Lipsky PE (2007) Modulatory effects of 1,25-dihydroxyvitamin D3 on human B cell differentiation. J Immunol 179:1634–1647. doi:10.1016/0003-9969(93)90126-7
175. Daniel C, Sartory NA, Zahn N, Radeke HH, Stein JM (2008) Immune modulatory treatment of trinitrobenzene sulfonic acid colitis with calcitriol is associated with a change of a T helper (Th) 1/Th17 to a Th2 and regulatory T cell profile. J Pharmacol Exp Ther 324:23–33. doi:10.1124/jpet.107.127209
176. Bhalla AK, Amento EP, Krane SM (1986) Differential effects of 1, 25-dihydroxyvitamin D3 on human lymphocytes and monocyte/macrophages: inhibition of interleukin-2 and augmentation of interleukin-1 production. Cell Immunol 98:311–322. doi:10.1016/0008-8749(86)90291-1
177. Boonstra A, Barrat FJ, Crain C, Heath VL, Savelkoul HF, O'Garra A (2001) 1α,25-Dihydroxyvitamin d3 has a direct effect on naive CD4(+) T cells to enhance the development of Th2 cells. J Immunol 167:4974–4980
178. Dietrich T, Kaumudi J, Joshipura KJ, Dawson-Hughes B, Bischoff-Ferrari HA (2004) Association between serum concentrations of 25-hydroxyvitamin D3 and periodontal disease in the US population. Am J Clin Nutr 80:108–113
179. US Department of Health and Human Services, National Center for Health Statistics (1996) NHANES III reference manuals and reports (CDROM). Centers for Disease Control and Prevention, Hyattsville
180. Drury TF, Winn DM, Snowden CB, Kingman A, Kleinman DV, Lewis B (1996) An overview of the oral health component of the 1988-1991 National Health and Nutrition Examination Survey (NHANES III-Phase 1). J Dent Res 75:620–630
181. Tomar SL, Asma S (2000) Smoking-attributable periodontitis in the United States: findings from NHANES III. National Health and Nutrition Examination Survey. J Periodontol 71:743–751. doi:10.1902/jop.2000.71.5.743
182. Amarasena N, Yoshihara A, Hirotomi T, Takano N, Miyazaki H (2008) Association between serum calcium and periodontal disease progression in non-institutionalized elderly. Gerodontology 25:245–250. doi:10.1111/j.1741-2358.2007.00211.x
183. Nishida M, Grossi SG, Dunford RG, Ho AW, Trevisan M, Genco RJ (2000) Calcium and the risk for periodontal disease. J Periodontol 71:1057–1066. doi:10.1902/jop.2000.71.7.1057
184. Takahashi O, Yoshihara A, Nakamura K, Miyazakia H (2012) Association between periodontitis and systemic bone mineral density in Japanese community-dwelling postmenopausal women. J Dent 40:304–311. doi:10.1016/j.jdent.2012.01.005
185. Famili P, Cauley J, Suzuki JB, Weyant R (2005) Longitudinal study of periodontal disease and edentulism with rates of bone loss in older women. J Periodontol 76:11–15. doi:10.1902/jop.2005.76.1.11

186. Adegboye ARA, Fiehn N-E, Twetman S, Christensen LB, Heitmann BL (2010) Low calcium intake is related to increased risk of tooth loss in men. J Nutr 140:1864–1868. doi:10.3945/jn.109.117770
187. Adegboye ARA, Christensen LB, Holm-Pedersen P, Avlund K, Boucher BJ, Heitmann BL (2012) Intake of dairy products in relation to periodontitis in older Danish adults. Nutrients 4:1219–1229. doi:10.3390/nu4091219
188. Al-Zahrani MS (2006) Increased intake of dairy products is related to lower periodontitis prevalence. J Periodontol 77:289–294. doi:10.1902/jop.2006.050082
189. Miley DD, Garcia MN, Hildebolt CF, Shannon WD, Couture RA, Anderson Spearie CL, Dixon DA, Langenwalter EM, Mueller C, Civitelli R (2009) Cross-sectional study of vitamin D and calcium supplementation effects on chronic periodontitis. J Periodontol 80:1433–1439. doi:10.1902/jop.2009.090077
190. Wical KE, Brussee P (1979) Effects of a calcium and vitamin D supplement on alveolar ridge resorption in immediate denture patients. J Prosthet Dent 41:4–11. doi:10.1016/0022-3913(79)90347-0
191. Shoji K, Ohtsuka-Isoya M, Horiuchi H, Shinoda H (2000) Bone mineral density of alveolar bone in rats during pregnancy and lactation. J Periodontol 71:1073–1078. doi:10.1902/jop.2000.71.7.1073
192. Shoji K, Ohtsuka-Isoya M, Shimauchi H, Shinoda H (2007) Effects of lactation on alveolar bone loss in experimental periodontitis. J Periodontol 78:152–156. doi:10.1902/jop.2007.060037
193. Messer HH, Goebel NK, Wilcox L (1981) A comparison of bone loss from different skeletal sites during acute calcium deficiency in mice. Arch Oral Biol 26:1001–1004. doi:10.1016/0003-9969(81)90110-2
194. Wang PL, Shirasu S, Shinohara M, Murakawa N, Endo M, Sakata S, Okamura M, Daito M, Ohura K (1998) Salivary amylase activity of rats fed a low calcium diet. Jpn J Pharmacol 78:279–283. doi:10.1254/jjp.78.279
195. Laires MJ, Monteiro CP, Bicho M (2004) Role of cellular magnesium in health and human disease. Front Biosci 1:262–276. doi:10.2741/1223
196. Yoshihara A, Iwasaki M, Miyazaki H (2011) Mineral content of calcium and magnesium in the serum and longitudinal periodontal progression in Japanese elderly smokers. J Clin Periodontol 38:992–997. doi:10.1111/j.1600-051X.2011.01769.x
197. Meisel P, Schwahn C, Luedemann J, John U, Kroemer HK, Kocher T (2005) Magnesium deficiency is associated with periodontal disease. J Dent Res 84:937–941. doi:10.1177/154405910508401012
198. Erdemir EO, Erdemir A (2006) The detection of salivary minerals in smokers and non-smokers with chronic periodontitis by the inductively coupled plasma-atomic emission spectrophotometry technique. J Periodontol 77:990–995. doi:10.1902/jop.2006.050202
199. Cutler CW, Kalmar JR, Arnold RR (1991) Antibody-dependent alternate pathway of complement activation in opsonophagocytosis of Porphyromonas gingivalis. Infect Immun 59:2105–2109
200. Barnard JP, Stinson AW (1999) Influence of environmental conditions on hydrogen peroxide formation by Streptococcus gordonii. Infect Immun 67:6558–6564
201. Strålfors A, Thilander H, Bergenholt A (1967) Simultaneous inhibition of caries and periodontal disease in hamsters by disinfection, tooth-brushing or phosphate addition. Arch Oral Biol 12:1367–1371. doi:10.1016/9969(67)90175-6
202. Yamaoka M, Uematsu T, Shiba T, Matsuura T, Ono Y, Ishizuka M, Naramoto H, Takahashi M, Sugiura-Tomita M, Iguchi K, Yamashita S, Furusawa K (2008) Effect of inorganic polyphosphate in periodontitis in the elderly. Gerodontology 25:10–17. doi:10.1111/j.1741-2358.2007.00185.x
203. Takami Y, Nakagaki H, Morita I, Tsuboi S, Takami S, Suzuki N, Niwa H, Ogura Y (2003) Blood test values and community periodontal index scores in medical checkup recipients. J Periodontol 74:1778–1784. doi:10.1902/jop.2003.74.12.1778

204. Craig RG, Yip JK, So MK, Boylan RJ, Socransky SS, Haffajee AD (2003) Relationship of destructive periodontal disease to the acute-phase response. J Periodontol 74:1007–1016. doi:10.1902/jop.2003.74.7.1007
205. Hatipoglu H, Hatipoglu MG, Cagirankaya LB, Caglayan F (2012) Severe periodontal destruction in a patient with advanced anemia: a case report. Eur J Dent 6:95–100
206. Hutter JW, Van der Velden U, Varoufaki A, Huffels RAM, Hoek FJ, Loos BG (2001) Lower numbers of erythrocytes and lower levels of hemoglobin in periodontitis patients compared to control subjects. J Clin Periodontol 28:930–936. doi:10.1034/j.1600-051x.2001.028010930.x
207. Gokhale SR, Sumanth S, Padhye AM (2010) Evaluation of blood parameters in patients with chronic periodontitis for signs of anemia. J Periodontol 81:1202–1206. doi:10.1902/jop.2010.100079
208. Prakash S, Dhingra K, Priya S (2012) Similar hematological and biochemical parameters among periodontitis and control group subjects. Eur J Dent 6:287–294
209. Lainson PA, Brady PP, Fraleigh CM (1968) Anemia, a systemic cause of periodontal disease? J Periodontol 39:35–38
210. Chawla TN, Kapoor KK, Teotia SP, Singh NK (1971) Anemia and periodontal disease – a correlative study. J Indian Dent Assoc 43:67–78
211. Siegel EH (1945) Total erythrocyte, leukocyte and differential white cell counts of blood in chronic periodontal disease. J Dent Res 24:270–271
212. Lee GR (1983) The anemia of chronic disease. Semin Hematol 20:61–80
213. Johansson I, Fagernäs C (1994) Effect of iron-deficiency anemia on saliva secretion rate and composition in the rat. Arch Oral Biol 39:51–56. doi:10.1016/0003-9969(94)90034-5
214. Tovaru S, Parlatescu I, Dumitriu AS, Bucur A, Kaplan I (2010) Oral complications associated with D-penicillamine treatment for Wilson disease: a clinicopathologic report. J Periodontol 81:1231–1236. doi:10.1902/jop.2010.090736
215. Spacciapoli P, Buxton D, Rothstein D, Friden P (2001) Antimicrobial activity of silver nitrate against periodontal pathogens. J Periodontal Res 36:108–113. doi:10.1034/j.1600-0765.2001.360207.x
216. Tamura M, Ochiai K (2009) Zinc and copper play a role in coaggregation inhibiting action of Porphyromonas gingivalis. Oral Microbiol Immunol 24:56–63. doi:10.1111/j.1399-302X.2008.00476.x
217. Olczak T, Maszczak-Seneczko D, Smalley JW, Olczak M (2012) Gallium(III), cobalt(III) and copper(II) protoporphyrin IX exhibit antimicrobial activity against *Porphyromonas gingivalis* by reducing planktonic and biofilm growth and invasion of host epithelial cells. Arch Microbiol 194:719–724. doi:10.1007/s00203-012-0804-3
218. De Souza AP, Gerlach RF, Line SRP (2000) Inhibition of human gingival gelatinases (MMP-2 and MMP-9) by metal salts. Dent Mater 16:103–108. doi:10.1016/S0109-5641(99)00084-6
219. Üçkardeş Y, Tekçiçek M, Özmert EN, Yurdakök K (2009) The effect of systemic zinc supplementation on oral health in low socioeconomic level children. Turk J Pediatr 51:424–428
220. Johnson DA, Alvares OF (1984) Zinc deficiency-induced changes in rat parotid salivary proteins. J Nutr 114:1955–1964
221. Orbak R, Kara C, Özbek E, Tezel A, Demir T (2007) Effects of zinc deficiency on oral and periodontal diseases in rats. J Periodontal Res 42:138–143. doi:10.1111/j.1600-0765.2006.00939.x
222. Di Paola R, Emanuela Mazzon E, Rotondo R, Dattola F, Britti D, De Majo M, Genovese T, Cuzzocrea S (2005) Reduced development of experimental periodontitis by treatment with M40403, a superoxide dismutase mimetic. Eur J Pharmacol 516:151–157. doi:10.1016/j.ephar.2005.04.039

Chapter 18
Effects of Antioxidants on Periodontal Disease

Takaaki Tomofuji, Daisuke Ekuni, Shinsuke Mizutani, and Manabu Morita

18.1 Introduction

Reactive oxygen/nitrogen species (ROS/RNS) exert deleterious effects by oxidizing biologically essential molecules such as lipids, proteins, carbohydrates, and DNA [1]. ROS/RNS induce oxidative damage in cellular membranes, tissues, and enzymes, which may eventually contribute to disorders and diseases such as periodontal disease, atherosclerosis, neurological diseases, and cancer [2]. On the other hand, ROS/RNS may act also as a cellular signaling messenger in physiological settings with important regulatory functions [3–9]. Hydrogen peroxide is considered to be the most important signaling messenger based on the specificity of its production, reaction, and removal [8]. ROS/RNS have both harmful and beneficial effects, as ROS/RNS are produced in a tightly controlled manner as regulators of gene expression, activators of receptors and nuclear transcription factors, and inducers of adaptive responses [2].

Mammals have evolved an elaborate defense network against oxidative stress, in which multiple antioxidant compounds and enzymes exert their respective roles [10, 11]. Humans produce numerous antioxidants, such as superoxide dismutases, catalases, reduced glutathione, and peroxiredoxins [1], and obtain some other actual (vitamins C and E) or putative (flavonoids and carotenoids) antioxidants from the diet [11]. One of the biggest research topics at present is that peroxiredoxins appear to be the most important scavengers of hydroxyl peroxide in vivo [12–15]. Among these, radical scavenging antioxidants, referred to simply antioxidants, play their roles by scavenging reactive free radicals to protect biologically essential molecules from oxidative modification [2]. The beneficial effects of these antioxidants have been

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supported by epidemiological studies [16]. However, many randomized, cross-over, intervention studies and meta-analyses on the effects of antioxidants on chronic diseases have given contradictory results [17]. This discrepancy may be ascribed to the complex effects of oxidative stress on pathogenesis, the role of antioxidants in human health, individuality, and aging [11, 18].

Periodontal disease is a chronic condition. The reported prevalence varies between 10 and 60 % in adults, depending on diagnostic criteria [19–21]. Periodontal disease is initiated by overgrowth of certain bacterial species, with a majority of Gram-negative, anaerobic bacteria growing in subgingival sites, although the mechanisms of disease progression are complex. The development of disease depends on the interaction between bacterial products and host response [22]. Oxidative stress is also involved in the initiation and progression of periodontal disease. Thus, antioxidant effects on periodontal disease are a notable topic in periodontal research [23–29]. Many in vitro and animal model studies have studied the effects of antioxidants and antioxidant potential on periodontal disease [23–63]. However, there have been few randomized controlled trials (RCTs) on the effects in humans [64–66], and there have been numerous contradictory results. Here, we discuss the effects of antioxidants on periodontal disease, limitations and possible future trials. With regard to vitamins C and E, they are discussed in detail in Chap. 19.

18.2 Antioxidants

Halliwell and Gutteridge defined an antioxidant as "any substance that delays, prevents or removes oxidative damage in a target molecule" [1]. Antioxidants can be complex molecules such as superoxide dismutases, catalases, and peroxiredoxins, or simpler molecules such as uric acid and glutathione [18].

In the late 1950s, it was proposed that aging is a result of progressive changes caused by cumulative free radical damage, and it is hypothesized that antioxidant molecules slow down the aging process and prolong lifespan [18]. In several cases in rodents, a statistically significant effect of antioxidants on lifespan has been reported [67, 68]. The supplement and nutraceutical industries have rapidly taken these concepts on board [18]. In recent studies, many compounds and plant extracts have been found to have considerable antioxidant activity in vitro, as demonstrated by assays such as oxygen radical absorbance capacity, 1,1-diphenyl-2-picrylhydrazyl, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonate), and ferric reducing antioxidant power [1, 10, 18, 69, 70]. Foods and beverages rich in antioxidants have often been associated with decreased risk of developing several diseases [71–80].

18.2.1 Role of Antioxidants

The role of antioxidants is inhibition of free radical mediated oxidation of biological molecules in the pathogenesis of diseases. For example, flavonoids have powerful

antioxidant activities in vitro, being able to scavenge a wide range of reactive oxygen, nitrogen and chlorine species, such as superoxide, hydroxyl radical, peroxyl radicals, hypochlorous acid, and peroxynitrous acid [71]. They can also chelate metal ions, often decreasing metal ion prooxidant activity [81–87].

In addition to these direct antioxidant effects, many antioxidant substances act as a cellular mediators to enhance the expression of antioxidant and detoxifying enzymes via the Nrf2–Keap1 system [88, 89]. The physiological importance of this system has been demonstrated by the experimental evidence that Nrf2 knockout mice are more prone to oxidative stress [90].

18.2.2 Flavonoids

Polyphenolic flavonoids are scavenging antioxidants, similarly to vitamins C and E [91]. These are absorbed following dietary intake of, in particular, vegetables, red wine, cocoa, and tea [92–94]. There are over 4,000 known flavonoids [92], including catechin, resveratrol, curcumin, quercetin, and genistein.

Cocoa has become a material of interest as a therapeutic natural product due to its flavonoid content [94]. Cocoa is a product derived from the beans of the *Theobroma cacao* plant. It has been consumed since 600 BC and its use spread rapidly to Western Europe in sixteen century [95]. Cocoa powder is a rich source of fiber (26–40 %), proteins (15–20 %), carbohydrates (about 15 %), and lipids (10–24 %), and it contains minerals (for example, calcium, magnesium, potassium) and vitamins (A, E, B, and folic acid) [94]. Cocoa powder contains up to 70 mg polyphenols/g (expressed as catechin) [96] and provides more phenolic antioxidants than beverages and fruits such as tea and blueberries [97, 98]. Cocoa contains the monomers (−)-epicatechin and catechin, and various polymers derived from these monomers, known as procyanidins, which are the major flavonoids in cocoa and chocolate products [99]. Procyanidins scavenge radicals, such as peroxynitrites [100, 101]. Quercetin may also contribute to cocoa's antioxidant activity by neutralizing radicals and chelating metal ions [102, 103]. Methylxanthines can also contribute to its antioxidant properties [104]. Several in vitro studies have confirmed the antioxidant capacity of cocoa flavonoids and their metabolites [105–109]. In vivo studies use whole cocoa powder, as it is difficult to isolate large amounts of cocoa polyphenols [94]. The intake of cocoa increases total antioxidant capacity and decreases lipid oxidation products in rat plasma and human plasma from healthy subjects [110–112]. A cocoa-enriched diet increases the antioxidant capacity of rat tissues to varying degrees, with the activity in thymus > spleen > liver [113]. Cocoa improves antioxidant defenses in experimentally induced oxidative stress in rats and humans [114, 115]. Several reviews on the impact of cocoa on the cardiovascular system or the beneficial effects of dietary flavonoids on health have compiled interventional studies in human subjects [116–120]. The various manners and rates in which flavonoids are absorbed have also been reviewed [94, 121]. Data on the distribution of flavonoid metabolites in tissues after cocoa intake are limited [94]. Absorbed flavonoids are

widely distributed and can be detected in numerous organs, including lymphoid tissues, at a concentration of nmol/g tissue [122, 123].

Green tea (from the plant *Camellia sinensis*) has important biological and pharmacological properties [124]. Green tea has been used for centuries in China, Japan, and Thailand as a traditional medicine with various applications [125]. The beneficial effects of green tea have been attributed to the presence of phenolic compounds that are powerful antioxidants and free iron scavengers [126]. In addition to polyphenols, green tea contains additional antioxidants such as carotenoids, tocopherols (vitamin E derivatives), and vitamin C, and also contains minerals that function as co-factors in antioxidant enzymes: zinc, selenium, and manganese [126]. The main polyphenols in green tea are catechins. Catechins are effective in neutralizing several types of radical such as peroxyl, peroxynitrite, superoxide, and 1,1-diphenyl-2-picryl-hydrazyl [127, 128]. The four main catechins are: epigallocatechin 3 gallate (EGCG), which constitutes about 59 % of total catechins; epigallocatechin (EGC) which constitutes about 19 %; epicatechin 3 gallate (ECG), which constitutes about 13.6 %; and epicatechin (EC), which constitutes about 6.4 % [129]. EGCG is the most abundant and potent. It is widely believed that green tea has health benefits; various types of cancer chemoprevention, weight loss, and protective effects against cardiovascular diseases (coronary heart disease and stroke) and neurodegenerative diseases (Alzheimer's disease and Parkinson's disease) [124, 125, 130–136].

Curcumin has a wide range of pharmacological activities, including antitumor, antioxidant, antiamyloid, and anti-inflammatory properties [137]. Curcumin (diferuloylmethane) is a major chemical component of the Asian spice known as turmeric (*Curcuma longa* Linn.) [138]. Bioavailability studies in laboratory rodents (mouse, rat), as well as in humans, have characterized the rate and concentration at which curcumin is absorbed, appears in the plasma, and reaches its target site [139–152]. The oral bioavailability of curcumin is low due to a relatively low intestinal (small intestines) absorption and rapid metabolism in the liver, followed by elimination through the gall bladder [142, 153, 154]. Over the past decade, several studies have substantiated the potential prophylactic or therapeutic value of curcumin. The presence of phenolic, β-diketone, and methoxy groups contribute to the free-radical-scavenging activity of curcumin [139]. The radical-scavenging properties of curcumin are mainly derived from its phenolic structure [155, 156]. Curcumin may induce endogenous antioxidant defense mechanisms (e.g., through gene regulatory mechanisms) and redox-regulated transcription factor Nrf2 [nuclear factor (erythroid-derived 2)-like 2] plays a key role in this process [139]. Nrf2 is a transcription factor that regulates the gene expression of antioxidant and phase II enzymes [139]. In response to an inducer, such as curcumin, the binding between Keap1 and Nrf2 is disrupted, and the reactive cysteine in Keap1 is altered either by oxidation or covalent modification [157, 158]. Furthermore, no studies have reported any toxicity associated with the use of curcumin in either animals or humans [159].

Resveratrol (*trans*-3,4′,5-trihydroxystilbene, $C_{14}H_{12}O_3$) is a plant-derived polyphenolic phytoalexin produced by the enzyme stilbene synthase in response to

infection by the pathogen *Botrytis cinerea* and to a variety of stress conditions [136]. Resveratrol is found in the roots of Japanese Knotweed (*Poligonum cuspidatum*), which has been used in traditional Asian herb medicine for hundreds of years in the treatment of inflammation [160]. Grape is also a source of resveratrol, and grapevine (*Vitis vinifera*) and wine are now considered to be a key source for health-promoting secondary metabolites, particularly antioxidant polyphenols such as resveratrol [161–163]. It has broad-spectrum beneficial health effects including anti-infective, antioxidant, cardioprotective functions, and cancer chemopreventive properties [164–173]. There is a lack of information on resveratrol bioavailability in vivo, in particular following oral administration [174]. The concentrations of resveratrol detected in tissue or at the cellular sites of action do not appear to be sufficiently adequate to demonstrate efficacy in human [175, 176].

Quercetin (3,3′,4′,5,7-pentahydroxyl-flavone) is a ubiquitous molecule and a polyphenolic antioxidant found in various fruits and vegetables, and is highly concentrated in onions, broccoli, apples, grapes (red wine), and soybeans [136]. Quercetin has a broad spectrum of beneficial properties, including anti-inflammatory effects, benefits for human endurance exercise capacity, atherosclerosis, thrombosis, hypertension, arrhythmia, and modulation of cancer-related multidrug resistance [177–183]. Quercetin is metabolized by the intestinal microflora to its corresponding hydroxyphenylacetic acids [184]. The magnitude of this process in relation to deglycosidation/metabolization is currently unknown [185]. Free plasma quercetin is detected at a concentration of 12 μM in humans after intravenous administration of 100 mg of quercetin [186]. In another case, a meal rich in plants (with 87 mg of quercetin) yielded mean plasma concentrations of 373 nM at 3 h post-ingestion [187]. These results suggest that one acute administration of quercetin does not reach the effective threshold of pharmacological plasma concentration [185]. However, chronic administration of quercetin represents a different situation [188–190] and in humans (50–150 mg orally for 2 weeks) significantly increases plasma concentrations of quercetin [187].

Isoflavones in soy-rich foods have contributed to relatively lower rates of prostate and breast cancers in Asian countries such as China and Japan than in the Western population [191]. Genistein (4,5,7-trihydroxyisoflavone) has been identified as the predominant isoflavone in foods enriched with soybean and in other legumes, including peas, lentils, and beans [192]. Many important biological effects of genistein consumption have been elucidated with respect to its anti-cancer properties [192]. Genistein has other health benefits, such as lowering the incidence of cardiovascular diseases, prevention of osteoporosis, attenuation of post-menopausal problems, and decreasing body mass and fat tissue [192–194]. After intake and ingestion, genistein along with other isoflavones is conjugated with glycoside and metabolized by enzymes in the intestine [192]. Genistein is metabolized to dihydrogenistein and 6′-hydroxy-*O*-desmethylangolensin. Genistein and their metabolites have been detected in plasma, prostatic fluid, breast aspartate and cyst fluid, urine, and feces [195–198]. Plasma levels of genistein in people consuming a soy-rich diet were 1–5 μM after metabolism and excretion [195].

18.2.3 Asian Traditional Medicines

Traditional oriental herbal remedies, particularly those developed as sophisticated formulae, such as traditional Chinese and Japanese (Kampo) medicines, would be an interesting target of study for their preventive and therapeutic effects on oxidative stress-related disorders [199]. The treatment rule is strictly defined in traditional medicine theory [200]. The concept includes the hypothesis that there is certain common factor involved in different pathological conditions, as well as some common properties present among different prescriptions [199]. This common element is oxidative stress, and thus the common characteristic associated with many prescriptions is assumed to be antioxidant activity [199]. In contrast to Western medicines, therapeutic strategies using Kampo are based on the recovery of distorted physiological balance in patients by stimulating their inherent recovery potential from the disease condition using characteristic herbal combinations [199]. Although the mechanisms remain unclear, Asian traditional medicines have some potential in the process of oxidative tissue damages including ROS generation. For example, there have been some studies in which the antioxidant activities of several Kampo and Chinese medicine formulae have been used to treat symptoms or conditions related to brain disorders such as apoplexy and migraine, those including Zokumei-To (Xu Ming Tang), Chouto-San (Gou Teng San), Reikeijutsukan-To (Ling Gui Shu Gan Tang), and Keishibukuryougan-ka-Yokuinin (Gui Zhi Fu Ling Wan jia Yiyiren) [199, 201]. These formulae have essentially high antioxidant activity, and the scavenging activity is higher against superoxide radicals than hydroxyl radicals [201]. On the other hand, Shengmai San (SMS), which have a long history of use in the treatment of coronary heart diseases [202], has higher hydroxyl radical scavenging activity than superoxide radical scavenging activity [202]. In addition to these studies, many other reports have indicated high the antioxidant potential of traditional formulae and their component herbs [203–206].

18.3 Effects of Antioxidants on Periodontal Disease

Oxidative stress is involved in the initiation and progression of periodontal disease. Research into the effects of antioxidants on periodontal disease has been conducted. Here, we discuss some of the effects of antioxidants (other than vitamins C and E) on periodontal disease (see Chap. 19).

18.3.1 Flavonoids

18.3.1.1 Cocoa

In an animal model, only the effects of cocoa consumption on periodontitis have been reported [26]. In this 4-week study, 24 male Wistar rats (age, 8 weeks) were used.

The rats were randomly divided into three groups of eight rats each: the control group, in which animals were fed standard chow and received no treatment; the periodontitis group, in which animals were fed powdered standard chow, and experimental periodontitis was induced by placing a 3-0 cotton ligature in a subgingival position around the mandibular first molars; and the cocoa group, in which animals were fed chow containing 10 % cocoa (containing 42 mg/g polyphenols), and experimental periodontitis was induced. Serum levels of ROS in the periodontitis group increased in a time-dependent manner, and these values were significantly higher than in the control group at 2 and 4 weeks ($P < 0.01$). Serum levels of ROS in the cocoa group were significantly lower than those in the periodontitis group at 2 and 4 weeks ($P < 0.01$). In contrast, serum levels of antioxidant power in the periodontitis group showed a time-dependent decrease when compared to the control group ($P < 0.01$). Serum levels of antioxidant power in the cocoa group were significantly higher than in the periodontitis group at 2 and 4 weeks ($P < 0.01$). Furthermore, the consumption of a cocoa-enriched diet decreased 8-hydroxydeoxyguanosine (8-OHdG) levels and increased the ratio of reduced form glutathione (GSH)/oxidized form glutathione (GSSG) in rat gingiva. Alveolar bone loss and polymorphonuclear leukocyte infiltration after ligature placement were also inhibited by cocoa intake.

18.3.1.2 Catechins

There have been various clinical trials, animal studies, and in vitro studies demonstrating the effects of green tea or catechins on periodontal disease or disease-related cell function. Some clinical trials are shown in Table 18.1 [207–209].

Catechins have been shown to possess potent antioxidant activity several times higher than that of vitamins C and E [93]. In a periodontitis model, which use application of 25 μg/μL lipopolysaccharide (LPS) from *Escherichia coli* (Sigma Chemical Co., St. Louis, MO) and 2.25 U/μL proteases from *Streptomyces griseus* to the palatal gingival sulcus of both maxillary first molars, the antioxidant effects have been demonstrated. Topical application of a green tea catechin-containing dentifrice reduced levels of expression of hexanoyl-lysine and nitrotyrosine in rat periodontal lesions to a greater degree than control dentifrice [25]. Gingiva to which green tea catechin-containing dentifrice was applied also showed lower levels of inflammation and tumor necrosis factor (TNF)-α, as compared to controls. Another in vivo study has shown that alveolar bone resorption and interleukin (IL)-1β expression induced by LPS in rat gingival tissue are significantly decreased by injection or oral administration of green tea catechin, although their antioxidant effects have not been investigated [41].

In addition to their direct antioxidant capacity, green tea catechins have antibacterial capacity [210–212] and may be useful for helping to prevent periodontal disease. For example, green tea catechins applied in periodontal pockets decrease the pocket depth and the proportion of Gram-negative anaerobic rods, while the same catechins show an in vitro bactericidal effect against *Porphyromonas gingivalis* (*P. gingivalis*) and *Prevotella* spp. [208]. Wine catechins also have strong antimicrobial activity

Table 18.1 Intervention studies regarding the effects of flavonoids on periodontal disease

Author (year)	Subjects/age	Study design	Indices associated with PD	Results
Chava and Vedula (2012) [207]	30 patients/38.9 ± 10.67 years	Split mouth design	GI, CAL, PPD	GI, CAL and PPD were improved in the green tea group
	Green tea catechin: 30 sites	1 % (w/v; 1 g/100 mL), solution of green tea extract		
	Control: 30 sites	Placebo gel for 4 weeks		
Hirasawa et al. (2002) [208]	6 volunteers/41–64 years	Case–control study	PPD, number of bacteria	Local delivery strip system decreased the pocket depth and the proportion of Gram-negative anaerobic rods
	Green tea catechin: 6 sites	Green tea catechin strips (5 %)/ week		
	Control: 6 sites	Placebo strips for 8 weeks		
Krahwinkel and Willershausen (2000) [209]	47 volunteers/25.76 years	RCT	Approximal plaque index, Sulcus bleeding index	No differences were observed between groups
	Green tea catechin: 24 subjects	Chewing 8 candies with green tea extract/day		
	Control: 25 subjects	Chewing placebo candies/day for 3 weeks		

CAL clinical attachment level, *GI* gingival index, *PPD* probing pocket depth, *RCT* randomized controlled trial

against *Porphyromonas gingivalis* (*P. gingivalis*) and *Prevotella intermedia* (*P. intermedia*) [213, 214].

Catechins have direct effects on host immune response. In vitro studies have shown that catechins (EGCG/ECG) decrease the production of the proinflamatory cytokines IL-1β, TNF-α, and IL-6, and enhance the production of the anti-inflammatory cytokine IL-10 [215–217]. Catechins also inhibit CC chemokine ligand 10 and 20 in human gingival fibroblasts, which plays a pivotal role in the recruitment of helper T cells and thus in the development of periodontal disease [218, 219]. Furthermore, EGCG significantly inhibits the survival of osteoclasts differentiated from RAW264.7 cells, and induces the apoptosis of osteoclasts or inhibits osteoclast formation stimulated by *P. gingivalis* [220, 221].

18.3.1.3 Curcumin

Although no clinical trials have demonstrated the effects of curcumin, the direct antioxidant capacity has been shown in an in vitro study [222]. Curcumin dose-dependently

inhibited thrombin-induced connective tissue growth factor expression through c-Jun NH_2-terminal kinase (JNK) suppression in human gingival fibroblasts via a mechanism that includes oxidative stress [222]. The results of this study suggest that curcumin can effectively inhibit the development of gingival overgrowth. However, it was reported that curcumin is able to induce ROS generation in human gingival fibroblasts [223, 224].

In rats following ligature-induced experimental periodontitis, receptor activator of nuclear factor-κB ligand (RANKL), receptor activator of nuclear factor-κB (RANK), osteoprotegerin (OPG), TNF-α and IL-6 expression levels were lower in the curcumin-treated group than in the experimental periodontitis group [225]. In another study, using the same ligature model, alveolar bone resorption was not affected by either dose of curcumin, but curcumin effectively inhibited cytokine gene expression at both the mRNA and protein levels, and produced dose-dependent inhibition of nuclear factor-κB (NF-κB) in gingival tissues [226]. In the LPS-injected periodontitis model, curcumin effectively inhibited cytokine expression [IL-6, TNF-α, and prostaglandin E2 (PGE2) synthase] on rat gingival tissue, but NF-κB was inhibited only by the lower dose of curcumin, whereas p38 mitogen-activated protein kinase activation (MAPK) was not affected [227].

Some in vitro studies have indicated the anti-inflammatory reactivity of curcumin. Curcumin strongly suppressed the production of IL-6 at both the gene transcription and translation levels in *P. intermedia* LPS-activated RAW264.7 cells [228]. Curcumin may contribute to blockade of the host-destructive processes mediated by IL-6 and appears to have potential therapeutic value in the treatment of inflammatory periodontal disease. Curcumin dose-dependently inhibited levels of TNF-α and IL-1β in RAW264.7 cells, and the level of monocyte chemoattractant protein 1 in human gingival fibroblasts stimulated with *P. gingivalis* LPS [229, 230].

18.3.1.4 Resveratrol

There have been no clinical trials, but some animal and cell culture studies have been conducted for resveratrol in periodontology. Resveratrol may promote immunomodulatory effects on the host response. In a rat ligature model, daily administration of 10 mg/kg resveratrol was performed [231]. Therapies were administered systemically for 30 days or for 19 days before periodontitis induction, followed by a further 11 days. The immuno-enzymatic assay of the gingival tissue showed a lower concentration of IL-17 in the resveratrol group than the control group, while no differences in IL-1β and IL-4 levels were observed. The study concludes that continuous administration of resveratrol may decrease periodontal breakdown induced experimentally in rats.

In human gingival fibroblasts, resveratrol decreased LPS and nicotine-induced cytotoxicity, ROS and PGE2 production, and expression of cyclooxygenase-2 [232]. Resveratrol inhibited nicotine and LPS-mediated protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K), p38, extracellular signal-regulated kinase, JNK, MAPK, and NF-κB activation. In another model, resveratrol significantly inhibited the increased production of vascular endothelial growth factor (VCAM) by human

gingival fibroblasts and decreased vascular permeability in response to vesicles and outer membrane proteins from *Aggregatibacter actinomycetemcomitans* and *P. gingivalis*, suggesting a therapeutic role in pathogenic bacteria-induced periodontal inflammation [38]. In human periodontal ligament cells, resveratrol treatment decreased NO expression induced by LPS from *P. gingivalis* and decreased the production of pro-inflammatory cytokines [233]. However, the cytotoxicity of hydrogen peroxide against S-G gingival epithelial cells was reduced by resveratrol, and no activity of resveratrol to function as an antioxidant was noted under these conditions [50].

Antibacterial effects of resveratrol were also observed in vitro. After adding resveratrol, the periodontal bacteria, *A. actinomycetemcomitans* and *P. gingivalis* showed significant decreases in viable count after 1 h, while no colony forming units were observed after 24 h [34]. These results suggest that resveratrol possesses significant properties on periodontal pathogens.

Furthermore, resveratrol significantly inhibited *P. gingivalis* LPS-induced adhesion of leukocytes to endothelial cells and to the aortic endothelium by down-regulating the cell adhesion molecules, intercellular adhesion molecule-1 and VCAM-1 [234], and partially reversed the inhibition of bone formation by the *P. gingivalis* LPS in rat bone marrow cells [235].

18.3.1.5 Quercetin

Only one in vitro study has shown the direct antioxidant capacity of quercetin in periodontology. Nitrite-induced fluorescence increase in the bacterial fraction of saliva was completely inhibited by quercetin and complete inhibition continued until almost all quercetin had been oxidized [236]. These results suggest that quercetin is able to protect the human oral cavity from damage induced by reactive nitrogen species and that the protective function of quercetin may be significant when the antioxidant capacity of saliva is decreased by periodontal disease.

The anti-inflammatory and antibacterial properties of quercetin have also been reported. In an animal model, quercetin (75 mg/kg) reduced 5 mg/mL LPS-induced osteoclasts and inhibited ligature-induced alveolar bone loss and inflammation [237]. In an in vitro model, quercetin demonstrated an inhibitory effect on MAPK activation, cyclooxygenase-2 (COX-2) expression, IL-1β and PGE2 synthesis on LPS-activated transduction mechanism regulation in human gingival fibroblasts [238]. Quercetin possesses antimicrobial properties on periodontal pathogens (*A. actinomycetemcomitans*, *Actinomyces viscosus*, *Fusobacterium nucleatum*, *Actinomyces naeslundii*, and *P. gingivalis*) in vitro [40, 239].

18.3.1.6 Genistein

In addition to the roles of inhibitor of tyrosine kinases, genistein has antioxidant capacity. Genistein suppressed both iNOS activity and nitrite production in an *A. actinomycetemcomitans*-stimulated murine macrophage cell line (RAW264.7 cells)

[240], and that by LPS-stimulated human gingival fibroblasts [241]. Furthermore, genistein demonstrated an inhibitory effect on MAPK activation on LPS-activated transduction mechanism regulation in human gingival fibroblasts [238, 242] and plays a role in the regulation of MAPK activation via G protein-coupled receptor 30 in periodontal ligament cells [243].

18.3.1.7 Other Flavonoids

There have been several studies into the effects of flavonoids that are not described above. For example, baicalin (7-glucuronic acid, 5,6-dihydroxy-flavone), which is a flavonoid compound purified from the medicinal plant, *Scutellaria baicalensis* Georgi, has been reported to possess anti-inflammatory and antioxidant activities, and to protect against tissue damage in ligature-induced periodontitis in rats [53, 244]. Nobiletin (5,6,7,8,3′,4′-hexamethoxy flavone) and tangeretin (5,6,7,8,4′-pentamethoxy flavone) are polymethoxy flavonoids abundantly present in orange peel, and were reported to restore alveolar bone mass in a mouse experimental model for periodontitis by inhibiting LPS-induced bone resorption [245]. Proanthocyanidins, the most abundant flavonoids extracted from red cranberry fruits, have been reported to possess antimicrobial, antiadhesion, antioxidant, and anti-inflammatory properties [246]. Cranberry proanthocyanidins inhibit *P. gingivalis* adherence to human oral epithelial cells [247], inhibit matrix metalloproteinase (MMP)-1, -3, -7, -8, -9, and -13 production by LPS-stimulated macrophages [248], and inhibit the maturation process of preosteoclastic cells [249]. Luteolin (3′,4′,5,7-tetrahydroxyflavone) is a flavone found at high concentrations in celery, green pepper, parsley, perilla leaf and seeds, and chamomile [250]. It suppresses the production of NO and IL-6 in murine macrophage-like RAW264.7 cells stimulated with LPS from *P. intermedia* [251] and LPS-induced NF-κB translocation in human gingival fibroblasts [241]. Apigenin (4′,5,7-trihydroxyflavone) possesses anti-inflammatory activity in human periodontal ligament cells and works through a novel mechanism involving the action of heme oxygenase-1 [252]. Mangiferin (C2-β-D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone) is widely distributed in higher plants, has antioxidant capacity [253], and reduces the alveolar bone loss of rats with experimental periodontitis [29]. Kaempferol (3,4,5,7-tetrahydroxyflavone), a flavonoid glycoside which is particularly abundant in fruits, vegetables, and beverages such as tea [254], attenuated the NF-κB nuclear binding activity in rat gingival tissue [255]. Pycnogenol is a water-soluble mixture of flavonoid compounds extracted from French maritime pine bark and chewing gum containing this compound minimizes gingival bleeding and plaque formation in humans [256].

18.3.2 Other Antioxidants

Molecular hydrogen, which selectively reduces cytotoxic ROS and oxidative stress, is considered to be a novel antioxidant [257]. Drinking water containing a therapeutic

dose of hydrogen (hydrogen-rich water) represents an alternative mode of delivery for molecular hydrogen. In a rat ligature model, hydrogen-rich water intake inhibited increases in serum ROS levels and lowered expression of 8-OHdG and nitrotyrosine in periodontal tissue [24]. It also suppressed both periodontal disease progression and initiation of atherosclerosis in rat experimental periodontitis [258].

In rat periodontitis models, the positive effects of antioxidants, such as thymoquinone (2-isopropyl-5-methyl-1,4-benzoquinone) [54], verbascoside [55], calcium gluconate [29], aminoguanidine [36], *N*-acetylcysteine [60], and tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl) [61] on periodontitis have been observed. On the other hand, in human RCTs, the effects of vitamin C [64], lycopene [65], and fruit/vegetable/berry juice powder [66] have been reported. In a study of lycopene [65], one hundred and ten subjects, including 50 smokers, 50 nonsmokers and 10 controls participated. Subjects in the smoker and nonsmoker groups had contralateral sites treated with lycopene gel and placebo. When compared with placebo, lycopene-treated sites in smokers and nonsmokers showed significant reductions in probing pocket depth and clinical attachment gain. However, there were no significant differences in clinical parameters when lycopene-treated sites in smokers and nonsmokers were compared, except for the reduction in 8-OHdG levels. In another study [66], subjects with chronic periodontitis were randomly assigned to one of three groups: fruit/vegetable, fruit/vegetable/berry, or placebo. These supplements were taken daily during non-surgical debridement and maintenance, and outcomes were assessed at 2, 5, and 8 months after completion. Clinical outcomes improved in all groups at 2 months, with additional improvement in probing pocket depth versus placebo for the fruit/vegetable group. Gingival crevicular fluid volumes diminished more strongly in the fruit/vegetable/berry groups than placebo at 2 months, but not at later times. The percentage of bleeding on probing (5 months) and cumulative plaque scores (8 months) were lower in the fruit/vegetable group.

Traditional medicine approaches include the use of mouthwash and topical application of various herbal agents [206]. Some studies have reported their antioxidant capacity. For example, neem (*Azadirachta indica*) has been reported to contain gallic acid, gallocatechin, epigallocatechin, and catechin, all of which can reduce the oxidative burst from polymorphonuclear leukocytes [259]. Gels and mouthwashes including neem improve clinical parameters in gingivitis [260–263]. Triphala, a potent rasayana, is derived from three fruits: amalaki (*Phyllanthus emblica*), haritaki (*Terminalia chebula*), and bibhitaki (*Terminalia bellerica*). It inhibits MMP-9 activity, and may prevent connective tissue destruction in periodontal disease [264]. Sesame oil was used for oil pulling and improved gingival parameters [265]. Polyunsaturated fatty acids in sesame oil affect lipid peroxidation and exhibit anti-inflammatory properties [266]. When some traditional Chinese medicines, such as Guchiwan and Guchigao, were used, dental plaque scores, gingival index, and periodontal index, as well as the IL-8 levels in gingival crevicular fluid were improved [267, 268]. These drugs also have antibacterial activity [269, 270], antioxidant effects [271], and osteoclast inhibiting activity [272].

Antioxidants also include minerals (iron, copper, selenium, and zinc). However, there is little information on the effects of minerals on periodontal disease. One

study reports the effects of vitamin E and selenium on collagen degradation [273]. In experimental granulation tissue induced by cellulose sponges in rats, administering pharmacological doses of both vitamin E and selenium reduced the breakdown of collagen. This suggests that these radicals play a role in collagen destruction by granulation tissues, as in periodontitis.

18.4 Limitation

Foods and beverages rich in antioxidants have often been associated with decreased risk of developing several diseases [71–80]. However, it remains unclear whether these products help to maintain human health and delay disease onset, or whether the protective effects are direct or indirect. It is necessary before making any claims of possible benefit from consuming antioxidant-rich foods or beverages to establish whether these in vitro antioxidant activities actually occur in vivo.

When high-dose antioxidant supplements are used, they generally do no good and sometimes cause harm [274–276]. Low-dose mixtures, such as in multivitamin/multimineral tablets, can sometimes do good, but may be beneficial only for people who are deficient in certain micronutrients by poor dietary habits [277, 278]. In periodontal disease, there have been few RCTs on the effects in humans [64–66], although many cell culture and animal model studies have been reported. Furthermore, these human reports have contradictory and confusing results. A review suggests that there is fair evidence that high polyphenol intake has a preventive effect against periodontal disease, but such effects have not been directly observed. Thus, we need further clinical investigations in order to determine the effects of antioxidants on human periodontal health.

18.5 Conclusion

Many animal and in vitro studies have determined the effects of antioxidants on periodontal disease. However, there have been few RCTs on the effects in humans and the results remain controversial. The use of adjunctive antioxidants thus requires further investigation.

References

1. Halliwell B, Gutteridge JMC (2007) Free radicals in biology and medicine, 4th edn. Oxford University Press, Oxford
2. Niki E (2012) Do antioxidants impair signaling by reactive oxygen species and lipid oxidation products? FEBS Lett 586:3767–3770. doi:10.1016/j.febslet.2012.09.025
3. Saran M, Bors W (1989) Oxygen radicals acting as chemical messengers: a hypothesis. Free Radic Res Commun 7:213–220

4. Suzuki YJ, Forman HJ, Sevanian A (1997) Oxidants as stimulators of signal transduction. Free Radic Biol Med 22:269–285
5. Finkel T (2011) Signal transduction by reactive oxygen species. J Cell Biol 194:7–15. doi:10.1083/jcb.201102095
6. Janssen-Heininger YMW, Mossman BT, Heintz NH et al (2008) Redox-based regulation of signal transduction: principles, pitfalls, and promises. Free Radic Biol Med 45:1–17. doi:10.1016/j.freeradbiomed.2008.03.011
7. Winterbourn CC (2008) Reconciling the chemistry and biology of reactive oxygen species. Nat Chem Biol 4:278–286. doi:10.1038/nchembio.85
8. Forman HJ, Maiorino M, Ursini F (2010) Signaling functions of reactive oxygen species. Biochemistry 49:835–842. doi:10.1021/bi9020378
9. Murphy MP, Holmgren A, Larsson N et al (2011) Unraveling the biological roles of reactive oxygen species. Cell Metab 13:361–366. doi:10.1016/j.cmet.2011.03.010
10. Niki E (2011) Assessment of antioxidant capacity in vitro and in vivo. Free Radic Biol Med 49:503–515. doi:10.1016/j.freeradbiomed.2010.04.016
11. Halliwell B (2012) Free radicals and antioxidants: updating a personal view. Nutr Rev 70:257–265. doi:10.1111/j.1753-4887.2012.00476.x
12. Rhee SG, Woo HA (2011) Multiple functions of peroxiredoxins: peroxidases, sensors and regulators of the intracellular messenger H2O2, and protein chaperones. Antioxid Redox Signal 15:781–794. doi:10.1089/ars.2010.3393
13. Woo HA, Yim SH, Shin DH et al (2010) Inactivation of peroxiredoxin I by phosphorylation allows localized H2O2 accumulation for cell signaling. Cell 140:517–528. doi:10.1016/j.cell.2010.01.009
14. Barranco-Medina S, Lázaro JJ, Dietz KJ (2009) The oligomeric conformation of peroxiredoxins links redox state to function. FEBS Lett 583:1809–1816. doi:10.1016/j.febslet.2009.05.029
15. Fisher AB (2011) Peroxiredoxin 6: a bifunctional enzyme with glutathione peroxidase and phospholipase A2 activities. Antioxid Redox Signal 15:831–844. doi:10.1089/ars.2010.3412
16. Gey KF (1998) Vitamins E plus C and interacting conutrients required for optimal health. A critical and constructive review of epidemiology and supplementation data regarding cardiovascular disease and cancer. Biofactors 7:113–174
17. Cordero Z, Drogan D, Weikert C, Boeing H (2010) Vitamin E and risk of cardiovascular diseases: a review of epidemiologic and clinical trial studies. Crit Rev Food Sci Nutr 50:420–440. doi:10.1080/10408390802304230
18. Gutteridge JM, Halliwell B (2010) Antioxidants: molecules, medicines, and myths. Biochem Biophys Res Commun 393:561–564. doi:10.1016/j.bbrc.2010.02.071
19. Madianos PN, Bobetsis YA, Kinane DF (2005) Generation of inflammatory stimuli: how bacteria set up inflammatory responses in the gingiva. J Clin Periodontol 32:57–71
20. Komiya-Ito A, Ishihara K, Tomita S, Kato T, Yamada S (2010) Investigation of subgingival profile of periodontopathic bacteria using polymerase chain reaction. Bull Tokyo Dent Coll 51:139–144
21. Xiong X, Buekens P, Fraser WD, Beck J, Offenbacher S (2006) Periodontal disease and adverse pregnancy outcomes: a systematic review. BJOG 113:135–143
22. Page RC (1998) The pathobiology of periodontal diseases may affect systemic diseases: inversion of a paradigm. Ann Periodontol 3:108–120
23. Parrish JH Jr, DeMarco TJ, Bissada NF (1977) Vitamin E and periodontitis in the rat. Oral Surg Oral Med Oral Pathol 44:210–218
24. Kasuyama K, Tomofuji T, Ekuni D, Tamaki N, Azuma T, Irie K, Endo Y, Morita M (2011) Hydrogen-rich water attenuates experimental periodontitis in a rat model. J Clin Periodontol 38:1085–1090. doi:10.1111/j.1600-051X.2011.01801.x
25. Maruyama T, Tomofuji T, Endo Y, Irie K, Azuma T, Ekuni D, Tamaki N, Yamamoto T, Morita M (2011) Supplementation of green tea catechins in dentifrices suppresses gingival oxidative stress and periodontal inflammation. Arch Oral Biol 56:48–53. doi:10.1016/j.archoralbio.2010.08.015

26. Tomofuji T, Ekuni D, Irie K, Azuma T, Endo Y, Tamaki N, Sanbe T, Murakami J, Yamamoto T, Morita M (2009) Preventive effects of a cocoa-enriched diet on gingival oxidative stress in experimental periodontitis. J Periodontol 80:1799–1808. doi:10.1902/jop.2009.090270
27. Ekuni D, Firth JD, Nayer T, Tomofuji T, Sanbe T, Irie K, Yamamoto T, Oka T, Liu Z, Vielkind J, Putnins EE (2009) Lipopolysaccharide-induced epithelial monoamine oxidase mediates alveolar bone loss in a rat chronic wound model. Am J Pathol 175:1398–1409. doi:10.2353/ajpath.2009.090108
28. Tomofuji T, Ekuni D, Sanbe T, Irie K, Azuma T, Maruyama T, Tamaki N, Murakami J, Kokeguchi S, Yamamoto T (2009) Effects of vitamin C intake on gingival oxidative stress in rat periodontitis. Free Radic Biol Med 46:163–168. doi:10.1016/j.freeradbiomed.2008.09.040
29. Duang XY, Wang Q, Zhou XD, Huang DM (2011) Mangiferin: a possible strategy for periodontal disease to therapy. Med Hypotheses 76:486–488. doi:10.1016/j.mehy.2010.11.029
30. Narotzki B, Levy Y, Aizenbud D, Reznick AZ (2013) Green tea and its major polyphenol EGCG increase the activity of oral peroxidases. Adv Exp Med Biol 756:99–104. doi:10.1007/978-94-007-4549-0_13
31. Zeidán-Chuliá F, Rybarczyk-Filho JL, Gursoy M, Könönen E, Uitto VJ, Gursoy OV, Cakmakci L, Moreira JC, Gursoy UK (2012) Bioinformatical and in vitro approaches to essential oil-induced matrix metalloproteinase inhibition. Pharm Biol 50:675–686. doi:10.3109/13880209.2012.677847
32. Velliyagounder K, Ganeshnarayan K, Velusamy SK, Fine DH (2012) In vitro efficacy of diallyl sulfides against the periodontopathogen Aggregatibacter actinomycetemcomitans. Antimicrob Agents Chemother 56:2397–2407. doi:10.1128/AAC.00020-12
33. Palmer LJ, Cooper PR, Ling MR, Wright HJ, Huissoon A, Chapple IL (2012) Hypochlorous acid regulates neutrophil extracellular trap release in humans. Clin Exp Immunol 167:261–268. doi:10.1111/j.1365-2249.2011.04518.x
34. O'Connor DJ, Wong RW, Rabie AB (2011) Resveratrol inhibits periodontal pathogens in vitro. Phytother Res 25:1727–1731. doi:10.1002/ptr.3501
35. San Miguel SM, Opperman LA, Allen EP, Zielinski J, Svoboda KK (2011) Bioactive antioxidant mixtures promote proliferation and migration on human oral fibroblasts. Arch Oral Biol 56:812–822. doi:10.1016/j.archoralbio.2011.01.001
36. Herrera BS, Martins-Porto R, Maia-Dantas A, Campi P, Spolidorio LC, Costa SK, Van Dyke TE, Gyurko R, Muscara MN (2011) iNOS-derived nitric oxide stimulates osteoclast activity and alveolar bone loss in ligature-induced periodontitis in rats. J Periodontol 82:1608–1615. doi:10.1902/jop.2011.100768
37. Yamada M, Kubo K, Ueno T, Iwasa F, Att W, Hori N, Ogawa T (2010) Alleviation of commercial collagen sponge- and membrane-induced apoptosis and dysfunction in cultured osteoblasts by an amino acid derivative. Int J Oral Maxillofac Implants 25:939–946
38. Núñez MJ, Novío S, Balboa J, Seoane J, Suárez JA, Freire-Garabal M (2010) Effects of resveratrol on expression of vascular endothelial growth factor in human gingival fibroblasts stimulated by periodontal pathogens. Acta Odontol Scand 68:239–247. doi:10.3109/00016357.2010.494269
39. Staudte H, Güntsch A, Völpel A, Sigusch BW (2010) Vitamin C attenuates the cytotoxic effects of Porphyromonas gingivalis on human gingival fibroblasts. Arch Oral Biol 55:40–45. doi:10.1016/j.archoralbio.2009.11.009
40. Geoghegan F, Wong RW, Rabie AB (2010) Inhibitory effect of quercetin on periodontal pathogens in vitro. Phytother Res 24:817–820. doi:10.1002/ptr.3014
41. Nakamura H, Ukai T, Yoshimura A, Kozuka Y, Yoshioka H, Yoshinaga Y, Abe Y, Hara Y (2010) Green tea catechin inhibits lipopolysaccharide-induced bone resorption in vivo. J Periodontal Res 45:23–30. doi:10.1111/j.1600-0765.2008.01198.x
42. Ohnishi T, Bandow K, Kakimoto K, Machigashira M, Matsuyama T, Matsuguchi T (2009) Oxidative stress causes alveolar bone loss in metabolic syndrome model mice with type 2 diabetes. J Periodontal Res 44:43–51. doi:10.1111/j.1600-0765.2007.01060.x
43. Geoghegan F, Tsui VW, Wong RW, Rabie AB (2008) Inhibitory effect of quercetin on periodontal pathogens. Ann R Australas Coll Dent Surg 19:157–158. doi:10.1002/ptr.3014

44. Argentin G, Cicchetti R (2006) Evidence for the role of nitric oxide in antiapoptotic and genotoxic effect of nicotine on human gingival fibroblasts. Apoptosis 11:1887–1897
45. Inaba H, Tagashira M, Kanda T, Ohno T, Kawai S, Amano A (2005) Apple- and hop-polyphenols protect periodontal ligament cells stimulated with enamel matrix derivative from Porphyromonas gingivalis. J Periodontol 76:2223–2229
46. Chang YC, Lai CC, Lin LF, Ni WF, Tsai CH (2005) The up-regulation of heme oxygenase-1 expression in human gingival fibroblasts stimulated with nicotine. J Periodontal Res 40:252–257
47. Battino M, Ferreiro MS, Armeni T, Politi A, Bompadre S, Massoli A, Bullon P (2005) In vitro antioxidant activities of antioxidant-enriched toothpastes. Free Radic Res 39:343–350
48. Battino M, Ferreiro MS, Fattorini D, Bullon P (2002) In vitro antioxidant activities of mouthrinses and their components. J Clin Periodontol 29:462–467
49. Shokri F, Heidari M, Gharagozloo S, Ghazi-Khansari M (2000) In vitro inhibitory effects of antioxidants on cytotoxicity of T-2 toxin. Toxicology 146:171–176
50. Babich H, Reisbaum AG, Zuckerbraun HL (2000) In vitro response of human gingival epithelial S-G cells to resveratrol. Toxicol Lett 114:143–153
51. Babich H, Zuckerbraun HL, Hirsch ST, Blau L (1999) In vitro cytotoxicity of the nitric oxide donor, S-nitroso-N-acetyl-penicillamine, towards cells from human oral tissue. Pharmacol Toxicol 84:218–225
52. Govindaraj J, Emmadi P, Deepalakshmi, Rajaram V, Prakash G, Puvanakrishnan R (2010) Protective effect of proanthocyanidins on endotoxin induced experimental periodontitis in rats. Indian J Exp Biol 48:133–142
53. Cai X, Li C, Du G, Cao Z (2008) Protective effects of baicalin on ligature-induced periodontitis in rats. J Periodontal Res 43:14–21. doi:10.1111/j.1600-0765.2007.00989.x
54. Ozdemir H, Kara MI, Erciyas K, Ozer H, Ay S (2012) Preventive effects of thymoquinone in a rat periodontitis model: a morphometric and histopathological study. J Periodontal Res 47:74–80. doi:10.1111/j.1600-0765.2011.01406.x
55. Paola RD, Oteri G, Mazzon E, Crisafulli C, Galuppo M, Toso RD, Pressi G, Cordasco G, Cuzzocrea S (2011) Effects of verbascoside, biotechnologically purified by Syringa vulgaris plant cell cultures, in a rodent model of periodontitis. J Pharm Pharmacol 63:707–717. doi:10.1111/j.2042-7158.2011.01262.x
56. Ku SK, Cho HR, Sung YS, Kang SJ, Lee YJ (2011) Effects of calcium gluconate on experimental periodontitis and alveolar bone loss in rats. Basic Clin Pharmacol Toxicol 108:241–250. doi:10.1111/j.1742-7843.2010.00646.x
57. Lohinai Z, Benedek P, Fehér E, Györfi A, Rosivall L, Fazekas A, Salzman AL, Szabó C (1998) Protective effects of mercaptoethylguanidine, a selective inhibitor of inducible nitric oxide synthase, in ligature-induced periodontitis in the rat. Br J Pharmacol 123:353–360
58. Paquette DW, Rosenberg A, Lohinai Z, Southan GJ, Williams RC, Offenbacher S, Szabó C (2006) Inhibition of experimental gingivitis in beagle dogs with topical mercaptoalkylguanidines. J Periodontol 77:385–391
59. Kador PF, O'Meara JD, Blessing K, Marx DB, Reinhardt RA (2011) Efficacy of structurally diverse aldose reductase inhibitors on experimental periodontitis in rats. J Periodontol 82:926–933. doi:10.1902/jop.2010.100442
60. Toker H, Ozdemir H, Eren K, Ozer H, Sahin G (2009) N-acetylcysteine, a thiol antioxidant, decreases alveolar bone loss in experimental periodontitis in rats. J Periodontol 80:672–678. doi:10.1902/jop.2009.080509
61. Di Paola R, Mazzon E, Zito D, Maiere D, Britti D, Genovese T, Cuzzocrea S (2005) Effects of Tempol, a membrane-permeable radical scavenger, in a rodent model periodontitis. J Clin Periodontol 32:1062–1068
62. Di Paola R, Mazzon E, Rotondo F, Dattola F, Britti D, De Majo M, Genovese T, Cuzzocrea S (2005) Reduced development of experimental periodontitis by treatment with M40403, a superoxide dismutase mimetic. Eur J Pharmacol 516:151–157
63. Petelin M, Pavlica Z, Ivanusa T, Sentjurc M, Skaleric U (2000) Local delivery of liposome-encapsulated superoxide dismutase and catalase suppress periodontal inflammation in beagles. J Clin Periodontol 27:918–925

64. Abou Sulaiman AE, Shehadeh RM (2010) Assessment of total antioxidant capacity and the use of vitamin C in the treatment of non-smokers with chronic periodontitis. J Periodontol 81:1547–1554. doi:10.1902/jop.2010.100173
65. Chandra RV, Sandhya YP, Nagarajan S, Reddy BH, Naveen A, Murthy KR (2012) Efficacy of lycopene as a locally delivered gel in the treatment of chronic periodontitis: smokers vs nonsmokers. Quintessence Int 43:401–411
66. Chapple IL, Milward MR, Ling-Mountford N, Weston P, Carter K, Askey K, Dallal GE, De Spirt S, Sies H, Patel D, Matthews JB (2012) Adjunctive daily supplementation with encapsulated fruit, vegetable and berry juice powder concentrates and clinical periodontal outcomes: a double-blind RCT. J Clin Periodontol 39:62–72. doi:10.1111/j.1600-051X.2011.01793.x
67. Harman D, Eddy DE (1979) Free radical theory of aging: beneficial effect of adding antioxidants to the maternal mouse diet on life span of offspring: possible explanation of the sex difference in longevity. Age 2:109–122
68. Comfort A (1979) The biology of senescence, 3rd edn. Elsevier Science, New York
69. Benzie IF, Szeto YT (1999) Total antioxidant capacity of teas by the ferric reducing/antioxidant power assay. J Agric Food Chem 47:633–636
70. Prior RL, Wu X, Schaich K (2005) Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J Agric Food Chem 53:4290–4302
71. Halliwell B, Rafter J, Jenner A (2005) Health promotion by flavonoids, tocopherols, tocotrienols, and other phenols: direct or indirect effects? Antioxidant or not? Am J Clin Nutr 81:268S–276S
72. Huxley RR, Neil HAW (2003) The relation between dietary flavonol intake and coronary heart disease mortality: a meta-analysis of prospective cohort studies. Eur J Clin Nutr 57:904–908
73. Hertog MGL, Hollman PCH (1996) Potential health effects of the dietary flavonol quercetin. Eur J Clin Nutr 50:63–71
74. Hertog MGL, Feskens EJM, Hollman PCH, Katan MB, Kromhout D (1993) Dietary antioxidant flavonoids and the risk of coronary heart diseases: the Zutphen Elderly Study. Lancet 342:1007–1011
75. Sesso HD, Gaziano M, Buring JE, Hennekens CH (1999) Coffee and tea intake and the risk of myocardial infarction. Am J Epidemiol 149:162–167
76. Hertog MGL, Bueno-de-Mesquita HB, Fehily AM, Sweetnam PM, Elwood PC, Kromhout D (1996) Fruit and vegetable consumption and cancer mortality in the Caerphilly Study. Cancer Epidemiol Biomarkers Prev 5:673–677
77. Yochum L, Kushi LH, Meyer K, Folsom AR (1999) Dietary flavonoid intake and risk of cardiovascular disease in postmenopausal women. Am J Epidemiol 149:943–949
78. Hirvonen T, Pietinen P, Virtanen M, Ovaskainen ML, Häkkinen S, Albanes D, Virtamo J (2001) Intake of flavonols and flavones and risk of coronary heart disease in male smokers. Epidemiology 12:62–67
79. Geleijnse JM, Launer LJ, Van der Kuip DAM, Hofman A, Witteman JCM (2002) Inverse association of tea and flavonoid intakes with incident myocardial infarction: the Rotterdam Study. Am J Clin Nutr 75:880–886
80. Mukamal KJ, Maclure M, Muller JE, Sherwood JB, Mittleman MA (2002) Tea consumption and mortality after acute myocardial infarction. Circulation 105:2476–2481
81. Silva MM, Santos MR, Caroco G, Rocha R, Justino G, Mira L (2002) Structure-antioxidant activity relationships of flavonoids: a re-examination. Free Radic Res 36:1219–1227
82. Pannala AS, Rice-Evans CA, Halliwell B, Singh S (1997) Inhibition of peroxynitrite-mediated tyrosine nitration by catechin polyphenols. Biochem Biophys Res Commun 232:164–168
83. Paya M, Halliwell B, Hoult JRS (1992) Interaction of a series of coumarins with reactive oxygen species: scavenging of superoxide, hypochlorous acid and hydroxyl radicals. Biochem Pharmacol 44:205–214
84. Boersma BJ, Patel RP, Kirk M, Jackson PL, Muccio D, Darley-Usmar VM, Barnes S (1999) Chlorination and nitration of soy isoflavones. Arch Biochem Biophys 368:265–275

85. Halliwell B (2000) Antioxidant activity and other biological effects of flavonoids. In: Rice-Evans C (ed) Wake up to flavonoids. Royal Society of Medicine Press, London, pp 13–23
86. Mira L, Fernandez MT, Santos M, Rocha R, Florêncio MH, Jennings KR (2002) Interactions of flavonoids with iron and copper ions: a mechanism for their antioxidant activity. Free Radic Res 36:1199–1208
87. Ketsawatsakul U, Whiteman M, Halliwell B (2000) A re-evaluation of the peroxynitrite scavenging activity of some dietary phenolics. Biochem Biophys Res Commun 279:692–699
88. Talalay P (1989) Mechanisms of induction of enzymes that protect against chemical carcinogenesis. Adv Enzyme Regul 28:237–250
89. Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M, Nabeshima Y (1997) An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun 236:313–322
90. Ramos-Gomez M, Kwak MK, Dolan PM, Itoh K, Yamamoto M, Talalay P, Kensler TW (2001) Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in Nrf2 transcription factor-deficient mice. Proc Natl Acad Sci U S A 98:3410–3415
91. Battino M, Bullon P, Wilson M, Newman H (1999) Oxidative injury and inflammatory periodontal diseases: the challenge of anti-oxidants to free radicals and reactive oxygen species. Crit Rev Oral Biol Med 10:458–476
92. Chapple IL, Matthews JB (2007) The role of reactive oxygen and antioxidant species in periodontal tissue destruction. Periodontol 2000 43:160–232
93. Rice-Evans C (1999) Implications of the mechanisms of action of tea polyphenols as antioxidants in vitro for chemoprevention in humans. Proc Soc Exp Biol Med 220:262–266
94. Ramiro-Puig E, Castell M (2009) Cocoa: antioxidant and immunomodulator. Br J Nutr 101:931–940. doi:10.1017/S0007114508169896
95. Dillinger TL, Barriga P, Escárcega S, Jimenez M, Salazar Lowe D, Grivetti LE (2000) Food of the gods: cure for humanity? A cultural history of the medicinal and ritual use of chocolate. J Nutr 130:2057S–2072S
96. Vinson JA, Proch J, Zubik L (1999) Phenol antioxidant quantity and quality in foods: cocoa, dark chocolate, and milk chocolate. J Agric Food Chem 47:4821–4824
97. Lee KW, Kim YJ, Lee HJ, Lee CY (2003) Cocoa has more phenolic phytochemicals and a higher antioxidant capacity than teas and red wine. J Agric Food Chem 51:7292–7295
98. Vinson JA, Proch J, Bose P, Muchler S, Taffera P, Shuta D, Samman N, Agbor GA (2006) Chocolate is a powerful ex vivo and in vivo antioxidant, an antiatherosclerotic agent in an animal model, and a significant contributor to antioxidants in the European and American diets. J Agric Food Chem 54:8071–8076
99. Gu L, House SE, Wu X, Ou B, Prior RL (2006) Procyanidin and catechin contents and antioxidant capacity of cocoa and chocolate products. J Agric Food Chem 54:4057–4061
100. Arteel GE, Sies H (1999) Protection against peroxynitrite by cocoa polyphenol oligomers. FEBS Lett 462:167–170
101. Counet C, Collin S (2003) Effect of the number of flavanol units on the antioxidant activity of procyanidin fractions isolated from chocolate. J Agric Food Chem 51:6816–6822
102. Formica JV, Regelson W (1995) Review of the biology of quercetin and related bioflavonoids. Food Chem Toxicol 33:1061–1080
103. Lamuela-Raventós RM, Andrés-Lacueva C, Permanyer J, Izquierdo-Pulido M (2001) More antioxidants in cocoa. J Nutr 131:834–835
104. Azam S, Hadi N, Khan NU, Hadi SM (2003) Antioxidant and prooxidant properties of caffeine, theobromine and xanthine. Med Sci Monit 9:BR325–BR330
105. Yilmaz Y, Toledo RT (2004) Major flavonoids in grape seeds and skins: antioxidant capacity of catechin, epicatechin, and gallic acid. J Agric Food Chem 52:255–260
106. Spencer JP, Schroeter H, Rechner AR, Rice-Evans C (2001) Bioavailability of flavan-3-ols and procyanidins: gastrointestinal tract influences and their relevance to bioactive forms in vivo. Antioxid Redox Signal 3:1023–1039

107. Natsume M, Osakabe N, Yasuda A, Baba S, Tokunaga T, Kondo K, Osawa T, Terao J (2004) In vitro antioxidative activity of (2)-epicatechin glucuronide metabolites present in human and rat plasma. Free Radic Res 38:1341–1348
108. Erlejman AG, Fraga CG, Oteiza PI (2006) Procyanidins protect Caco-2 cells from bile acid- and oxidant-induced damage. Free Radic Biol Med 41:1247–1256
109. Lee KW, Kundu JK, Kim SO, Chun KS, Lee HJ, Surh YJ (2006) Cocoa polyphenols inhibit phorbol ester-induced superoxide anion formation in cultured HL-60 cells and expression of cyclooxygenase-2 and activation of NF-kB and MAPKs in mouse skin in vivo. J Nutr 136:1150–1155
110. Baba S, Osakabe N, Natsume M, Yasuda A, Takizawa T, Nakamura T, Terao J (2000) Cocoa powder enhances the level of antioxidative activity in rat plasma. Br J Nutr 84:673–680
111. Wang JF, Schramm DD, Holt RR, Ensunsa JL, Fraga CG, Schmitz HH, Keen CL (2000) A dose–response effect from chocolate consumption on plasma epicatechin and oxidative damage. J Nutr 130:2115S–2119S
112. Lecumberri E, Mateos R, Ramos S, Alía M, Rúperez P, Goya L, Izquierdo-Pulido M, Bravo L (2006) Characterization of cocoa fiber and its effect on the antioxidant capacity of serum in rats. Nutr Hosp 21:622–628
113. Ramiro-Puig E, Urpí-Sardà M, Pérez-Cano FJ, Franch A, Castellote C, Andrés-Lacueva C, Izquierdo-Pulido M, Castell M (2007) Cocoa-enriched diet enhances antioxidant enzyme activity and modulates lymphocyte composition in thymus from young rats. J Agric Food Chem 55:6431–6438
114. Mateos R, Lecumberri E, Ramos S, Goya L, Bravo L (2005) Determination of malondialdehyde (MDA) by high-performance liquid chromatography in serum and liver as a biomarker for oxidative stress. Application to a rat model for hypercholesterolemia and evaluation of the effect of diets rich in phenolic antioxidants from fruits. J Chromatogr B Analyt Technol Biomed Life Sci 827:76–82
115. Fraga CG, Actis-Goretta L, Ottaviani JI, Carrasquedo F, Lotito SB, Lazarus S, Schmitz HH, Keen CL (2005) Regular consumption of a flavanol-rich chocolate can improve oxidant stress in young soccer players. Clin Dev Immunol 12:11–17
116. Cooper KA, Donovan JL, Waterhouse AL, Williamson G (2008) Cocoa and health: a decade of research. Br J Nutr 99:1–11
117. Fisher ND, Hollenberg NK (2005) Flavanols for cardiovascular health: the science behind the sweetness. J Hypertens 23:1453–1459
118. Osakabe N (2005) Cacao polyphenols and atherosclerosis. J Clin Biochem Nutr 37:67–72
119. Heptinstall S, May J, Fox S, Kwik-Uribe C, Zhao L (2006) Cocoa flavanols and platelet and leukocyte function: recent in vitro and ex vivo studies in healthy adults. J Cardiovasc Pharmacol 47:S197–S205
120. Hodgson JM, Croft KD (2006) Dietary flavonoids: effects on endothelial function and blood pressure. J Sci Food Agric 86:2492–2498
121. Hackman RM, Polagruto JA, Zhu QY (2008) Flavanols: digestion, absorption and bioactivity. Phytochem Rev 7:195–208
122. Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L (2004) Polyphenols: food sources and bioavailability. Am J Clin Nutr 79:727–747
123. de Boer VC, Dihal AA, van der Woude H, Arts IC, Wolffram S, Alink GM, Rietjens IM, Keijer J, Hollman PC (2005) Tissue distribution of quercetin in rats and pigs. J Nutr 135:1718–1725
124. Mak JC (2012) Potential role of green tea catechins in various disease therapies: progress and promise. Clin Exp Pharmacol Physiol 39:265–273. doi:10.1111/j.1440-1681.2012.05673.x
125. Pandey M, Gupta S (2009) Green tea and prostate cancer: from bench to clinic. Front Biosci 1:13–25
126. Morel I, Lescoat G, Cogrel P, Sergent O, Pasdeloup N, Brissot P, Cillard P, Cillard J (1993) Antioxidant and iron-chelating activities of the flavonoids catechin, quercetin and diosmetin on iron-loaded rat hepatocyte cultures. Biochem Pharmacol 45:13–19

127. Hatano T, Miyatake H, Natsume M, Osakabe N, Takizawa T, Ito H, Yoshida T (2002) Proanthocyanidin glycosides and related polyphenols from cacao liquor and their antioxidant effects. Phytochemistry 59:749–758
128. Pollard SE, Kuhnle GG, Vauzour D, Vafeiadou K, Tzounis X, Whiteman M, Rice-Evans C, Spencer JP (2006) The reaction of flavonoid metabolites with peroxynitrite. Biochem Biophys Res Commun 350:960–968
129. Narotzki B, Reznick AZ, Aizenbud D, Levy Y (2012) Green tea: a promising natural product in oral health. Arch Oral Biol 57:429–435. doi:10.1016/j.archoralbio.2011.11.017
130. McKay DL, Blumberg JB (2002) The role of tea in human health: an update. J Am Coll Nutr 21:1–13
131. Johnson JJ, Bailey HH, Mukhtar H (2010) Green tea polyphenols for prostate cancer chemoprevention: a translational perspective. Phytomedicine 17:3–13. doi:10.1016/j.phymed.2009.09.011
132. Andrade JP, Assunção M (2012) Protective effects of chronic green tea consumption on age-related neurodegeneration. Curr Pharm Des 18:4–14
133. Islam MA (2012) Cardiovascular effects of green tea catechins: progress and promise. Recent Pat Cardiovasc Drug Discov 7:88–99
134. Davinelli S, Sapere N, Zella D, Bracale R, Intrieri M, Scapagnini G (2012) Pleiotropic protective effects of phytochemicals in Alzheimer's disease. Oxid Med Cell Longev 2012:386527. doi:10.1155/2012/386527
135. Hurt RT, Wilson T (2012) Geriatric obesity: evaluating the evidence for the use of flavonoids to promote weight loss. J Nutr Gerontol Geriatr 31:269–289. doi:10.1080/21551197.2012.698222
136. Cimino S, Sortino G, Favilla V, Castelli T, Madonia M, Sansalone S, Russo GI, Morgia G (2012) Polyphenols: key issues involved in chemoprevention of prostate cancer. Oxid Med Cell Longev 2012:632959. doi:10.1155/2012/632959
137. Maheshwari RK, Singh AK, Gaddipati J, Srimal RC (2006) Multiple biological activities of curcumin: a short review. Life Sci 78:2081–2087
138. Grynkiewicz G, Ślifirski P (2012) Curcumin and curcuminoids in quest for medicinal status. Acta Biochim Pol 59:201–212
139. Esatbeyoglu T, Huebbe P, Ernst IM, Chin D, Wagner AE, Rimbach G (2012) Curcumin—from molecule to biological function. Angew Chem Int Ed Engl 51:5308–5332. doi:10.1002/anie.201107724
140. Pan MH, Huang TM, Lin JK (1999) Biotransformation of curcumin through reduction and glucuronidation in mice. Drug Metab Dispos 27:486–494
141. Cheng AL, Hsu CH, Lin JK, Hsu MM, Ho YF, Shen TS, Ko JY, Lin JT, Lin BR, Ming-Shiang W, Yu HS, Jee SH, Chen GS, Chen TM, Chen CA, Lai MK, Pu YS, Pan MH, Wang YJ, Tsai CC, Hsieh CY (2001) Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. Anticancer Res 21:2895–2900
142. Shoba G, Joy D, Joseph T, Majeed M, Rajendran R, Srinivas PS (1998) Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. Planta Med 64:353–356
143. Sharma RA, McLelland HR, Hill KA, Ireson CR, Euden SA, Manson MM, Pirmohamed M, Marnett LJ, Gescher AJ, Steward WP (2001) Pharmacodynamic and pharmacokinetic study of oral Curcuma extract in patients with colorectal cancer. Clin Cancer Res 7:1894–1900
144. Sharma RA, Euden SA, Platton SL, Cooke DN, Shafayat A, Hewitt HR, Marczylo TH, Morgan B, Hemingway D, Plummer SM, Pirmohamed M, Gescher AJ, Steward WP (2004) Phase I clinical trial of oral curcumin: biomarkers of systemic activity and compliance. Clin Cancer Res 10:6847–6854
145. Lao CD, Ruffin MT 4th, Normolle D, Heath DD, Murray SI, Bailey JM, Boggs ME, Crowell J, Rock CL, Brenner DE (2006) Dose escalation of a curcuminoid formulation. BMC Complement Altern Med 6:10
146. Garcea G, Jones DJ, Singh R, Dennison AR, Farmer PB, Sharma RA, Steward WP, Gescher AJ, Berry DP (2004) Detection of curcumin and its metabolites in hepatic tissue and portal blood of patients following oral administration. Br J Cancer 90:1011–1015

147. Garcea G, Berry DP, Jones DJ, Singh R, Dennison AR, Farmer PB, Sharma RA, Steward WP, Gescher AJ (2005) Consumption of the putative chemopreventive agent curcumin by cancer patients: assessment of curcumin levels in the colorectum and their pharmacodynamic consequences. Cancer Epidemiol Biomarkers Prev 14:120–125
148. Ireson C, Orr S, Jones DJ, Verschoyle R, Lim CK, Luo JL, Howells L, Plummer S, Jukes R, Williams M, Steward WP, Gescher A (2001) Characterization of metabolites of the chemopreventive agent curcumin in human and rat hepatocytes and in the rat in vivo, and evaluation of their ability to inhibit Phorbol ester-induced prostaglandin E2 production. Cancer Res 61:1058–1064
149. Asai A, Miyazawa T (2000) Occurrence of orally administered curcuminoid as glucuronide and glucuronide/sulfate conjugates in rat plasma. Life Sci 67:2785–2793
150. Schiborr C, Eckert GP, Rimbach G, Frank J (2010) A validated method for the quantification of curcumin in plasma and brain tissue by fast narrow-bore high-performance liquid chromatography with fluorescence detection. Anal Bioanal Chem 397:1917–1925
151. Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB (2007) Bioavailability of curcumin: problems and promises. Mol Pharm 4:807–818
152. Vareed SK, Kakarala M, Ruffin MT, Crowell JA, Normolle DP, Djuric Z, Brenner DE (2008) Pharmacokinetics of curcumin conjugate metabolites in healthy human subjects. Cancer Epidemiol Biomarkers Prev 17:1411–1417. doi:10.1158/1055-9965.EPI-07-2693
153. Wahlström B, Blennow G (1978) A study on the fate of curcumin in the rat. Acta Pharmacol Toxicol 43:86–92
154. Yang KY, Lin LC, Tseng TY, Wang SC, Tsai TH (2007) Oral bioavailability of curcumin in rat and the herbal analysis from curcuma longa by LC-MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci 853:183–189
155. Priyadarsini KI, Maity DK, Naik GH, Kumar MS, Unnikrishnan MK, Satav JG, Mohan H (2003) Role of phenolic O-H and methylene hydrogen on the free radical reactions and antioxidant activity of curcumin. Free Radic Biol Med 35:475–484
156. Ak T, Gülçin I (2008) Antioxidant and radical scavenging properties of curcumin. Chem Biol Interact 174:27–37. doi:10.1016/j.cbi.2008.05.003
157. Surh YJ (2003) Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer 3:768–780
158. Slocum SL, Kensler TW (2011) Nrf2: control of sensitivity to carcinogens. Arch Toxicol 85:273–284. doi:10.1007/s00204-011-0675-4
159. Goel A, Aggarwal BB (2010) Curcumin, the golden spice from Indian saffron, is a chemosensitizer and radiosensitizer for tumors and chemoprotector and radioprotector for normal organs. Nutr Cancer 62:919–930. doi:10.1080/01635581.2010.509835
160. Donnez D, Jeandet P, Clément C, Courot E (2009) Bioproduction of resveratrol and stilbene derivatives by plant cells and microorganisms. Trends Biotechnol 27:706–713. doi:10.1016/j.tibtech.2009.09.005
161. Paredes-López O, Cervantes-Ceja ML, Vigna-Pérez M, Hernández-Pérez T (2010) Berries: improving human health and healthy aging, and promoting quality life - a review. Plant Foods Hum Nutr 65:299–308. doi:10.1007/s11130-010-0177-1
162. Castrejón ADR, Eichholz I, Rohn S, Kroh LW, Huyskens-Keil S (2008) Phenolic profile and antioxidant activity of high bush blueberry (Vaccinium corymbosum L.) during fruit maturation and ripening. Food Chem 109:564–572. doi:10.1016/j.foodchem.2008.01.007
163. Vitrac X, Moni JP, Vercauteren J, Deffieux G, Mérillon JM (2002) Direct liquid chromatography analysis of resveratrol derivatives and flavanonols in wines with absorbance and fluorescence detection. Anal Chim Acta 458:103–110. doi:10.1016/S0003-2670(01)01498-2
164. Gupta SC, Kannappan R, Reuter S, Kim JH, Aggarwal BB (2011) Chemosensitization of tumors by resveratrol. Ann N Y Acad Sci 1215:150–160. doi:10.1111/j.1749-6632.2010.05852.x
165. Frei B (2004) Efficacy of dietary antioxidants to prevent oxidative damage and inhibit chronic disease. J Nutr 134:3196–3198
166. Kundu JK, Surh YJ (2008) Cancer chemopreventive and therapeutic potential of resveratrol: mechanistic perspectives. Cancer Lett 269:243–261. doi:10.1016/j.canlet.2008.03.057

167. Kundu JK, Shin YK, Kim SH, Surh YJ (2006) Resveratrol inhibits Phorbol ester-induced expression of COX-2 and activation of NFkappaB in mouse skin by blocking IkappaB kinase activity. Carcinogenesis 27:1465–1474
168. Subbaramaiah K, Chung WJ, Michaluart P, Telang N, Tanabe T, Inoue H, Jang M, Pezzuto JM, Dannenberg AJ (1998) Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. J Biol Chem 273:21875–21882
169. Carluccio MA, Ancora MA, Massaro M, Carluccio M, Scoditti E, Distante A, Storelli C, De Caterina R (2007) Homocysteine induces VCAM-1 gene expression through NF-kappaB and NAD(P)H oxidase activation: protective role of Mediterranean diet polyphenolic antioxidants. Am J Physiol Heart Circ Physiol 293:2344–2354
170. Csiszar A, Smith K, Labinsky N, Orosz Z, Rivera A, Ungvari Z (2006) Resveratrol attenuates TNF-alpha-induced activation of coronary arterial endothelial cells: role of NF-kappaB inhibition. Am J Physiol Heart Circ Physiol 291:1694–1699
171. Shankar S, Nall D, Tang SN, Meeker D, Passarini J, Sharma J, Srivastava RK (2011) Resveratrol inhibits pancreatic cancer stem cell characteristics in human and KrasG12D transgenic mice by inhibiting pluripotency maintaining factors and epithelialmesenchymal transition. PLoS One 6:e16530. doi:10.1371/journal.pone.0016530
172. Vergara D, Simeone P, Toraldo D, Del Boccio P, Vergaro V, Leporatti S, Pieragostino D, Tinelli A, De Domenico S, Alberti S, Urbani A, Salzet M, Santino A, Maffia M (2012) Resveratrol downregulates Akt/GSK and ERK signalling pathways in OVCAR-3 ovarian cancer cells. Mol Biosyst 8:1078–1087. doi:10.1039/c2mb05486h
173. Sharma S, Chopra K, Kulkarni SK (2007) Effect of insulin and its combination with resveratrol or curcumin in attenuation of diabetic neuropathic pain: participation of nitric oxide F TNF-alpha. Phytother Res 21:278–283
174. Giovinazzo G, Ingrosso I, Paradiso A, De Gara L, Santino A (2012) Resveratrol biosynthesis: plant metabolic engineering for nutritional improvement of food. Plant Foods Hum Nutr 67:191–199
175. Walle T, Hsieh F, DeLegge MH, Oatis JE Jr, Walle UK (2004) High absorption but very low bioavailability of oral resveratrol in humans. Drug Metab Dispos 32:1377–1382
176. Walle T (2011) Bioavailability of resveratrol. Ann N Y Acad Sci 1215:9–15. doi:10.1111/j.1749-6632.2010.05842.x
177. Hertog MG, Hollman PC, Katan MB, Kromhout D (1993) Intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands. Nutr Cancer 20:21–29
178. Bischoff SC (2008) Quercetin: potentials in the prevention and therapy of disease. Curr Opin Clin Nutr Metab Care 11:733–740. doi:10.1097/MCO.0b013e32831394b8
179. Chen C, Zhou J, Ji C (2010) Quercetin: a potential drug to reverse multidrug resistance. Life Sci 87:333–338. doi:10.1016/j.lfs.2010.07.004
180. Kressler J, Millard-Stafford M, Warren GL (2011) Quercetin and endurance exercise capacity: a systematic review and meta-analysis. Med Sci Sports Exerc 43:2396–2404. doi:10.1249/MSS.0b013e31822495a7
181. Mendoza EE, Burd R (2011) Quercetin as a systemic chemopreventative agent: structural and functional mechanisms. Mini Rev Med Chem 11:1216–1221
182. Larson AJ, Symons JD, Jalili T (2010) Quercetin: a treatment for hypertension? a review of efficacy and mechanisms. Pharmaceuticals 3:237–250. doi:10.3390/ph3010237
183. Russo M, Spagnuolo C, Tedesco I, Bilotto S, Russo GL (2012) The flavonoid quercetin in disease prevention and therapy: facts and fancies. Biochem Pharmacol 83:6–15. doi:10.1016/j.bcp.2011.08.010
184. Vissiennon C, Nieber K, Kelber O, Butterweck V (2012) Route of administration determines the anxiolytic activity of the flavonols kaempferol, quercetin and myricetin - are they prodrugs? J Nutr Biochem 23:733–740. doi:10.1016/j.jnutbio.2011.03.017
185. Dajas F (2012) Life or death: neuroprotective and anticancer effects of quercetin. J Ethnopharmacol 143:383–396. doi:10.1016/j.jep.2012.07.005
186. Lamson DW, Brignall MS (2000) Antioxidants and cancer part3: quercetin. Altern Med Rev 5:196–208

187. Kelly GS (2011) Quercetin. Monograph. Altern Med Rev 16:172–194
188. Manach C, Morand C, Texier O, Favier ML, Agullo G, Demigné C, Régérat F, Rémésy C (1995) Quercetin metabolites in plasma of rats fed diets. J Nutr 125:1911–1922
189. Paulke A, Nöldner M, Schubert-Zsilavecz M, Wurglics M (2008) St. John's wort flavonoids and their metabolites show antidepressant activity and accumulate in brain after multiple oral doses. Pharmazie 63:296–302
190. Rangel-Ordóñez L, Nöldner M, Schubert-Zsilavecz M, Wurglics M (2010) Plasma levels and distribution of flavonoids in rat brain after single and repeated doses of standardized Ginkgo biloba extract EGb 761®. Planta Med 76:1683–1690. doi:10.1055/s-0030-1249962
191. Banerjee S, Li Y, Wang Z, Sarkar FH (2008) Multi-targeted therapy of cancer by genistein. Cancer Lett 269:226–242. doi:10.1016/j.canlet.2008.03.052
192. Perabo FG, Von Löw EC, Ellinger J, von Rücker A, Müller SC, Bastian PJ (2008) Soy isoflavone genistein in prevention and treatment of prostate cancer. Prostate Cancer Prostatic Dis 11:6–12
193. Si H, Liu D (2007) Phytochemical genistein in the regulation of vascular function: new insights. Curr Med Chem 14:2581–2589
194. Marini H, Minutoli L, Polito F, Bitto A, Altavilla D, Atteritano M, Gaudio A, Mazzaferro S, Frisina A, Frisina N, Lubrano C, Bonaiuto M, D'Anna R, Cannata ML, Corrado F, Adamo EB, Wilson S, Squadrito F (2007) Effects of the phytoestrogen genistein on bone metabolism in osteopenic postmenopausal women: a randomized trial. Ann Intern Med 146:839–847
195. Adlercreutz H, Markkanen H, Watanabe S (1993) Plasma concentrations of phyto-oestrogens in Japanese men. Lancet 342:1209–1210
196. Mills PK, Beeson WL, Phillips RL, Fraser GE (1989) Cohort study of diet lifestyle and prostate cancer in Adventist men. Cancer 64:598–604
197. Knight DC, Eden JA (1996) A review of the clinical effects of phytoestrogens. Obstet Gynecol 87:897–904
198. Zava DT, Dollbaum CM, Blen M (1998) Estrogen and progestin bioactivity of foods, herbs, and spices. Proc Soc Exp Biol Med 217:369–378
199. Konishi T (2009) Brain oxidative stress as basic target of antioxidant traditional oriental medicines. Neurochem Res 34:711–716. doi:10.1007/s11064-008-9872-9
200. Cheng JT (2000) Review: drug therapy in Chinese traditional medicine. J Clin Pharmacol 40:445–450
201. Liu J, Edamatsu R, Kabuto H, Mori A (1990) Antioxidant action of guilingji in the brain of rats with FeCl3-induced epilepsy. Free Radic Biol Med 9:451–454
202. Ou B, Huang D, Hampsch-woodill M, Flanagan JA (2003) When east meets west: the relationship between yin-yang and antioxidation-oxidation. FASEB J 17:127–129
203. Rausch WD, Liu S, Gille G, Radad K (2006) Neuroprotective effects of ginsenosides. Acta Neurobiol Exp 66:369–375
204. Tang SY, Whiteman M, Peng ZF, Jenner A, Young EL, Halliwell B (2004) Characterization of antioxidant and antiglycation properties and isolation of active ingredients from traditional Chinese medicines. Free Radic Biol Med 36:1575–1587
205. Stickel F, Schuppan D (2007) Herbal medicine in the treatment of liver diseases. Dig Liver Dis 39:293–304
206. Surathu N, Kurumathur AV (2011) Traditional therapies in the management of periodontal disease in India and China. Periodontol 2000 56:14–24. doi:10.1111/j.1600-0757.2010.00369.x
207. Chava VK, Vedula BD (2012) Thermo reversible green tea catechin gel for local application in chronic periodontitis - a 4 week clinical trial. J Periodontol. doi:10.1902/jop.2012.120425
208. Hirasawa M, Takada K, Makimura M, Otake S (2002) Improvement of periodontal status by green tea catechin using a local delivery system: a clinical pilot study. J Periodontal Res 37:433–438
209. Krahwinkel T, Willershausen B (2000) The effect of sugar-free green tea chew candies on the degree of inflammation of the gingiva. Eur J Med Res 5:463–467
210. Tamura M, Saito H, Kikuchi K, Ishigami T, Toyama Y, Takami M, Ochiai K (2011) Antimicrobial activity of Gel-entrapped catechins toward oral microorganisms. Biol Pharm Bull 34:638–643

211. Taylor PW, Hamilton-Miller JM, Stapleton PD (2005) Antimicrobial properties of green tea catechins. Food Sci Technol Bull 2:71–81
212. Okamoto M, Sugimoto A, Leung KP, Nakayama K, Kamaguchi A, Maeda N (2004) Inhibitory effect of green tea catechins on cysteine proteinases in Porphyromonas gingivalis. Oral Microbiol Immunol 19:118–120
213. Petti S, Scully C (2009) Polyphenols, oral health and disease: a review. J Dent 37:413–423. doi:10.1016/j.jdent.2009.02.003
214. Ho KY, Tsai CC, Huang JS, Chen CP, Lin TC, Lin CC (2001) Antimicrobial activity of tannin components from Vaccinium vitis-idaea L. J Pharm Pharmacol 53:187–191
215. Yang F, de Villiers WJ, McClain CJ, Varilek GW (1998) Green tea polyphenols block endotoxin-induced tumor necrosis factor-production and lethality in a murine model. J Nutr 128:2334–2340
216. Crouvezier S, Powell B, Keir D, Yaqoob P (2001) The effects of phenolic components of tea on the production of pro- and anti-inflammatory cytokines by human leukocytes in vitro. Cytokine 13:280–286
217. Hosokawa Y, Hosokawa I, Ozaki K, Nakanishi T, Nakae H, Matsuo T (2010) Tea polyphenols inhibit IL-6 production in tumor necrosis factor superfamily 14-stimulated human gingival fibroblasts. Mol Nutr Food Res 54(Suppl 2):S151–S158. doi:10.1002/mnfr.200900549
218. Hosokawa Y, Hosokawa I, Ozaki K, Nakanishi T, Nakae H, Matsuo T (2010) Catechins inhibit CXCL10 production from oncostatin M-stimulated human gingival fibroblasts. J Nutr Biochem 21:659–664. doi:10.1016/j.jnutbio.2009.04.005
219. Hosokawa Y, Hosokawa I, Ozaki K, Nakanishi T, Nakae H, Matsuo T (2009) Catechins inhibit CCL20 production in IL-17A-stimulated human gingival fibroblasts. Cell Physiol Biochem 24:391–396. doi:10.1159/000257431
220. Yun JH, Kim CS, Cho KS, Chai JK, Kim CK, Choi SH (2007) (−)-Epigallocatechin gallate induces apoptosis, via caspase activation, in osteoclasts differentiated from RAW 264.7 cells. J Periodontal Res 42:212–218
221. Yun JH, Pang EK, Kim CS, Yoo YJ, Cho KS, Chai JK, Kim CK, Choi SH (2004) Inhibitory effects of green tea polyphenol (−)-epigallocatechin gallate on the expression of matrix metalloproteinase-9 and on the formation of osteoclasts. J Periodontal Res 39:300–307
222. Chen YW, Yang WH, Wong MY, Chang HH, Yen-Ping Kuo M (2012) Curcumin inhibits thrombin-stimulated connective tissue growth factor (CTGF/CCN2) production through c-Jun NH2-terminal kinase suppression in human gingival fibroblasts. J Periodontol 83:1546–1553. doi:10.1902/jop.2012.110641
223. Atsumi T, Tonosaki K, Fujisawa S (2006) Induction of early apoptosis and ROS-generation activity in human gingival fibroblasts (HGF) and human submandibular gland carcinoma (HSG) cells treated with curcumin. Arch Oral Biol 51:913–921
224. Atsumi T, Fujisawa S, Tonosaki K (2005) Relationship between intracellular ROS production and membrane mobility in curcumin- and tetrahydrocurcumin-treated human gingival fibroblasts and human submandibular gland carcinoma cells. Oral Dis 11:236–242
225. Zhou T, Chen D, Li Q, Sun X, Song Y, Wang C (2013) Curcumin inhibits inflammatory response and bone loss during experimental periodontitis in rats. Acta Odontol Scand 71:349–356. doi:10.3109/00016357.2012.682092
226. Guimarães MR, Coimbra LS, de Aquino SG, Spolidorio LC, Kirkwood KL, Rossa C Jr (2011) Potent anti-inflammatory effects of systemically administered curcumin modulate periodontal disease in vivo. J Periodontal Res 46:269–279
227. Guimarães MR, de Aquino SG, Coimbra LS, Spolidorio LC, Kirkwood KL, Rossa C Jr (2012) Curcumin modulates the immune response associated with LPS-induced periodontal disease in rats. Innate Immun 18:155–163. doi:10.1177/1753425910392935
228. Kim SJ (2011) Curcumin suppresses the production of interleukin-6 in Prevotella intermedia lipopolysaccharide-activated RAW 264.7 cells. J Periodontal Implant Sci 41:157–163. doi:10.5051/jpis.2011.41.3.157
229. Chen D, Nie M, Fan MW, Bian Z (2008) Anti-inflammatory activity of curcumin in macrophages stimulated by lipopolysaccharides from Porphyromonas gingivalis. Pharmacology 82:264–269. doi:10.1159/000161127

230. Watanabe A, Takeshita A, Kitano S, Hanazawa S (1996) CD14-mediated signal pathway of Porphyromonas gingivalis lipopolysaccharide in human gingival fibroblasts. Infect Immun 64:4488–4494
231. Casati MZ, Algayer C, Cardoso da Cruz G, Ribeiro FV, Casarin RC, Pimentel SP, Cirano FR (2013) Resveratrol decreases periodontal breakdown and modulate local levels of cytokines during periodontitis in rats. J Periodontol. doi:10.1902/jop.2013.120746
232. Park GJ, Kim YS, Kang KL, Bae SJ, Baek HS, Auh QS, Chun YH, Park BH, Kim EC (2012) Effects of sirtuin 1 activation on nicotine and lipopolysaccharide-induced cytotoxicity and inflammatory cytokine production in human gingival fibroblasts. J Periodontal Res. doi:10.1111/jre.12030
233. Rizzo A, Bevilacqua N, Guida L, Annunziata M, Romano Carratelli C, Paolillo R (2012) Effect of resveratrol and modulation of cytokine production on human periodontal ligament cells. Cytokine 60:197–204. doi:10.1016/j.cyto.2012.06.004
234. Park HJ, Jeong SK, Kim SR, Bae SK, Kim WS, Jin SD, Koo TH, Jang HO, Yun I, Kim KW, Bae MK (2009) Resveratrol inhibits Porphyromonas gingivalis lipopolysaccharide-induced endothelial adhesion molecule expression by suppressing NF-kappaB activation. Arch Pharm Res 32:583–591. doi:10.1007/s12272-009-1415-7
235. Andreou V, D'Addario M, Zohar R, Sukhu B, Casper RF, Ellen RP, Tenenbaum HC (2004) Inhibition of osteogenesis in vitro by a cigarette smoke-associated hydrocarbon combined with Porphyromonas gingivalis lipopolysaccharide: reversal by resveratrol. J Periodontol 75:939–948
236. Takahama U, Hirota S, Oniki T (2006) Quercetin-dependent scavenging of reactive nitrogen species derived from nitric oxide and nitrite in the human oral cavity: interaction of quercetin with salivary redox components. Arch Oral Biol 51:629–639
237. Cheng WC, Huang RY, Chiang CY, Chen JK, Liu CH, Chu CL, Fu E (2010) Ameliorative effect of quercetin on the destruction caused by experimental periodontitis in rats. J Periodontal Res 45:788–795. doi:10.1111/j.1600-0765.2010.01301.x
238. Gutiérrez-Venegas G, Jiménez-Estrada M, Maldonado S (2007) The effect of flavonoids on transduction mechanisms in lipopolysaccharide-treated human gingival fibroblasts. Int Immunopharmacol 7:1199–1210
239. Li M, Xu Z (2008) Quercetin in a lotus leaves extract may be responsible for antibacterial activity. Arch Pharm Res 31:640–644. doi:10.1007/s12272-001-1206-5
240. Sosroseno W, Bird PS, Seymour GJ (2011) Nitric oxide production by a murine macrophage cell line (RAW264.7 cells) stimulated with Aggregatibacter actinomycetemcomitans surface-associated material. Anaerobe 17:246–251. doi:10.1016/j.anaerobe.2011.06.006
241. Gutiérrez-Venegas G, Kawasaki-Cárdenas P, Arroyo-Cruz SR, Maldonado-Frías S (2006) Luteolin inhibits lipopolysaccharide actions on human gingival fibroblasts. Eur J Pharmacol 541:95–105
242. Gutiérrez-Venegas G, Kawasaki-Cárdenas P, Cruz-Arroyo SR, Pérez-Garzón M, Maldonado-Frías S (2006) Actinobacillus actinomycetemcomitans lipopolysaccharide stimulates the phosphorylation of p44 and p42 MAP kinases through CD14 and TLR-4 receptor activation in human gingival fibroblasts. Life Sci 78:2577–2583
243. Luo LJ, Liu F, Lin ZK, Xie YF, Xu JL, Tong QC, Shu R (2012) Genistein regulates the IL-1 beta induced activation of MAPKs in human periodontal ligament cells through G protein-coupled receptor 30. Arch Biochem Biophys 522:9–16. doi:10.1016/j.abb.2012.04.007
244. Kubo M, Matsuda H, Tanaka M, Kimura Y, Okuda H, Higashino M, Tani T, Namba K, Arichi S (1984) Studies on Scutellariae radix: VII. Anti-arthritic and anti-inflammatory actions of methanolic extract and flavonoid components from Scutellariae radix. Chem Pharm Bull (Tokyo) 32:2724–2729
245. Tominari T, Hirata M, Matsumoto C, Inada M, Miyaura C (2012) Polymethoxy flavonoids, nobiletin and tangeretin, prevent lipopolysaccharide-induced inflammatory bone loss in an experimental model for periodontitis. J Pharmacol Sci 119:390–394
246. Feghali K, Feldman M, La VD, Santos J, Grenier D (2011) Cranberry proanthocyanidins: natural weapons against periodontal diseases. J Agric Food Chem 60:5728–5735. doi:10.1021/jf203304v

247. La VD, Howell AB, Grenier D (2010) Anti-Porphyromonas gingivalis and anti-inflammatory activities of A-type cranberry proanthocyanidins. Antimicrob Agents Chemother 54:1778–1784. doi:10.1128/AAC.01432-09
248. La VD, Howell AB, Grenier D (2009) Cranberry proanthocyanidins inhibit MMP production and activity. J Dent Res 88:627–632. doi:10.1177/0022034509339487
249. Tanabe S, Santos J, La VD, Howell AB, Grenier D (2011) A-type cranberry proanthocyanidins inhibit the RANKL-dependent differentiation and function of human osteoclasts. Molecules 16:2365–2374. doi:10.3390/molecules16032365
250. Lo'pez-La'zaro M (2009) Distribution and biological activities of the flavonoid luteolin. Mini Rev Med Chem 9:31–59
251. Choi EY, Jin JY, Choi JI, Choi IS, Kim SJ (2011) Effects of luteolin on the release of nitric oxide and interleukin-6 by macrophages stimulated with lipopolysaccharide from Prevotella intermedia. J Periodontol 82:1509–1517. doi:10.1902/jop.2011.100759
252. Jeong GS, Lee SH, Jeong SN, Kim YC, Kim EC (2009) Anti-inflammatory effects of apigenin on nicotine- and lipopolysaccharide-stimulated human periodontal ligament cells via heme oxygenase-1. Int Immunopharmacol 9:1374–1380. doi:10.1016/j.intimp.2009.08.015
253. Dar A, Faizi S, Naqvi S, Roome T, Zikr-ur-Rehman S, Ali M, Firdous S, Moin ST (2005) Analgesic and antioxidant activity of mangiferin and its derivatives: the structure activity relationship. Biol Pharm Bull 28:596–600
254. Anton R (1988) Flavonoids and traditional medicine. Prog Clin Biol Res 280:423–439
255. Kim HK, Park HR, Lee JS, Chung TS, Chung HY, Chung J (2007) Down-regulation of iNOS and TNF-alpha expression by kaempferol via NF-kappaB inactivation in aged rat gingival tissues. Biogerontology 8:399–408
256. Kimbrough C, Chun M, dela Roca G, Lau BH (2002) PYCNOGENOL chewing gum minimizes gingival bleeding and plaque formation. Phytomedicine 9:410–413
257. Ohsawa I, Ishikawa M, Takahashi K, Watanabe M, Nishimaki K, Yamagata K, Katsura K, Katayama Y, Asoh S, Ohta S (2007) Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. Nat Med 13:688–694
258. Ekuni D, Tomofuji T, Endo Y, Kasuyama K, Irie K, Azuma T, Tamaki N, Mizutani S, Kojima A, Morita M (2012) Hydrogen-rich water prevents lipid deposition in the descending aorta in a rat periodontitis model. Arch Oral Biol 57:1615–1622. doi:10.1016/j.archoralbio.2012.04.013
259. Biswas K, Chattopadhyay I, Banerjee RK, Bandyopadhyay U (2002) Biological activities and medicinal properties of neem (Azadirachta indica). Curr Sci 82:1336–1345
260. Botelho MA, Santos RA, Martins JG, Carvalho CO, Paz MC, Azenha C, Ruela RS, Queiroz DB, Ruela WS, Marinho G, Ruela FI (2008) Efficacy of a mouthrinse based on leaves of the neem tree (Azadirachta indica) in the treatment of patients with chronic gingivitis: a doubleblind, randomized, controlled trial. J Med Plants Res 2:341–346
261. Pai MR, Acharya LD, Udupa N (2004) The effect of two different dental gels and a mouthwash on plaque and gingival scores: a six-week clinical study. Int Dent J 54:219–223
262. Sharma S, Saimbi CS, Koirala B, Shukla R (2008) Effect of various mouthwashes on the levels of interleukin-2 and interferongamma in chronic gingivitis. J Clin Pediatr Dent 32:111–114
263. Vanka A, Tandon S, Rao SR, Udupa N, Ramkumar P (2001) The effect of indigenous Neem Azadirachta indica [correction of (Adirachta indica)] mouth wash on Streptococcus mutans and lactobacilli growth. Indian J Dent Res 12:133–144
264. Abraham S, Kumar MS, Sehgal PK, Nitish S, Jayakumar ND (2005) Evaluation of the inhibitory effect of triphala on PMN-type matrix metalloproteinase (MMP-9). J Periodontol 76:497–502
265. Asokan S, Emmadi P, Chamundeswari R (2009) Effect of oil pulling on plaque induced gingivitis: a randomized, controlled, triple-blind study. Indian J Dent Res 20:47–51
266. Sankar D, Sambandam G, Rao R, Pugalendi KV (2005) Modulation of blood pressure, lipid profiles and redox status in hypertensive patients taking different edible oils. Clin Chim Acta 355:97–104

267. Zhang JZ, Yang XX, Tong YH (1992) Clinical study on using Guchiwan and spiromycin to treat periodontal disease. Chin J Integr Chin Western Med 12:83–85
268. Song H, Zhao RF, Zhou YJ (1996) The effect of Guchigao on gingival crevicular fluid IL-8 in periodontitis patients. In: Proceedings of the 3rd national conference on integrated Chinese and western medicine stomatology, Xianen, pp 25–28
269. Mullally BH, James JA, Coulter WA, Linden GJ (1995) The efficacy of a herbal-based toothpaste on the control of plaque and gingivitis. J Clin Periodontol 22:686–689
270. Van der Weijden GA, Timmer CJ, Timmerman MF, Reijerse E, Mantel MS, van der Velden U (1998) The effect of herbal extracts in an experimental mouthrinse on established plaque and gingivitis. J Clin Periodontol 25:399–403
271. Wu SJ, Ng LT, Lin CC (2004) Antioxidant activities of some common ingredients of traditional Chinese medicine, Angelica sinensis, Lycium barbarum and Poria cocos. Phytother Res 18:1008–1012
272. Chan Y, Lai CH, Yang HW, Lin YY, Chan CH (2003) The evaluation of Chinese herbal medicine effectiveness on periodontal pathogens. Am J Chin Med 31:751–761
273. Asman B, Wijkander P, Hjerpe A (1994) Reduction of collagen degradation in experimental granulation tissue by vitamin E and selenium. J Clin Periodontol 21:45–47
274. Bjelakovic G, Nikolova D, Simonetti RG, Gluud C (2004) Antioxidant supplements for prevention of gastrointestinal cancers: a systematic review and etaanalysis. Lancet 364:1219–1228
275. Bjelakovic G, Nikolova D, Simonetti RG, Gluud C (2008) Systematic review: primary and secondary prevention of gastrointestinal cancers with antioxidant supplements. Aliment Pharmacol Ther 28:689–703
276. Dietrich M, Traber MG, Jacques PF, Cross CE, Hu Y, Block G (2006) Does gamma-tocopherol play a role in the primary prevention of heart disease and cancer? A review. J Am Coll Nutr 25:292–299
277. Halliwell B (2009) The wanderings of a free radical. Free Radic Biol Med 46:531–542. doi:10.1016/j.freeradbiomed.2008.11.008
278. Hercberg S, Ezzedine K, Guinot C, Preziosi P, Galan P, Bertrais S, Estaquio C, Briançon S, Favier A, Latreille J, Malvy D (2007) Antioxidant supplementation increases the risk of skin cancers in women but not in men. J Nutr 137:2098–2105

Chapter 19
Role of Vitamin C and Vitamin E in Periodontal Disease

Hideki Nagata

19.1 Introduction

Vitamin C is a water-soluble organic substance that cannot be synthesized by the body; therefore, it must be obtained from an individual's daily diet. Also known as ascorbic acid, it is involved in wound healing and collagen production by preventing iron-dependent oxidation of lysyl and prolyl hydroxylase. In human and animal studies on vitamin C deficiency, ascorbate supplementation increased collagen synthesis and decreased polymorphonuclear neutrophil (PMN) chemotaxis [4, 5, 9]. High vitamin C levels are accumulated in granulocytes, mononuclear leucocytes, and platelets [28], and neutrophil polymorphonuclear leucocytes and macrophages contain an intracellular ascorbate concentration that is 10–40 times higher than that in the plasma [58]. Chapple and Matthews [19] summarized the following functions of vitamin C: (1) scavenging water-soluble peroxyl radicals; (2) scavenging superoxide and perhydroxyl radicals; (3) preventing damage mediated by hydroxyl radicals on uric acid; (4) scavenging hypochlorous acid; (5) decreasing heme breakdown and subsequent Fe^{2+} release, thereby preventing Fenton reactions; (6) scavenging single oxygen and hydroxyl radicals; (7) re-forming α-tocopherol from its radical; (8) protecting against reactive oxygen species (ROS) released from cigarette smoke; (9) reducing C-reactive protein-mediated expression of monocyte adhesion molecules; (10) decreasing pro-inflammatory gene expression through effects on the nuclear factor-κB transcription factor.

Vitamin E, which comprises related compounds named tocopherols or tocotrienols, is a fat-soluble vitamin with primary function of antioxidation. It is essential for maintaining cell membrane integrity against lipid peroxidation by peroxyl

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radical scavenging. In cells, a major amount of vitamin E is situated in the membranes, adjacent to unsaturated fatty acids that are vulnerable to attack by free radicals [65].

Dietary antioxidants such as vitamin C and vitamin E have been shown to exert protective effects against diseases involving chronic inflammation [38, 65], one of them being periodontal disease. Although periodontal disease is caused by periodontopathic bacteria, it is recognized as a multifactorial disease. The presence of ROS has been suggested to play a central part in tissue damage associated with chronic inflammatory conditions such as periodontal disease [30]. Periodontal disease is associated with an increased production of ROS, which, if not buffered by sufficient antioxidants, can cause damage to the host cells and periodontal tissues [50]. Waddington et al. [87] reviewed a potential role of ROS in the pathogenesis of periodontal disease, and indicated that ROS may play a part in the direct degradation of connective tissue components and modification of their structures; this is likely to lead to a loss in periodontal function. ROS may also lead to altered metabolic activity in the connective tissues, although the precise contribution of ROS has not been fully elucidated. It has been reported that plasma ascorbic acid has a strong inverse relation with biomarkers of oxidative damage [14]. Increased levels of these biomarkers have been found in patients with periodontal disease [79], and initial periodontal treatment has been reported to decrease the levels of 8-hydroxydeoxyguanosine (8-OHdG), which is one of these biomarkers [80]. Studies investigating the association of vitamin C and vitamin E as antioxidants with periodontal disease have been performed for many years, and several reviews have been published [10, 19, 26, 54, 60, 67, 71, 75, 76, 84, 85, 89]. In this chapter, the studies published after 1980 are presented to review the role of vitamin C and vitamin E in periodontal disease.

19.2 Association of Vitamin C Levels with Periodontal Disease

19.2.1 Association of Vitamin C Levels with Gingivitis

For a very long time, an association between vitamin C deficiency and the clinical features of gingivitis has been reported and accepted. Melnick et al. [48] showed a significant association between decreased plasma ascorbate levels and acute necrotizing ulcerative gingivitis (ANUG) in a case–control study including 60 patients with a history of ANUG infection and 60 age–race–sex-matched controls. Leggott et al. [40] investigated the effects of controlled ascorbic acid depletion and supplementation on periodontal health and found that measures of gingival inflammation were directly related to ascorbic acid levels. The same authors performed another study [41] and reported that gingival bleeding increased significantly after the period of ascorbic acid depletion and returned to baseline values after the period of ascorbic acid repletion, even though no significant changes in plaque accumulation,

probing pocket depth (PPD), or clinical attachment level (CAL) were observed. Therefore, it is considered that vitamin C intake may influence gingival status and vitamin C supplementation may be useful for preventing gingivitis.

19.2.2 Animal Studies on the Effects of Vitamin C on Periodontal Disease

To investigate the effects of vitamin C on periodontal disease, studies using animal models have been conducted. Alvares et al. [5] evaluated the effects of chronic subclinical ascorbic acid deficiency on periodontal health in a monkey model and found that gingival index (GI) [46] score and PPD were significantly greater in the ascorbate deficient animals than in the controls. Recent animal studies indicated the efficacy of vitamin C in improving periodontal disease. Sanbe et al. [69] examined the effects of vitamin C on bone resorption in rats fed a high-cholesterol diet and showed that vitamin C intake decreased the effects of a high-cholesterol diet on alveolar bone density and osteoclast differentiation and decreased periodontal 8-OHdG expression. In a ligature-induced rat periodontitis model, Tomofuji et al. [82] demonstrated that vitamin C intake induced an increase in plasma vitamin C levels, resulting in an improvement in gingival 8-OHdG levels and the reduced form glutathione (GSH):oxidized form glutathione (GSSG) ratio. Moreover, gene expression for interleukin-1α and interleukin-1β was down-regulated by more than twofold after vitamin C intake, suggesting that systemic administration of vitamin C can be clinically beneficial in improving periodontitis-induced oxidative stress through down-regulation of inflammatory gene expression. In the same ligature-induced periodontitis rat model, Ekuni et al. [27] found that vitamin C intake significantly increased plasma vitamin C levels and the GSH:GSSG ratio. More recently, Akman et al. [3] showed that α-lipoic acid and vitamin C treatment exhibited beneficial effects on the mesial/distal periodontal bone support in regions of ligature-induced periodontitis around the teeth of a rat model and concluded that α-lipoic acid and vitamin C treatment exerted therapeutic effects on inhibition of alveolar bone resorption. From these results, vitamin C appears to possess a potent therapeutic effect on periodontal disease in animal models.

19.2.3 Association of Vitamin C Levels in Body Fluids with Periodontal Disease

19.2.3.1 Association of Plasma/Serum Vitamin C Levels with Periodontal Disease

A number of studies analyzing the association of plasma/serum vitamin C levels with periodontal disease have been reported, and most of them indicate that plasma/serum

vitamin C levels are decreased in patients with periodontal disease. Väänänen et al. [83] compared the periodontal condition of 75 dentulous subjects (plasma vitamin C levels, <25 μmol/L) with that of 75 control subjects (plasma vitamin C levels, ≥50 μmol/L) matched for age, sex, and number of teeth. The proportion of sites with bleeding on probing (BOP) and a PPD of ≥4 mm was significantly higher in the study group than in the control group. In the analysis of covariance, the influence of low plasma ascorbic acid levels on the presence of both gingival inflammation and deep pockets remained statistically significant when age, sex, vocational education, brushing of teeth, smoking status, and dental plaque were standardized. Pussinen et al. [64] investigated the association of plasma vitamin C levels with periodontal disease by measuring the antibodies to periodontopathic bacteria in 431 men from Finland and Russia. They found that the antibody levels to *Porphyromonas gingivalis* were negatively correlated with plasma vitamin C levels; this association remained significant in a linear regression model after adjustment for age, number of teeth and fillings, serum carbohydrate-deficient transferrin levels, and number of cigarettes smoked/day. *P. gingivalis*-seropositivity decreased with an increase in vitamin C levels. Amarasena et al. [7] also analyzed the relationship between serum vitamin C levels and periodontitis as estimated by CAL in 413 community-dwelling Japanese individuals aged 70 years. They found that serum vitamin C levels were inversely related to CAL and that CAL was 4 % greater in subjects with lower serum vitamin C levels than in those with higher serum vitamin C levels using multiple linear regression analysis, notwithstanding the factors of smoking status, diabetes, oral hygiene, sex, or number of teeth present. The authors concluded that serum vitamin C levels may have a relatively weak but significant relationship with periodontitis in the elderly population. Panjamurthy et al. [61] assessed the degree of oxidative stress in the plasma of 25 patients with chronic periodontitis and 25 healthy subjects and reported that enzymatic antioxidant activities were significantly higher while the nonenzymatic antioxidant levels were significantly lower in the plasma, erythrocytes, erythrocyte membranes, and gingival tissues of the patients with periodontal disease than in those of the healthy subjects. Vitamin C levels in the plasma of patients with periodontal disease were significantly lower than those in the plasma of the healthy subjects. A similar result was observed by Staudte et al. [78], who reported significantly decreased plasma vitamin C levels in patients with chronic periodontitis compared with those in healthy controls. In analysis of 11,480 subjects in the Third National Health and Nutrition Examination Survey (NHANES III), Chapple et al. [20] examined the association of serum antioxidant levels with an altered relative risk for periodontal disease and found a strong and consistent inverse association between serum vitamin C levels and the prevalence of periodontitis using multiple logistic regression analysis adjusted for age, sex, race/ethnicity, body mass index, cigarette smoking status, oral contraceptive and hormone replacement therapy use, diabetes, poverty income ratio, and education, with the association being stronger in patients with severe disease. Higher serum antioxidant levels were associated with a lower odds ratio (OR) for severe periodontitis [OR, 0.53; 95 % confidence interval (CI), 0.42–0.68]. Even in never-smokers, the protective effect was more pronounced (OR, 0.38; 95 % CI, 0.26–0.63). The authors concluded that

increased serum antioxidant levels are associated with a decreased relative risk of periodontitis, even in never-smokers. In the JAVA project conducted by Amaliya et al. [6], the negative association between plasma vitamin C levels and CAL was demonstrated in 123 Indonesian subjects. Subjects with vitamin C deficiency (<2.0 mg/L) exhibited greater attachment loss compared with that in subjects with depleted (2.0–3.9 mg/L) or normal (≥4.0 mg/L) plasma vitamin C levels. Thomas et al. [81] confirmed the finding that lower serum vitamin C levels are associated with periodontitis. Furthermore, they indicated that serum vitamin C levels increased in patients with diabetes and periodontitis compared with those in patients with periodontitis in the absence of diabetes. In a recent study [39], vitamin C levels in the plasma, PMNs, and peripheral blood mononuclear cells (PBMCs) of 21 patients with untreated periodontitis and 21 healthy controls matched for age, sex, race, and smoking status were measured. Plasma vitamin C levels were lower in patients with periodontitis compared with those in controls, while vitamin C levels in PMNs and PBMCs exhibited no differences between the patients and controls. In the patient group, PPD appeared to be negatively associated with vitamin C levels in PMNs. Iwasaki et al. [33] examined the longitudinal relationship of serum ascorbic acid levels with periodontal disease in 224 Japanese individuals aged 71 years. Participants were classified by tertiles of serum ascorbic acid. The number of teeth present at baseline, mean CAL at baseline, sex, education, diabetes, smoking status, brushing frequency, use of devices for inter-dental cleaning, and pattern of dental visits were tested as potential confounders in the multivariate models. The multivariate adjusted relative risks in the highest, middle, and lowest tertiles were 1.00 (reference), 1.12 (95 % CI, 1.01–1.26), and 1.30 (95 % CI, 1.16–1.47), and it was concluded that low serum ascorbic acid levels may be a risk factor for periodontal disease in Japanese elderly individuals. Recent studies, published after 2000, concerning the association of plasma/serum vitamin C levels with periodontal disease are summarized in Table 19.1.

Taken together, most studies clearly demonstrated the relationship between low serum/plasma vitamin C levels and increased risk of periodontal disease. However, it should be noticed that serum vitamin C levels reflect only the current nutritional status and not the lifelong history, and they are affected by many factors other than dietary intake of vitamin C, including diurnal variability, medication, inflammations, and stress. Furthermore, it should be considered that PMNs and PBMCs are able to accumulate vitamin C and may contain 10–40 times higher vitamin C levels than those in the plasma.

19.2.3.2 Association of Antioxidant Levels in the Saliva and Gingival Crevicular Fluid with Periodontal Disease

With regard to the association of periodontal disease and salivary vitamin C levels, Diab-Ladki et al. [25] examined antioxidant activities in the saliva of 20 healthy individuals and 17 patients with periodontal disease. Stimulated saliva from healthy individuals was significantly more effective in scavenging a wide variety of free

Table 19.1 Association of plasma/serum vitamin C levels and dietary vitamin C intake with periodontal disease

Authors (year) [reference]	Subjects/age	Study design	Definition of PD	Result
Vitamin C level in plasma/serum				
Pussinen et al. (2003) [64]	431 males/25–64 years	Cross-sectional study	Antibodies against periodonto-pathic bacteria	Inverse relationship between plasma VC levels and *P. gingivalis* antibody level but not *A. actinomycetemcomitans* antibody level
Amarasena et al. (2005) [7]	413 subjects/70 years	Cross-sectional study	CAL	Inverse relationship between serum VC level and CAL
Panjamurthy et al. (2005) [61]	25 male PD patients 25 male controls/25–35 years	Case–control study	PPD	Lower plasma VC levels in PD patients
Staudte et al. (2005) [78]	58 PD patients 22 controls/22–75 years	Case–control study	PPD	Lower plasma VC levels in PD patients
Amaliya et al. (2007) [6]	123 subjects/33–43 years	Cross-sectional study	CAL	Inverse relationship between plasma VC levels and CAL
Chapple et al. (2007) [20]	11,480 subjects/>20 years	Cross-sectional study	CAL/PPD	Inverse relationship between serum VC levels and PD
Thomas et al. (2010) [81]	20 patients with type II DM and PD 20 healthy subjects with PD 20 healthy subjects without PD/not given	Case–control study	CAL	Lower serum VC levels in PD patients Lower serum VC levels in DM patients with PD
Iwasaki et al. (2012) [33]	224 subjects/71 years	Longitudinal study	CAL	Inverse relationship between serum VC levels and PD
Kuzmanova et al. (2012) [39]	21 PD patients 21 healthy subjects/≥21 years	Case–control study	Radiographic bone loss	Lower plasma VC levels in PD patients
Dietary vitamin C intake				
Nishida et al. (2000) [55]	12,419 subjects/20–90+ years	Cross-sectional study	CAL	Inverse relationship between dietary VC intake and CAL in current and former smokers
Iwasaki et al. (2012) [34]	264 subjects/75 years	Longitudinal study	CAL	Inverse relationship between dietary VC intake and PD

PD periodontal disease, *DM* diabetes mellitus, *VC* vitamin C, A. actinomycetemcomitans, *Aggregatibacter actinomycetemcomitans*

radicals generated in vitro compared with stimulated saliva from patients with periodontal disease. The total antioxidant activity of saliva was significantly decreased in the patients despite the fact that the levels of three main antioxidants, namely uric acid, ascorbic acid, and albumin, were not significantly affected. The authors concluded that periodontal disease is associated with an imbalance between oxidants and antioxidants. Sculley and Langley-Evans [72] investigated 129 patients and concluded that periodontal disease is associated with decreased salivary antioxidant levels and increased oxidative damage within the oral cavity. On the other hand, Buduneli et al. [16] evaluated the effects of gingival inflammation on salivary antioxidant levels in patients with gingivitis. Whole saliva samples and clinical periodontal recordings were obtained at baseline from 20 patients with gingivitis and 20 healthy subjects and at 1 month following the initial phase of treatment in the patients. Salivary total glutathione levels were decreased following therapy in the patients who smoked, while salivary ascorbate levels and total antioxidant capacities were unaffected by successful periodontal treatment, irrespective of smoking status.

Gümüş et al. [29] investigated the effects of diabetes on salivary antioxidant capacity. They measured the salivary levels of GSH, ascorbic acid, and the total antioxidant capacity in 16 patients with type I diabetes mellitus, 25 patients with type II diabetes mellitus, and 24 systematically healthy patients, all with inflammatory periodontal disease. Salivary GSH levels were lower in patients with type I diabetes mellitus than in the other group, but no significant differences were observed in salivary vitamin C levels and total antioxidant capacity among the three groups.

With regard to vitamin C levels in gingival crevicular fluid (GCF), Holmes [31] investigated the effects of smoking and/or vitamin C levels on GCF flow. Ten smoking and ten non-smoking male dental students were evaluated. GCF flow was significantly lesser in the smokers than in the non-smokers. One month of (500 mg) twice daily vitamin C supplementation resulted in a significant decrease in GCF flow in both the smokers and non-smokers. In dogs, Pavlica et al. [62] reported that the total antioxidant capacity in GCF is related to the degree of severity of periodontal disease.

Ascorbic acid levels in GCF were compared with those in plasma by Meyle and Kapitza [49]. GCF samples were collected from clinically healthy gingival sites of 21 healthy volunteers and assayed for ascorbic acid levels, together with blood plasma samples. The mean ascorbic acid level in GCF was significantly higher than that in plasma. With regard to the antioxidant levels in body fluids, Brock et al. [15] examined antioxidant capacities in saliva, GCF, plasma, and serum in subjects with periodontal health and disease. GCF antioxidant levels were significantly lower in the patients with periodontitis than in the healthy controls. Salivary, plasma, and serum total antioxidant capacities were also lower in patients with periodontitis, however, the difference was only significant for plasma levels. In the healthy subjects, GCF antioxidant levels were significantly higher than those in paired serum or plasma samples.

19.2.3.3 Effect of Smoking on Vitamin C Levels in Body Fluids

Smoking affects the antioxidant levels in the body, including vitamin C levels. Keith and Mossholder [36] reported that smokers had lower dietary intakes and plasma ascorbic acid levels compared with non-smokers, even after adjusting for vitamin C intake. Schectman et al. [70] showed that, compared with never-smokers, smokers of 20 cigarettes daily had the lowest vitamin C dietary intake and serum vitamin C levels while smokers of 1–19 cigarettes daily had decreased vitamin C intake and serum vitamin C levels. This inverse association of vitamin C levels and vitamin C intake with smoking was independent of age, sex, body weight, race, and alcoholic beverage consumption. Following further adjustment for dietary vitamin C intake, the negative correlation between cigarette smoking and serum vitamin C levels persisted. Zhou et al. [93] also investigated plasma vitamin C levels in 1,225 smokers and 524 non-smokers and found that the average plasma vitamin C level in the smoking group was significantly decreased compared with that in the non-smoking group. The average plasma vitamin C level increased in a group of 73 smokers who stopped smoking completely for 6 months, although it was still significantly lower than that in the matched non-smoker group. However, after smoking cessation for 1 year, the average plasma vitamin C level was not significantly different from that in the control group. The effects of smoking cessation on plasma vitamin C levels were confirmed by Polidori et al. [63], who indicated that smoking cessation for 4 weeks was followed by a marked increase in plasma antioxidant levels including vitamin C levels, and substantially improved plasma resistance toward oxidative challenge. A similar result was observed in GCF by Seri et al. [73], who evaluated ascorbic acid levels in GCF of 25 smokers and 16 non-smokers with clinically healthy gingiva. Smokers were found to have significantly lower vitamin C levels compared with non-smokers. From these results, it is evident that smoking decreases antioxidant capacity, including vitamin C levels in body fluids such as plasma/serum and GCF. This may be one of the reasons why smoking is a major risk factor for periodontitis.

19.2.4 Human Studies on the Effects of Vitamin C on Periodontal Disease

19.2.4.1 Association of Dietary Vitamin C Intake with Periodontal Disease

Meta-analysis revealed a moderate relationship between dietary vitamin C intake measured by Food Frequency Questionnaire and Dietary Recalls/diary and plasma vitamin C levels [24]. A number of epidemiological and case–control human studies that evaluated the association of dietary vitamin C intake with periodontal disease have been reported. On the basis of the First National Health and Nutrition Examination Survey study that included 8,609 subjects, Ismail et al. [32] found a

significant association, albeit weak, between vitamin C deficiency and periodontal disease. For subjects who reported nil consumption of vitamin supplements, a significant linear relationship was found between Periodontal Index (PI) [68] scores and dietary ascorbic acid adequacy levels in the regression model. For subjects who reported vitamin supplement consumption, dietary ascorbic acid adequacy levels did not explain any of the variance in PI scores after controlling for age, sex, race, income, education, and oral hygiene status. Blignaut and Grobler [13] compared the periodontal condition of workers in citrus fruit-producing farms with that of workers in grain-producing farms. They concluded that a PPD of ≥4 mm occurred less frequently in individuals who consumed large amounts of different fruits and, by far, least frequently in individuals who consumed citrus fruit. Using the data of 12,419 subjects aged 20–90+ years who were included in the NHANES III study, Nishida et al. [55] evaluated the role of dietary vitamin C intake as a contributing risk factor for periodontal disease. They found a relationship between decreased dietary vitamin C intake and increased risk for periodontal disease (OR, 1.19; 95 % CI, 1.05–1.33). Both current and former smokers with a low dietary intake of vitamin C showed an increased risk of periodontal disease. There was a dose–response relationship between dietary vitamin C intake and periodontal disease, with ORs, 1.30 and 1.16 for individuals who consumed 0–29 mg and 100–179 mg of vitamin C/day, respectively, compared with individuals who consumed ≥180 mg of vitamin C/day. The authors concluded that dietary vitamin C intake was weakly but significantly correlated with periodontal disease as measured by CAL in current and former smokers.

With regard to longitudinal studies, Clark et al. [21] examined the short-term effects of pregnancy and dietary vitamin C intake on radiographic density and alveolar crest morphology of the mandible. Seventy-six women between 10 and 20 weeks' gestational age were recruited. Dietary vitamin C intake showed a positive correlation with bone density change. Iwasaki et al. [34] reported a retrospective cohort study with a follow-up period of 2 years that included 264 subjects aged 75 years and classified by tertile of antioxidant intake. Periodontal disease progression was considered as loss of attachment of ≥3 mm over the study course. A higher vitamin C intake was inversely associated with the number of teeth exhibiting periodontal disease progression. Multivariate-adjusted incidence rate ratios for the first, second, and third tertiles were 1.00, 0.76 (95 % CI, 0.60–0.97), and 0.72 (95 % CI, 0.56–0.93), suggesting that high vitamin C intake may mitigate periodontal disease. Recent studies, published after 2000, concerning the association of dietary vitamin C intake with periodontal disease are summarized in Table 19.1.

19.2.4.2 Intervention Studies on the Effects of Vitamin C on Periodontal Disease

Woolfe et al. [90] investigated the effect of ascorbic acid megadoses on gingival clinical parameters. The non-deficient individuals were divided into two groups matched for age, periodontal status, and oral hygiene level: one received 1 g/day of

ascorbic acid for 6 weeks and the other a placebo, and following 1 week of ascorbic acid/placebo intake, all subjects underwent scaling and root planing and received oral hygiene instructions. Correlations between clinical parameters and ascorbic acid levels revealed no significant differences between the vitamin C and placebo groups, suggesting that the use of megadoses of vitamin C in healthy human subjects does not have a predictable or strong effect on the gingival response to initial therapy. Vogel et al. [86] conducted a double-masked experimental gingivitis study of 24 dental students and determined the effects of supplementation with ascorbic acid megadoses on the clinical determinants of inflammatory progression in individuals with a mean daily ascorbate intake level of approximately twice the recommended daily allowance. They reported that although the group receiving ascorbate supplements demonstrated a significant increase in plasma vitamin C levels compared with the placebo group, no significant differences were found with respect to PMN chemotaxis or responses to experimental gingivitis between the two groups. Leggott et al. [40] examined the effects of controlled ascorbic acid depletion and supplementation on periodontal health. Eleven healthy male non-smokers aged 19–28 years consumed a rotating, 7-day, adequate diet, and it was found that gingival inflammation was directly related to ascorbic acid status whereas plaque accumulation and PPD were not. Jacob et al. [35] also showed that BOP decreased after normal (65 mg/day) ascorbic acid intake compared with that after deficient (5 mg/day) intake, as well as after supplementary (605 mg/day) ascorbic acid intake compared with that after normal intake in 11 young men aged 19–32 years. They suggested that ascorbic acid status may influence the early stages of gingival inflammation. The relationships among varying levels of ascorbate intake, periodontal status, and subgingival microflora were analyzed by Leggott et al. [41]. Twelve healthy male non-smokers aged 25–43 years consumed a rotating 4-day diet adequate in all nutrients except ascorbic acid. Following an initial baseline period during which the subjects received 250 mg of ascorbic acid/day, the subjects received 5 mg of ascorbic acid/day for a 32-day depletion period. Eight subjects were receiving either 60 or 250 mg of ascorbic acid/day for a 56-day repletion period. There were no significant changes in plaque accumulation, PPD, or CAL, while gingival bleeding increased significantly after the period of ascorbic acid depletion and returned to baseline values after the period of ascorbic acid repletion. No relationship could be demonstrated between the presence or proportion of periodontal micro-organisms and measures of gingival bleeding or ascorbate levels. Staudte et al. [78] examined plasma vitamin C levels and inflammatory measures in patients with periodontitis before and after the consumption of grapefruit. Fifty-eight patients with chronic periodontitis were assigned to the test group ($n=38$) and a diseased control group ($n=20$) and were compared with 22 healthy subjects. The test group consumed two grapefruits daily for 2 weeks. Plasma vitamin C levels at baseline were significantly decreased in the test group and diseased controls compared with those in the healthy controls, and smokers exhibited lower vitamin C levels compared with non-smokers. Following grapefruit consumption, plasma vitamin C levels increased significantly in the test group compared with those in the control group. Furthermore the sulcus bleeding index (SBI) [51] was decreased in the test group, while the plaque index

and PPD were unaffected, suggesting that the patients with periodontitis, particularly the smokers, were characterized by below-normal plasma vitamin C levels. The intake of grapefruit for 2 weeks led to an increase in plasma vitamin C levels and improved gingival inflammation. Lingström et al. [45] evaluated the effects of vitamin C in chewing gum on calculus formation. Thirty subjects, all calculus formers, chewed gum [vitamin C-containing, 60 mg, non-vitamin C-containing, and vitamin C + carbamide-containing (30 mg + 30 mg)] for a period of 3 months. One group did not chew any gum as a negative control. A significant decrease in the total calculus formation was observed in the subjects who chewed the vitamin C- and vitamin C + carbamide-containing gums compared with that in the negative controls; this decrease was most pronounced in the heavy calculus formers. However, no significant differences in calculus formation were observed between the subjects who chewed the vitamin C-containing gum and those who chewed the non-vitamin C-containing gum. A decreased amount of visible plaque was also observed after chewing of vitamin C- and non-vitamin C-containing gums; however, only the vitamin C-containing gum decreased the number of bleeding sites. Abou Sulaiman and Shehadeh [1] investigated plasma total antioxidant capacity in patients with chronic periodontitis and assessed the effects of vitamin C therapy as an adjunct to non-surgical periodontal treatment. Thirty patients with chronic periodontitis and 30 matched controls were analyzed. Patients from the chronic periodontitis group were randomly allocated into two groups: 15 patients received non-surgical treatment with an adjunctive dose of vitamin C (2,000 mg/day for 4 weeks) while 15 received non-surgical periodontal therapy alone. Plasma total antioxidant capacity was significantly lower in the patients than in the controls. The periodontal therapy increased plasma total antioxidant capacity and improved clinical measures in the patients; however, the adjunctive dose of vitamin C did not offer any additional effects.

Periodontal disease is one of the major causes of tooth loss. Studies investigating the association of vitamin C levels with dental status have been reported, and evidence indicating the association of vitamin C levels with tooth loss is increasing. Sheiham et al. [74] analyzed the relationship between dental status in individuals aged ≥65 years and intake of certain nutrients. They found that the intake of vitamin C was significantly lower in edentulous subjects and that plasma vitamin C levels exhibited large and statistically significant differences between the dentulous and edentulous subjects. Nowjack-Raymer and Sheiham [56] demonstrated that denture-wearers had lower serum vitamin C levels compared with individuals who had all their natural teeth. In another study, they reported that serum vitamin C levels were significantly lower in individuals with <28 teeth than in individuals with a complete set of teeth after adjusting for age, sex, race–ethnicity, socio-economic status, smoking status, calorie intake, and supplement use [57], suggesting the association of vitamin C deficiency with tooth loss. Marcenes et al. [47] analyzed the data obtained from a national survey in Great Britain and found that vitamin C intake was significantly lower in edentulous individuals than in dentulous individuals. Furthermore, the mean daily vitamin C intake was significantly higher in individuals with more teeth after adjusting for sex, social class, region of origin, and partial denture wearing. A similar result was obtained in the study reported by Wakai et al. [88],

who analyzed the data of 20,366 Japanese dentists and found that vitamin C intake decreased with an increase in the number of teeth lost after adjusting for age, sex, smoking status, physical activity, and history of diabetes. Yoshida et al. [91] investigated 182 community-dwelling elderly Japanese individuals aged 65–85 years and showed that vitamin C intake was significantly lower in the group with lost contacts and retained molar occlusion by way of removable partial dentures than in the group with contacts and retained molar occlusion by way of natural teeth. In contrast, Yoshihara et al. [92] conducted a study of 57 elderly Japanese subjects aged 74 years and indicated no significant association between the number of teeth present and vitamin C intake.

Judging from the above results, vitamin C supplementation appears to decrease gingival bleeding; however, the effects of vitamin C on PPD and CAL in humans remain questionable despite proven efficacy in animal models. Table 19.2 shows more recent intervention studies in humans, published after 2000, concerning the effects of vitamin C on periodontal disease.

19.3 Association of Vitamin E Levels with Periodontal Disease

19.3.1 Animal Studies Concerning the Effects of Vitamin E on Periodontal Disease

Studies concerning the association of vitamin E levels with periodontal disease are fewer than those concerning vitamin C. Kim and Shklar [37] demonstrated in a rat model that animals receiving vitamin E supplements experienced accelerated gingival wound healing. Cohen and Meyer [23] investigated the effects of dietary vitamin E supplementation on alveolar bone loss in rats and found that vitamin E supplementation had significant protective effects on bone loss induced by stress. This effect was most pronounced at sites most susceptible to loss. In another rat model, Åsman et al. [8] demonstrated that a combination of vitamin E and selenium decreased ROS-induced collagen degradation in experimental granulation tissue. On the other hand, Carvalho et al. [17] investigated the effects of vitamin E on alveolar bone loss in rats with ligature-induced experimental periodontitis and found that vitamin E therapy decreased inflammatory reactions, prevented malondialdehyde formation, and decreased immunoreactivity to the inducible isoforms of nitric oxide synthases; however, it did not decrease alveolar bone loss. This suggests that vitamin E has the potential to decrease oxidative damage and inflammatory responses in experimental periodontitis, but it does not prevent alveolar bone loss. Li et al. [42] evaluated the effects of ketoprofen with or without vitamin E on ligature-induced periodontitis in a monkey model. Ketoprofen positively altered alveolar bone activity without added or synergistic benefits from vitamin E.

Table 19.2 Intervention studies concerning the effects of vitamin C on periodontal disease

Author (year) [reference]	Subjects/age	Study design	Indices associated with PD	Result
Nagata et al. (2003) [52]	65 smokers VC: 17 subjects VE: 16 subjects VC+VE: 17 subjects Control: 15 subjects/27–75 years	RCT VC 1,000 mg/day VE 135 mg/day VC+VE Placebo tablet for 24 weeks	CAL, PPD, BOP, PII, I CTP	CAL and PPD were improved in the VC, VE, and VC+VE groups I CTP was improved in the VC+VE group
Staudte et al. (2005) [78]	58 PD patients(38 test, 20 control) 22 healthy controls/22–75 years	Case–control study. Two grapefruits/day for 2 weeks	PII, PPD, SBI	Plasma VC levels were increased in the test group SBI was decreased in the test group
Lingström et al. (2005) [45]	30 calculus formers/>20 years (53 ± 14)	Cross-over study Chewing gum (with/without 60 mg VC) for 3 months	Calculus score, PII, GBI	No differences were observed between groups
Nagata et al. (2006) [53]	42 non-smokers VC+VE: 19 subjects Control: 20 subjects/31–74years	RCT VC (1,000 mg/day)+VE (135 mg/day) Placebo tablet for 24 weeks	CAL, PPD, GI, BOP, PII, GSH	PPD, GI and BOP were improved in the VC+VE group GSH levels in GCF increased in the VC+VE group
Abou Sulaiman and Shehadeh (2010) [1]	30 PD patients 30 controls/23–65 years	Case–control study 15 patients received non-surgical treatment+VC (2,000 mg/day for 4 weeks) 15 patients received non-surgical treatment alone	PPD, CAL, BOP, PII, GI	Adjunctive dose of VC did not offer additional effects

PD periodontal disease, *PII* plaque index [77], *VC* vitamin C, *VE* vitamin E, *RCT* randomized controlled trial, *I CTP* cross-linked carboxyterminal telopeptide of type I collagen, *GBI* gingival bleeding index [2]

19.3.2 Human Studies Concerning the Effects of Vitamin E on Periodontal Disease

Human studies concerning the effects of vitamin E on periodontal disease have also been conducted; however, contradictory results have been reported. Royack et al. [66] investigated the effects of vitamin E on oxidative damage in human oral epithelial cells and demonstrated that cells pretreated with vitamin E before exposure to H_2O_2 also showed the presence of hydroxyl radicals; however, the relative levels were lower than those observed without vitamin E pretreatment, indicating that vitamin E provides initial protection from oxidative damage. Battino [11] investigated the vitamin E content in subjects with healthy gingiva and those with gingivitis using an immunohistochemical approach and showed that vitamin E was dramatically decreased despite the increased amount of cells present in the periodontally affected tissues. Panjamurthy et al. [61] also reported that the levels of vitamin E were significantly lower in the plasma and erythrocyte membranes of patients with periodontitis than in those of healthy subjects. Recently two longitudinal studies were reported by Iwasaki et al. [33, 34]. Two hundred twenty-four Japanese individuals aged 71 years were classified by tertiles of serum α-tocopherol levels. The number of teeth with a loss of CAL ≥3 mm at any site during the study period (1999–2007) was calculated as periodontal disease events. Multivariate adjusted relative risks in the highest, middle, and lowest tertiles were 1.00 (reference), 1.09 (95 % CI, 0.98–1.21), and 1.15 (95 % CI, 1.04–1.28), suggesting that low serum α-tocopherol levels may be a risk factor for periodontal disease in the Japanese elderly [33]. In a retrospective cohort study, they indicates that high intake of vitamin E may mitigate periodontal disease [34].

On the other hand, Chapple et al. [20] reported no relationship between serum vitamin E levels and the prevalence of periodontitis using multiple logistic regression analyses. Linden et al. [44] investigated the association between periodontal health and serum levels of various antioxidants, including vitamin E, in 1,258 Western European men aged 67–70 years. Subjects were divided into a low-threshold periodontitis, a high-threshold periodontitis, and the remaining populations according to periodontal status. There were no significant differences in serum α-tocopherol and γ-tocopherol levels in relation to periodontitis among groups.

Among intervention studies, a study by Cohen et al. [22] compared the effects of topical 5 % vitamin E gel delivering 800 mg of α-tocopherol and a placebo gel on established and developing plaque and periodontal disease in 48 adult subjects. No significant effects on plaque or gingivitis were observed in the placebo and vitamin E groups. Liede et al. [43] assessed the effects of α-tocopherol supplementation on gingival bleeding in 409 men aged 55–74 years. Gingival bleeding was more common in subjects who received α-tocopherol than in those did not, with a high prevalence of dental plaque. This suggests that α-tocopherol supplementation increases the risk of clinically important bleeding.

Regarding the effects of vitamin E on tooth loss, Yoshihara et al. [92] indicated that no significant differences existed between the number of teeth present and

Table 19.3 Human studies concerning the association of vitamin E with periodontal disease

Authors (year) [reference]	Subjects/age	Study design	Definition of PD	Result
Panjamurthy et al. (2005) [61]	25 male PD patients 25 male controls/25–35 years	Case–control study	PPD	Lower plasma VE levels in PD patients
Chapple et al. (2007) [20]	11,480 subjects/>20 years	Cross-sectional study	CAL/PPD	No relationship between serum VE levels and PD
Linden et al. (2009) [44]	1,258 men/60–70 years	Cross-sectional study	CAL	No relationship between serum VE levels and PD
Iwasaki et al. (2012) [33]	224 subjects/71 years	Longitudinal study	CAL	Inverse relationship between serum VE levels and PD
Iwasaki et al. (2012) [34]	264 subjects/75 years	Longitudinal study	CAL	Inverse relationship between dietary VE intake and PD

PD periodontal disease, *VE* vitamin E

vitamin E intake. This is in accordance with the result obtained by Yoshida et al. [91] investigated the correlation between dental and nutritional status among 182 elderly Japanese individuals aged 65–85 years. The subjects were divided into two groups according to occlusion, and no significant differences in vitamin E intake were observed between the group with lost contacts and that with retained contacts.

Concerning serum vitamin E levels during pregnancy, Cerná et al. [18] investigated 39 pregnant women and reported that serum vitamin E levels gradually increased from the third to the seventh month of pregnancy, following which it declined and increased again in a relatively sharp manner shortly before term, when it was at its peak. However, statistical evaluation did not prove significant differences. With regard to the effects of smoking on vitamin E levels in body fluids, Zhou et al. [93] demonstrated that plasma vitamin E levels were significantly decreased in smokers compared with those in non-smokers. In a group of smokers who stopped smoking completely for 6 months, the average plasma vitamin E level increased, although it was still significantly lower than that in the matched non-smokers. However, after smoking cessation for a year, the average plasma vitamin E level was not significantly different from that in the non-smokers. In contrast, Seri et al. [73] reported that GCF tocopherol levels in smokers were not significantly different from those in non-smokers.

Taken together, conflicting results have been reported on the association of vitamin E levels with periodontal disease; therefore, the effects of vitamin E on periodontal disease are currently viewed with skepticism. Recent human studies, published after 2000, concerning the effects of vitamin E on periodontal disease are summarized in Table 19.3.

19.4 Effect of Vitamin C + E Supplements on Periodontal Disease

A combined action of vitamin C and vitamin E appears to exist, and a synergy between vitamin C and vitamin E has been reported. For example, Bendich et al. [12] indicated in a guinea pig model that higher dietary vitamin C intake increased the vitamin E content in the lung at all levels of vitamin E intake. When ascorbic acid was depleted, no regeneration of vitamin E was possible, and a decrease in its concentration was observed [10]. Therefore, we investigated the effects of vitamin C + E supplements on periodontal disease in a randomized controlled trial. A total of 65 current smokers aged 27–75 years were randomly assigned to four groups that were studied for 24 weeks: the vitamin C group (1,000 mg/day), the vitamin E group (135 mg/day), the combination group (C + E: 1,000 + 135 mg/day), and the placebo group (placebo without vitamin C and vitamin E). Vitamin C levels in blood samples from the vitamin C and combination groups and vitamin E levels in blood samples from the vitamin E and combination groups significantly increased from each level at baseline. No differences were observed in CAL and PPD among groups according to subject-based analysis; however, site-based analysis revealed that CAL and PPD in the vitamin C, vitamin E, and combination groups showed statistically significant improvements during the trial compared with those in the placebo group. Furthermore, the rate of GCF samples wherein cross-linked carboxyterminal telopeptide of type I collagen as a biochemical marker of periodontal disease was decreased or maintained was significantly greater in the combination group than in the placebo group at 24 weeks. These results suggest that vitamin C + E supplements may maintain periodontal health status in smokers [52]. Using data obtained in the same study, Okamura et al. [59] investigated the effects of the intake of tablets containing vitamin C and vitamin E on blood antioxidative activity in smokers. Compared with the placebo group, thiobarbituric acid reactive substances as a marker of lipid peroxide significantly decreased in the vitamin E and combination groups and slightly decreased in the vitamin C group, suggesting that intake of chewable tablets containing vitamin C and vitamin E increased blood antioxidative activity. A similar effect was observed even in the non-smokers. Forty two non-smokers undergoing periodontal maintenance were randomly assigned to the combination group or the placebo group. Serum vitamin C and vitamin E levels in the combination group increased significantly at 24 weeks. Furthermore, GCF vitamin C and GSH levels significantly increased at 24 weeks compared with those at baseline in the combination group, while no change was observed in GCF vitamin E levels in the combination group. Subject-based analysis revealed that BOP and PPD were significantly improved at 24 weeks compared with those at baseline in the combination group, which also demonstrated a significant improvement in GI at 24 weeks relative to the placebo group. These results indicate that vitamin C + E supplements may prevent periodontal disease during the periodontal maintenance period by controlling oxidative status in the periodontal tissues, even in non-smokers [53]. These results are summarized in Table 19.2.

19.5 Conclusions

Numerous studies concerning the association of vitamin C with periodontal disease, including in vitro studies, animal studies, epidemiological studies, case–control studies, and intervention studies, have been conducted for a long time, and the evidence of an affirmative association has been accumulated. In vitamin C-deficient subjects, features of inflammation including bleeding and edema are observed. A number of studies clearly demonstrated the association of plasma/serum vitamin C levels with periodontal disease. Many epidemiological studies found significant associations between decreased vitamin C intake and an increased risk of periodontal disease. Numerous epidemiological and interventional studies also indicate the efficacy of vitamin C in preventing/decreasing gingival bleeding; however, the effects of vitamin C on PPD and CAL in humans are currently questionable despite proven efficacy in animal models. Concerning vitamin E, the number of studies on the association of vitamin E with periodontal disease is much lower than that of studies concerning vitamin C; furthermore, the results of these studies are contradictory. Therefore, the efficacy of vitamin E in preventing periodontal disease is currently viewed with skepticism. We investigated the effects of vitamin C + E supplements on periodontal disease in randomized controlled trials and found that these supplements significantly improved the clinical parameters and biomarkers of periodontal disease, even though the degree of improvement is not equivalent to that seen with periodontal therapy, including scaling and oral hygiene instructions. Data collected from the literature suggest that vitamin C and vitamin C + E supplements may decrease the risk of periodontal disease and may be potent nutrients for preventing gingivitis and maintaining healthy periodontal tissues. However, they cannot be used to treat periodontal disease currently; in other words, they may be useful as functional foods but not as therapeutic material. Current evidence is insufficient to evaluate the beneficial effects of vitamin C and vitamin E on periodontal disease and periodontal health, and the mechanism by which these vitamins improve periodontal disease has not been fully elucidated. Additional research is required to generate conclusive evidence on role of vitamin C and vitamin E in periodontal disease.

References

1. Abou Sulaiman AE, Shehadeh RM (2010) Assessment of total antioxidant capacity and the use of vitamin C in the treatment of non-smokers with chronic periodontitis. J Periodontol 81:1547–1554
2. Ainamo J, Bay I (1975) Problems and proposals for recording gingivitis and plaque. Int Dent J 25:229–235
3. Akman S, Canakci V, Kara A, Tozoglu U, Arabaci T, Dagsuyu IM (2013) Therapeutic effects of alpha lipoic acid and vitamin C on alveolar bone resorption after experimental periodontitis in rats: a biochemical, histochemical and stereologic study. J Periodontol 84(5):666–674
4. Alvares O, Siegel I (1981) Permeability of gingival sulcular epithelium in the development of scorbutic gingivitis. J Oral Pathol 10:40–48

5. Alvares O, Altman LC, Springmeyer S, Ensign W, Jacobson K (1981) The effect of subclinical ascorbate deficiency on periodontal health in nonhuman primates. J Periodontal Res 16:628–636
6. Amaliya, Timmerman MF, Abbas F, Loos BG, Van der Weijden GA, Van Winkelhoff AJ, Winkel EG, Van der Velden U (2007) Java project on periodontal diseases: the relationship between vitamin C and the severity of periodontitis. J Clin Periodontol 34:299–304
7. Amarasena N, Ogawa H, Yoshihara A, Hanada N, Miyazaki H (2005) Serum vitamin C-periodontal relationship in community-dwelling elderly Japanese. J Clin Periodontol 32:93–97
8. Åsman B, Wijkander P, Hjerpe A (1994) Reduction of collagen degradation in experimental granulation tissue by vitamin E and selenium. J Clin Periodontol 21:45–47
9. Aurer-Koželj J, Kralj-Klobučar N, Buzina R, Bačić M (1982) The effect of ascorbic acid supplementation on periodontal tissue ultrastructure in subjects with progressive periodontitis. Int J Vitam Nutr Res 52:333–341
10. Battino M, Ballon P, Wilson M, Newman H (1999) Oxidative injury and inflammatory periodontal diseases: the challenge of anti-oxidants to free radicals and reactive oxygen species. Crit Rev Oral Biol Med 10:458–476
11. Battino M, Bompadre S, Politi A, Fioroni M, Rubini C, Bullon P (2005) Antioxidant status (CoQ10 and Vit. E levels) and immunohistochemical analysis of soft tissues in periodontal diseases. Biofactors 25:213–217
12. Bendich A, D'Apolito P, Gabriel E, Machlin LJ (1984) Interaction of dietary vitamin C and vitamin E on guinea pig immune response to mitogens. J Nutr 114:1588–1593
13. Blignaut JB, Grobler SR (1992) High fruit consumption and the periodontal status of farm workers. Clin Prev Dent 14:25–28
14. Block G, Dietrich M, Norkus EP, Morrow JD, Hudes M, Caan B, Packer L (2002) Factors associated with oxidative stress in human populations. Am J Epidemiol 156:274–285
15. Brock GR, Butterworth CJ, Matthews JB, Chapple ILC (2004) Local and systemic total antioxidant capacity in periodontitis and health. J Clin Periodontol 31:515–521
16. Buduneli N, Kardeşler L, Işik H, Willis CS, Hawkins SI, Kinane DF, Scott DA (2006) Effects of smoking and gingival inflammation on salivary antioxidant capacity. J Clin Periodontol 33:159–164
17. Carvalho RDS, de Souza CM, Neves JC, Holanda-Pinto SA, Pinto LM, Brito GA, de Andrade GM (2013) Vitamin E does not prevent bone loss and induced anxiety in rats with ligature-induced periodontitis. Arch Oral Biol 58:50–58
18. Cerná H, Veselý J, Nastoupilová E, Lechner J, Fingerová H, Pohanka J (1990) Periodontium and vitamin E and A in pregnancy. Acta Univ Palacki Olomuc Fac Med 125:173–179
19. Chapple ILC, Matthews JB (2007) The role of reactive oxygen and antioxidant species in periodontal tissue destruction. Periodontol 2000 43:160–232
20. Chapple ILC, Milward MR, Dietrich T (2007) The prevalence of inflammatory periodontitis is negatively associated with serum antioxidant concentrations. J Nutr 137:657–664
21. Clark DE, Navia JM, Manson-Hing LR, Duncan HE (1990) Evaluation of alveolar bone in relation to nutritional status during pregnancy. J Dent Res 69(3):890–895
22. Cohen RE, Ciancio SG, Mather ML, Curro FA (1991) Effect of vitamin E gel, placebo gel and chlorhexidine on periodontal disease. Clin Prev Dent 13(5):20–24
23. Cohen ME, Meyer DM (1993) Effect of dietary vitamin E supplementation and rotational stress on alveolar bone loss in rice rats. Arch Oral Biol 38(7):601–606
24. Dehghan M, Akhtar-Danesh N, McMillan CR, Thabane L (2007) Is plasma vitamin C an appropriate biomarker of vitamin C intake? a systematic review and meta-analysis. Nutr J 6:41
25. Diab-Ladki R, Pellat B, Chahine R (2003) Decrease in the total antioxidant activity of saliva in patients with periodontal diseases. Clin Oral Investig 7:103–107
26. Dorsky R (2001) Nutrition and oral health. Gen Dent 49(6):576–582
27. Ekuni D, Tomofuji T, Sanbe T, Irie K, Azuma T, Maruyama T, Tamaki N, Murakami J, Kokeguchi S, Yamamoto T (2009) Vitamin C intake attenuates the degree of experimental atherosclerosis induced by periodontitis in the rat by decreasing oxidative stress. Arch Oral Biol 54:495–502

28. Evans RM, Currie L, Campbell A (1982) The distribution of ascorbic acid between various cellular components of blood, in normal individuals, and its relation to the plasma concentration. Br J Nutr 47:473–482
29. Gümüş P, Buduneli N, Çetinkalp Ş, Hawkins SI, Renaud D, Kinane DF, Scott DA (2009) Salivary antioxidants in patients with type 1 or 2 diabetes and inflammatory periodontal disease: a case–control study. J Periodontol 80:1440–1446
30. Hoffeld JT (1982) Oxygen radicals in inflammation and immunity. In: Genco RJ, Mergenhagen SE (eds) Host-parasite interactions in periodontal diseases. American Society for Microbiology, Washington, DC
31. Holmes LG (1990) Effects of smoking and/or vitamin C on crevicular fluid flow in clinically healthy gingival. Quintessence Int 21:191–195
32. Ismail AI, Burt BA, Eklund SA (1983) Relation between ascorbic acid intake and periodontal disease in the United States. J Am Dent Assoc 107:927–931
33. Iwasaki M, Manz MC, Taylor GW, Yoshihara A, Miyazaki H (2012) Relations of serum ascorbic acid and α-tocopherol to periodontal disease. J Dent Res 91:167–172
34. Iwasaki M, Maynihan P, Manz MC, Taylor GW, Yoshihara A, Muramatsu K, Watanabe R, Miyazaki H (2012) Dietary antioxidants and periodontal disease in community-based older Japanese: a 2-year follow-up study. Public Health Nutr 16:330–338
35. Jacob RA, Omaye AT, Skala JH, Leggott PJ, Rothman DL, Murray PA (1987) Experimental vitamin C depletion and supplementation in young men. Nutrient interactions and dental health effects. Ann N Y Acad Sci 498:333–346
36. Keith RE, Mossholder SB (1986) Ascorbic acid status of smoking and nonsmoking adolescent females. Int J Vitam Nutr Res 56:363–366
37. Kim JE, Shklar G (1983) The effect of vitamin E on the healing of gingival wounds in rats. J Periodontol 54(5):305–308
38. Knekt P, Ritz J, Pereira MA, O'Reilly EJ, Augustsson K, Fraser GE, Goldbourt U, Heitmann BL, Hallmans G, Liu S, Pietinen P, Spiegelman D, Stevens J, Virtamo J, Willett WC, Rinum EB, Ascherio A (2004) Antioxidant vitamins and coronary heart disease risk: a pooled analysis of 9 cohorts. Am J Clin Nutr 80:1508–1520
39. Kuzmanova D, Jansen IDC, Schoenmaker T, Nazmi K, Teeuw WJ, Bizzarro S, Loos BG, Nan der Velden U (2012) Vitamin C in plasma and leucocytes in relation to periodontitis. J Clin Periodontol 39:905–912
40. Leggott PJ, Robertson PB, Rothman DL, Murray PA, Jacob RA (1986) The effect of controlled ascorbic acid depletion and supplementation on periodontal health. J Periodontol 57:480–485
41. Leggott PJ, Robertson PB, Jacob RA, Zambon JJ, Walsh M, Armitage GC (1991) Effects of ascorbic acid depletion and supplementation on periodontal health and subgingival microflora in humans. J Dent Res 70:1531–1536
42. Li KL, Vogel R, Jeffcoat MK, Alfano MC, Smith MA, Collins JG, Offenbacher S (1996) The effect of ketoprofen creams on periodontal disease in rhesus monkeys. J Periodontal Res 31:525–532
43. Liede KE, Haukka JK, Saxén LM, Heinonen OP (1998) Increased tendency towards gingival bleeding caused by joint effect of α-tocopherol supplementation and acetylsalicylic acid. Ann Med 30:542–546
44. Linden GJ, McClean KM, Woodside JV, Patterson CC, Evans A, Young IS, Kee F (2009) Antioxidants and periodontitis in 60-70-year-old men. J Clin Periodontol 36:843–849
45. Lingström P, Fure S, Dinitzen B, Fritzne C, Klefbom C, Birkhed D (2005) The release of vitamin C from chewing gum and its effects on supragingival calculus formation. Eur J Oral Sci 113:20–27
46. Löe H, Silness J (1963) Periodontal disease in pregnancy. Acta Odontol Scand 21:533–551
47. Marcenes W, Steele JG, Sheiham A, Walls AWG (2003) The relationship between dental status, food selection, nutrient intake, nutritional status, and body mass index in older people. Cad Saude Publica 19(3):809–816
48. Melnick SL, Alvarez JO, Navia JM, Cogen RB, Roseman JM (1988) A case–control study of plasma ascorbate and acute necrotizing ulcerative gingivitis. J Dent Res 67:855–860

49. Meyle J, Kapitza K (1990) Assay of ascorbic acid in human crevicular fluid from clinically healthy gingival sites by high-performance liquid chromatography. Arch Oral Biol 35:319–323
50. Moynihan PJ (2005) The role of diet and nutrition in the etiology and prevention of oral diseases. Bull World Health Organ 83:694–699
51. Mühlemann HR, Son S (1971) Gingival sulcus bleeding – a leading symptom in initial gingivitis. Helv Odontol Acta 15:107–113
52. Nagata H, Ojima M, Shizukuishi S, Takemura A, Hinode D, Fukui M, Nakamura R (2003) Effect of vitamin C and vitamin E supplements on periodontal disease in smokers. J Dent Health 53:544–553 (in Japanese)
53. Nagata H, Shizukuishi S, Saho T, Nozaki T, Murakami S, Takeguchi-Hatanaka K, Katsuragi-Fuke K, Takashiba S, Takemura A (2006) The effect of vitamin C and E supplementation in non-smokers in periodontal maintenance. Japan J Conserv Dent 49:683–692 (in Japanese)
54. Neiva RF, Steigenga J, Al-Shammari KF, Wang HL (2003) Effects of specific nutrients on periodontal disease onset, progression and treatment. J Clin Periodontol 30:579–589
55. Nishida M, Grossi SG, Dunford RG, Ho AW, Trevisan M, Genco RJ (2000) Dietary vitamin C and the risk for periodontal disease. J Periodontol 71:1215–1223
56. Nowjack-Raymer RE, Sheiham A (2003) Association of edentulism and diet and nutrition in US adults. J Dent Res 82(2):123–126
57. Nowjack-Raymer RE, Sheiham A (2007) Numbers of natural teeth, diet, and nutritional status in US adults. J Dent Res 86(12):1171–1175
58. Oberritter H, Glatthaar B, Moser U, Schmidt KH (1986) Effect of functional stimulation on ascorbate content in phagocytes under physiological and pathological conditions. Int Arch Allergy Appl Immunol 81:46–50
59. Okamura S, Hinode D, Nagata H, Kondo K (2005) The effects of vitamin C and E intake on blood antioxidative activity in smokers. Health Sci 21(1):105–114 (in Japanese)
60. Palacios C, Joshipura KJ, Willett WC (2009) Nutrition and health: guidelines for dental practitioners. Oral Dis 15:369–381
61. Panjamurthy K, Manoharan S, Ramachandran CR (2005) Lipid peroxidation and antioxidant status in patients with periodontitis. Cell Mol Biol Lett 10:255–264
62. Pavlica Z, Petelin M, Nemec A, Erzen D, Skaleric U (2004) Measurement of total antioxidant capacity in gingival crevicular fluid and serum in dogs with periodontal disease. Am J Vet Res 65:1584–1588
63. Polidori MC, Mecocci P, Stahl W, Sies H (2003) Cigarette smoking cessation increases plasma levels of several antioxidant micronutrients and improves resistance towards oxidative challenge. Br J Nutr 90:147–150
64. Pussinen PJ, Laatikainen T, Alfthan G, Asikainen S, Jousilahti P (2003) Periodontitis is associated with a low concentration of vitamin C in plasma. Clin Diagn Lab Immunol 10:897–902
65. Rock CL, Jacob RA, Bowen PE (1996) Update on the biological characteristics on the antioxidant micronutrients: vitamin C, vitamin E, and the carotenoids. J Am Diet Assoc 96:693–702
66. Royack GA, Nguyen MP, Tong DC, Poot M, Oda D (2000) Response of human oral epithelial cells to oxidative damage and the effect of vitamin E. Oral Oncol 36:37–41
67. Rubinoff AB, Latner PA, Pasut LA (1989) Vitamin C and oral health. J Can Dent Assoc 55(9):705–707
68. Russell AL (1956) A system of classification and scoring for prevalence surveys of periodontal disease. J Dent Res 35(3):350–359
69. Sanbe T, Tomofuji T, Ekuni D, Azuma T, Tamaki N, Yamamoto T (2007) Oral administration of vitamin C prevents alveolar bone resorption induced by high dietary cholesterol in rats. J Periodontol 78:2165–2170
70. Schectman G, Byrd JC, Gruchow HW (1989) The influence of smoking on vitamin C status in adults. Am J Public Health 79:158–162
71. Schifferle RE (2005) Nutrition and periodontal disease. Dent Clin N Am 49:595–610
72. Sculley DV, Langley-Evans SC (2003) Periodontal disease is associated with lower antioxidant capacity in whole saliva and evidence of increased protein oxidation. Clin Sci 105:167–172

73. Seri M, D'Alessandro A, Seri S (1999) The effect of cigarette smoking on vitamin C and vitamin E levels of gingival crevicular fluid. Boll Soc Ital Biol Sper 75:21–25
74. Sheiham A, Steele JG, Marcenes W, Lowe C, Finch S, Bates CJ, Prentice A, Walls AWG (2001) The relationship among dental status, nutrient intake, and nutritional status in older people. J Dent Res 80:408–413
75. Shizukuishi S, Tanaka M, Nagata H (2011) Current evidence regarding association between periodontal disease and nutrition. J Dent Health 61:2–12 (in Japanese)
76. Shizukuishi S, Tanaka M, Nagata H (2011) Current evidence regarding functional foods for periodontal health. J Dent Health 61:190–202 (in Japanese)
77. Silness J, Löe H (1964) Periodontal disease in pregnancy. II. Correlation between oral hygiene and periodontal condition. Acta Odontol Scand 22:121–135
78. Staudte H, Sigusch BW, Glockmann E (2005) Grapefruit consumption improves vitamin C status in periodontitis patients. Br Dent J 199:2113–2217
79. Su H, Gornitsky M, Velly AM, Yu H, Benarroch M, Schipper HM (2009) Salivary DNA, lipid, and proteins oxidation in non-smokers with periodontal disease. Free Radic Biol Med 46:914–921
80. Sugano N, Yokoyama K, Oshikawa M, Kumagai K, Takane M, Tanaka H, Ito K (2003) Detection of *Streptococcus anginosus* and 8-hydroxy-deoxyguanosine in saliva. J Oral Sci 45:181–184
81. Thomas B, Kumari S, Ramitha K, Kumari MBA (2010) Comparative evaluation of micronutrient status in the serum of diabetes mellitus patients and healthy individuals with periodontitis. J Indian Soc Periodontol 14:46–49
82. Tomofuji T, Ekuni D, Sanbe T, Irie K, Azuma T, Maruyama T, Tamaki N, Murakami J, Kokeguchi S, Yamamoto T (2009) Effects of vitamin C intake on gingival oxidative stress in rat periodontitis. Free Radic Biol Med 46:163–168
83. Väänänen MK, Markkanen HA, Tuovinen VJ, Kullaa AM, Karinpää AM, Kumpusalo EA (1993) Periodontal health related to plasma ascorbic acid. Proc Finn Dent Soc 89:51–59
84. Van der Putten GJ, Vanobbergen J, De Visschere L, Schols J, De Baat C (2009) Association of some specific nutrient deficiencies with periodontal disease in elderly people: a systematic literature review. Nutrition 25:717–722
85. Van der Velden U, Kuzmanova D, Chapple ILC (2010) Micronutritional approaches to periodontal therapy. J Clin Periodontol 38(Suppl 11):142–158
86. Vogel RI, Lamster IB, Wechsler SA, Macedo B, Hartley LJ, Macedo JA (1986) The effects of megadoses of ascorbic acid on PMN chemotaxis and experimental gingivitis. J Periodontol 57:472–479
87. Waddington RJ, Moseley R, Embery G (2000) Reactive oxygen species: a potential role in the pathogenesis of periodontal diseases. Oral Dis 6:138–151
88. Wakai K, Naito M, Naito T, Kojima M, Nakagaki H, Umemura O, Yokota M, Hanada N, Kawamura T (2010) Tooth loss and intakes of nutrients and foods: a nationwide survey of Japanese dentists. Community Dent Oral Epidemiol 38:43–49
89. Woolfe SN, Hume WR, Kenney EB (1980) Ascorbic acid and periodontal disease: a review of the literature. J West Soc Periodontol Periodontal Abstr 28(2):44–56
90. Woolfe SN, Kenney EB, Hume WR, Carranza FA Jr (1984) Relationship of ascorbic acid levels of blood and gingival tissue with response to periodontal therapy. J Clin Periodontol 11:159–165
91. Yoshida M, Kikutani T, Yoshikawa M, Tsuga K, Kimura M, Akagawa Y (2011) Correlation between dental and nutritional status in community-dwelling elderly Japanese. Geriatr Gerontol Int 11:315–319
92. Yoshihara A, Watanabe R, Nishimuta M, Hanada N, Miyazaki H (2005) The relationship between dietary intake and the number of teeth in elderly Japanese subjects. Gerodontology 22:211–218
93. Zhou JF, Yan XF, Guo FZ, Sun NY, Qian ZJ, Ding DY (2000) Effects of cigarette smoking and smoking cessation on plasma constituents and enzyme activities related to oxidative stress. Biomed Environ Sci 13:44–55

Chapter 20
Salivary Biomarkers of Oxidative Stress Associated with Periodontal Diseases

Maria Greabu and Bogdan Calenic

20.1 Introduction

Oxidative stress is defined as an imbalance between antioxidant systems of the body and the production of reactive oxygen species (ROS), and associated with many systemic or tissue specific conditions. Initiation and development of periodontal disease are also connected with a progressive accumulation of ROS in oral fluids and tissues. Saliva plays an important role in counteracting the effects of oxidative stress through the presence of several antioxidant systems and can accurately reflect the oxidative stress status in a pathological situation. In this chapter, we summarize important salivary functions in the oral environment with a special focus on its diagnostic potential and on the antioxidant defense mechanisms that are connected with the pathogenesis of periodontitis.

20.2 Oxidative Stress: Implications in Periodontal Disease

Oxidative stress (OS) appears when the redox balance constituted of the prooxidants and antioxidants is broken in favor of the prooxidant or when there is a disruption in the redox signaling and control. ROS include free radical species and molecules which are capable of radical formation in the extra- and intracellular environments. ROS include normal metabolic products such as hydrogen peroxide, hydroxyl radicals, or superoxide radicals. ROS of endogenous origin are produced in both physiological and pathological processes such as aging, phagocytosis, diabetes, cancers, or oral conditions and have a myriad of cellular or tissue origins

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(mitochondria, endothelial cells, immune cells, peroxisomes, polymorphonuclear neutrophils). Typical exogenous sources for ROS include: cigarette smoke and ionizing radiation. In normal conditions, ROS often have beneficial effects for biological processes and are involved in key mechanisms such as: modulation of cellular redox state, modulation of metabolism, cell signaling, activation of gene-transcription factors, antibacterial roles by regulating bacterial growth, stimulation of pro-inflammatory cytokine release—by intracellular thiol depletion, and nuclear factor kB (NF-kB) activation. A solid body of literature demonstrates that this imbalance between antioxidant mechanisms and the production of ROS is closely associated with the initiation and development of several systemic or tissue specific diseases including: diabetes, cancer, cardiovascular diseases, asthma, chronic obstructive pulmonary diseases, or oral conditions [1–9].

Periodontal diseases including both gingivitis and periodontitis are the most widespread chronic conditions affecting populations worldwide [10]. Periodontitis is a nonreversible inflammatory disease affecting the supporting tissues of the teeth. Periodontal disease is initiated and developed mainly by oral microorganisms from the dental plaque that elicit an immune response in the tooth's neighboring tissues. These responses are usually followed by migration of oral epithelium, loss of collagen fibers, and alveolar bone resorption leading to formation of periodontal pockets. Loss of support leads to increased tooth mobility followed by tooth loss. Tissue destruction is a consequence of immune and inflammatory processes triggered by dental plaque, processes that include a progressive accumulation of ROS in both oral tissues and surrounding biological fluids. Current research can connect the pathogenesis of periodontitis with OS [11]. As pointed out, OS appears as an imbalance between free radicals and ROS levels and AO defense mechanisms. This process, characterized either by an increased ROS production or by a decreased AO activity, may play a key role in the initiation and development of oral conditions. Due to its accessibility oral environment may provide an ideal medium where antioxidant response to OS or OS mediated tissue damage can be studied. Recent data shows that ROS can directly influence cellular metabolism and behavior in many cell types including cells from oral tissues [12–17]. Other reports demonstrate positive associations between OS and systemic markers of inflammation linked to other general diseases such as cancer, diabetes, and heart failure [18–20]. OS may also be one possible link between the presence of periodontal diseases and systemic disorders such as the metabolic syndrome [21], cardiovascular diseases [22–24], diabetes [25], or cancer [26].

20.3 Saliva: General Characteristics and Functions

Saliva is a body fluid that can accurately reflect the status of OS in a particular situation as well as different markers defining oral or general pathologies. During the past two decades, saliva has been considered as an alternative diagnostic approach

for multiple oral and systemic diseases [3, 27–29]. From a clinical application point of view, saliva meets a number of characteristics that recommend it as one of the most suitable biological fluids for diagnosis. Most importantly saliva fulfills one key aspect in the process of any diagnosis sequence: it is noninvasive. Other qualities include: easy collection method; simple handling and storage procedures; correlations between markers found in blood and markers found in saliva; marker concentration does not depend on flow rate; small sample amounts needed for detection; the potential for performing dynamic studies; good sensitivity; noninvasive collection method which provides a good cooperation with the patients [30]. These aspects make saliva one of the top priority biomedical research challenges of the twenty-first century.

Saliva functions include protection and when needed repair of oral mucosa; roles in taste and digestive process; antibacterial, antiviral, and antifungal activity that protects oral cavity from insults coming from different microorganisms. Saliva physical and chemical properties as well as its components are also essential for protecting the teeth and the periodontal apparatus that supports the teeth. A thin saliva film containing proteins: mucins, statherins, proline-rich proteins and minerals such as calcium, phosphate, or fluoride ions is always covering the tooth surface preventing demineralization, promoting remineralization, and inhibiting bacterial adherence and growth [4]. A detailed overview of the salivary functions is included in Table 20.1.

Saliva is a complex mixture including an important number of inorganic and organic molecules acting as a possible “mirror of the body’s health.” Saliva composition is composed of salivary glands secretions, mucosal transudate, gingival crevicular fluid, nasal and pharynx mucous, food debris, epithelial and blood cells, oral microorganisms, and traces of exogenous chemicals and drugs. Saliva composition depends on a variety of biological factors, exogenous and endogenous insults, the time of day, stimulated or unstimulated saliva, healthy or diseased state, age of the subject. For example, unstimulated whole saliva is a mixture of mucous and serous secretions originating from minor salivary glands (8 %); sublingual gland (4 %); parotid gland (25 %); and submandibular gland (65 %). Despite its clear advantages as a diagnostic and prognostic fluid some authors argue that in the past saliva has been largely disregarded due to a set of limitations. Some drawbacks include individual and inter-individual physiological differences, type of saliva collected, and genetic variations. Recent proteomic studies have identified and characterized more than 2,400 compounds in salivary proteome of a wide range of local or systemic diseases. Twenty-one percent of the proteins discovered so far are connected to immune system; 9.7 % with molecular signaling pathways; 4.2 % to the cell proliferation process while 4.8 % are associated to cellular motility [31]. Nevertheless none of the detected protein has just one function; many of them have different biological roles. With the new available methodologies such as mass spectrometry and 2D electrophoresis coupled with high-performance liquid chromatography it seems fair to assume that the number of salivary markers will expand in the near future. Studies show that human saliva includes a plethora of proteins

Table 20.1 Salivary functions in the oral cavity

Function	Marker	Remarks	References
Taste	Gustin (carbonic anhydrase VI), zinc	Saliva (hypotonic fluid) dissolves the substances that are better perceived by taste buds	[61, 62]
Protection of oral tissues	Mucin 1, mucin 2, mucin 5B, mucin 7, epidermal growth factor	Lubrication, viscoelasticity, protection against infection, inflammation and mechanical wear	[62–64]
Buffer system	Urea, salivary proteins (sialin), acid carbonic–carbonate system, phosphate system	Saliva increases the pH preventing enamel demineralization	[37]
Protection of tooth surface	pH; concentration of: calcium, phosphate, flour ions	Lubrication (same as protection of oral tissues)—prevents enamel erosion Enamel remineralization/demineralization ion-exchange confer enamel less solubility and a higher integrity	[37, 65]
	Salivary flow rate	Oral clearance—dilution and removal of foreign substances	
Digestion	Ptyalin (alpha-amylase), lingual lipase, protease, DNAse, RNAse	Saliva is responsible for starch digestion and formation of bolus; salivary flow increases with size and hardness of the chewed object; strong association between saliva composition and flow and the frequency and duration of swallowing	[62, 64]
Antibacterial, antiviral and antifungal properties	IgA, lysozyme, lactoferrin, sialoperoxidase, proline-rich proteins, statherins, cystatins, histatins, agglutinin; mucins, lactoperoxidase, agglutinin, VEGh	Regulate agglutination, and promote or inhibit bacterial adhesion to hard surfaces	[37, 62, 64, 66]

such as immunoglobulins, statherins, proline-rich proteins, histatins, mucins, and enzymes [32–35]. It is also becoming increasingly clear that different salivary glands contribute to the salivary proteome with specific proteins and peptides. Thus Veerman et al. [36] show that cystatins, statherins, and proline-rich proteins can derive from all three major salivary glands. Other researchers report that mucins, cystatins, calgrunalin, amylase, statherins are secreted either from submandibular and/or sublingual glands [37, 38]. Other studies demonstrate that minor salivary glands are also involved in the secretion of amylase, histatins, or mucins [39]. Recent studies show that during fetal development salivary glands can release certain peptides that aid the development of oral cavity [40].

20.4 Salivary Biomarkers in Oral and Systemic Diseases

While there is no comprehensive definition of a biomarker, it can be defined as: "a biomarker is a cellular, biochemical, molecular or genetic alteration by which a normal, abnormal or simply biologic process can be recognized or monitored" [5].

Due to the anatomic proximity intense efforts have been undertaken to identify salivary biomarkers that can reflect oncological pathologies such as head and neck or oral cancers [41–43] (see Table 20.2). Saliva composition can also reflect

Table 20.2 Salivary biomarkers of different general and oral conditions

Disease	Salivary marker	Reference
General conditions		
Breast cancer	Her2, c-erbB-2, CA15-3	[67, 68]
Gastric cancer	Panel of four proteins	[31]
Pancreatic cancer	KRAS, MBD3L2, ACRV1, DPM1 miRNA	[69]
Lung cancer	HP, AZGP1, calprotectin	[70]
Cardiovascular system associated conditions	MMP-9, vascular cell adhesion molecule, troponin, myoglobin, creatine kinase MB, C reactive protein, free fatty acid, intercellular adhesion molecule, ischemia modified albumin, low density lipoprotein, soluble CD-40 ligand	[6]
Systemic sclerosis	Keratin 6, psoriasin, Arp2/3 complex	[71]
Alzheimer disease	Acetylcholinesterase	[51]
Physiological stress	Cortisol, alpha-amylase	[72]
Anorexia, bulimia	Cortisol, alpha amylase	[73]
Diabetes	Inflammatory markers, glucose, cortisol, salivary pH	[74, 75]
AIDS	HIV-1	[76]
Hepatitis	Virus detection	[77]
Monitoring drug abuse	Different drugs	[78]
Neuroendocrine functions	Oxytocin	[79]
Oral conditions		
Periodontal disease	Ig gamma 2, Ig alpha2, Vit. D-binding protein, alpha-amylase, zinc-alpha2 glycoprotein, lactotransferrin, elongation factor 2, 14-3-3 sigma; aspartate aminotransferase, alkaline phosphatase, lactate dehydrogenase, prostaglandin E2, calprotectin, cystatin S, lysozyme, IL1-beta, histatins, defensins, peroxidase, mucins, inflammatory, collagen breakdown and bone remodeling related biomarkers	[5, 6, 48, 52, 80–85]
Dental caries	Cystatin S, proline-rich proteins, lipocalin, cystatin SN, mucins, statherin, lactoferrin	[86–89]
Oral lichen planus	Palate, lung and nasal epithelium carcinoma associated protein	[90]
Sjögren syndrome	Albumin, alpha-actin-1, salivary amylase, calgranulin B	[50]
Head and neck region cancer	Maspin; stathmin; Dim1p; v-Ha-ras oncogene; tumor necrosis factor; pirin; alpha and beta defensins; endothelins, statherins, interleukin-8	[42, 44, 45, 91, 92]

cancerous changes of the glands. Saliva from patients with salivary tumors contains higher levels of stathmin or maspin, tumor necrosis factor, transketolase, Dim1p, v-Ha-ras oncogene, type I collagen pro alpha or pirin [44, 45]. Several experiments have also focused on the potential anti-carcinogenic properties of saliva [46]. Other tumors that can be detected using salivary biomarkers include breast cancer, gastric cancer, and larynx neoplasms [32, 47, 48]. Another condition intensely studied in association with ROS and saliva is Sjögren syndrome. The affection is an autoimmune disease which among other effects alters the composition of saliva and tears. Several studies showed that the protein print is different in human saliva from patients with Sjögren syndrome when compared to controls [49]. Whole saliva was also analyzed in relation with other diseases such as systemic sclerosis, Alzheimer disease, anorexia and bulimia, psychological stress [2, 50–52].

20.5 Saliva: Antioxidant Defense Systems and Oxidative Stress Biomarkers

Studies show that one important function of saliva is its potential to act as a defense mechanism against OS [7, 53, 54]. This is achieved through the presence of antioxidant systems such as: uric acid, albumin, ascorbate, glutathione and specialized enzymes like superoxide dismutase, glutathione peroxidase, or catalase. Out of all salivary glands, the parotid gland was proven to play a prominent role in secreting salivary AO.

Our group has analyzed major antioxidants and oxidative stress markers (Fig. 20.1) in connection with biomarkers in saliva from patients with various oral conditions (see Table 20.3).

Uric acid represents a major AO of saliva accounting for more than 85 % of the total antioxidant salivary activity. Its concentrations in saliva range, depending on experimental conditions, between 40 and 240 μM. It is a powerful scavenger with a mechanism of action based on reactivity with HO· and binding to iron and copper ions. In our studies salivary levels of uric acid were significantly decreased in chronic periodontitis patients as compared to controls. At the same time uric acid was found in a negative correlation with CTX I and MMP-8 both markers of bone resorption [55]. This finding shows that saliva reflects the associations that can exist between decreased concentrations of antioxidant mechanisms and development of periodontal disease. A previous study done by our group shows that uric acid levels were decreased not only in patients with periodontal disease but also in smokers when compared to no-smoker controls [56]. This study shows that smoking habit can bring the most important antioxidant of saliva to 1/3 of the levels found in non-smoking volunteers. Oral lichen planus is a chronic inflammatory disease of unknown origins with an increased cancerous potential. OS is involved in the development of oral lichen planus [57]. We reported that in oral lichen planus saliva sample was twice less than in healthy controls [56]. Chronic inflammation can thus deplete saliva of its antioxidant potential possibly making the oral tissues more

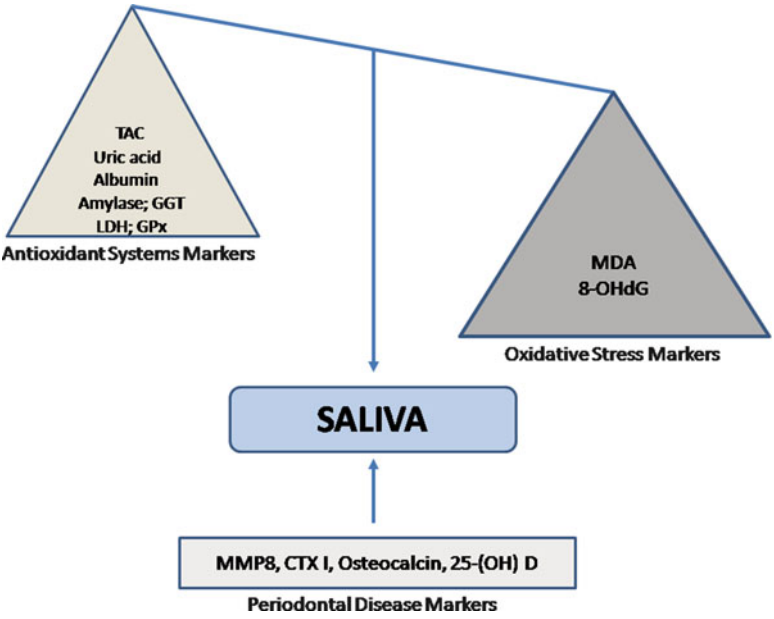

Fig. 20.1 Saliva can accurately reflect antioxidant, oxidative stress, and periodontal disease markers as well as possible functional associations between them. In chronic inflammatory condition such as periodontitis the balance between AO and ROS is disrupted in favor of OS, leading to increased oral tissues damage. The diagram contains major salivary antioxidants and OS markers as well periodontal biomarkers analyzed by our group

vulnerable to OS. Saliva is the first body fluid to enter in contact with cigarette smoke (CS). Saliva plays an important role in reducing the carcinogenic effects of molecules found in cigarette smoke. Our group assessed the direct effect of CS on salivary antioxidant mechanisms with a focus on uric acid. The results show that both CS and particulate phase can decrease the antioxidant capacity of saliva by significantly reducing the uric acid levels. Interestingly in the same experiment addition of vitamin C was shown to have a protective effect on uric acid [58].

Albumin can be detected both in saliva and gingival crevicular fluid. Compared to uric acid it can be found in a lower concentration of approximately 10 μM. It has both a preventive role, binding to metal ions and a scavenger role taking over AO functions of uric acid when needed. Its concentrations decrease in saliva of patients with periodontal disease or diabetes probably in connection to OS increments in the oral cavity. Consistent with other studies our results show that in saliva from patients with chronic periodontitis, albumin levels are significantly decreased when compared to controls which can reflect the OS involvement in the development of the disease [56]. When we compared saliva from smoking patients with no-smoking volunteers albumin levels were found to be similar showing that this marker may not be sensitive for smokers. Interestingly albumin concentration was higher but with no significant difference in oral lichen planus patients than in healthy controls.

Table 20.3 Antioxidant defense mechanisms, oxidative stress markers, and periodontal disease biomarkers detected in whole saliva

	Oral condition	Results	References
AO			
Uric acid (mg/mg albumin)	Chronic periodontitis	Significantly decreased Patients vs. healthy controls	[55]
TAC (nmol/mg albumin)	Chronic periodontitis	Significantly decreased Patients vs. healthy controls	[55]
GPx (U/mg albumin)	Chronic periodontitis	Significantly decreased Patients vs. healthy controls	[55]
Uric acid (mg/dL)	Smoking patients	Significantly decreased Smokers vs. controls	[56]
Albumin (g/dL)	Smoking patients	Significantly decreased Smokers vs. controls	[56]
TAC (mM)	Smoking patients	Significantly decreased Smokers vs. controls	[56]
GPx (U/L)	Cigarette smoke effect on saliva (ex vivo)	Significantly decreased Sample vs. control	[59]
GGT (U/L)	Cigarette smoke effect on saliva (ex vivo)	Significantly decreased Sample vs. control	[59]
TAC (mM)	Cigarette smoke effect on saliva (ex vivo)	Significantly decreased Sample vs. control	[59]
Uric acid (mg/dL)	Cigarette smoke effect on saliva (ex vivo)	Significantly decreased Sample vs. control	[58]
Amylase (U/L)	Cigarette smoke effect on saliva (ex vivo)	Significantly decreased Sample vs. control	[58]
LDH (U/L)	Cigarette smoke effect on saliva (ex vivo)	Significantly decreased Sample vs. control	[58]
OS markers			
8-HOdG (ng/mg albumin)	Chronic periodontitis	Significantly increased Patients vs. healthy controls	[55]
MDA (nmol/mg albumin)	Chronic periodontitis	Significantly increased Patients vs. healthy controls	[55]
Periodontal markers			
Osteocalcin (pg/mg albumin)	Chronic periodontitis	Significantly increased Patients vs. healthy controls	[55]
25–OH D (ng/mg albumin)	Chronic periodontitis	Significantly increased Patients vs. healthy controls	[55]
MMP-8 (ng/mg albumin)	Chronic periodontitis	Significantly increased Patients vs. healthy controls	[55]
CTX I (ng/mg albumin)	Chronic periodontitis	Significantly increased Patients vs. healthy controls	[55]

Reported data is associated with various oral conditions: chronic periodontitis, oral lichen planus, smokers. Abbreviations are adequately explained in the text

This increment should be associated with decreased levels of uric acid and can be seen as a possible compensatory mechanism for counteracting OS.

Total antioxidant capacity (TAC) includes all salivary antioxidant components having therefore an improved clinical significance in evaluating the AO salivary

status in both normal and pathological conditions. TAC levels depend on the analyzed oral fluid: thus it has different values in saliva and gingival crevicular fluid. TAC is significantly decreased in smokers, patients with periodontal affections, patients with oral cancer or diabetes. In a recent study our group demonstrated that saliva from chronic periodontitis patients had a decreased TAC value compared to normal controls [55]. However, TAC did not correlate with other analyzed periodontal biomarkers. In a previous study Miricescu et al. [56] showed that besides periodontal patients, TAC levels were also significantly lower in smoking patients when compared to controls. These results are also consistent with in vitro experiments performed by our group. Thus direct effect of CS on saliva significantly decreased TAC concentrations ($p < 0.01$) [59]. This suggests the conclusion that in a prolonged inflammatory state such as smoking habit or chronic periodontitis the antioxidant mechanisms of saliva are decreased making the oral tissues more vulnerable to OS and altering tissue homeostasis.

Although glutathione is found in relatively low concentrations, approximately 2 μM, it plays a major role in periodontal diseases through regulating pro-inflammatory cytokines such as TNFα, IL-8, and IL-6 which in turn activate bone resorption. The tripeptide is also involved in protecting the oral cavity from OS induced by metal ions from dental materials.

Ascorbic acid concentrations in saliva are similar to those found for albumin. Its levels are particularly high in gingival crevicular fluid where the concentration is three times higher than in plasma. Some reports show that an adequate intake may avoid cigarette smoke induced by oxidative damage. Our group also supports this assumption by showing that in vitro adding vitamin C to saliva directly exposed to CS maintains constant the levels of uric acid [58].

Salivary peroxidase plays a dual role acting both as an antibacterial and an antioxidant vector. The enzyme is of key importance in the oral defense mechanism, especially against the attack of ROS related to CS and the evolution of oral cancer. It has decreased activity in smokers probably due to the cyanide ions present in cigarette smoke. Salivary peroxidase also has a lower activity in saliva of patients with oral cancer [53].

Gamma-glutamyltransferase (GGT) is a potential OS marker not only in serum but also in saliva being a key enzyme in the glutathione metabolism. The enzyme modulates antioxidant/antitoxic defense with possible implications in tumor progression. An increased GGT activity may be an adaptive response to OS which can increase the translocation of glutathione precursors into cells. Smokers' saliva contains significantly lower concentrations of GGT. We have analyzed the effect of CS on saliva in relation to GGT in an in vitro study. As expected the results show that exposure to CS resulted in a statistically significant decrease of salivary GGT ($p < 0.01$) [59]. We have also studied salivary GGT together with TAC and uric acid in a group of non-ferrous metals mine workers. The results showed significant differences in all analyzed markers and suggested a possible usage for monitoring oxidant exposure in this group [60].

Glutathione peroxidase is a key selenium dependent enzyme of glutathione metabolism. The biological function of GPx is to protect the organism against OS through reducing lipid hydroperoxides and hydrogen peroxide. This enzyme plays

a major role in protection against low levels of OS. In one in vitro study we show that GPx levels in saliva are markedly decreased by prolonged incubation with CS. This suggests that GPx can be a potential marker for assessing OS caused by smoking [59]. These findings have been re-confirmed in a recent study on saliva from patients with chronic periodontitis. While no correlation could be found between GPx and other clinical or periodontal disease markers, the enzyme was significantly decreased in patients vs. healthy controls [55].

Other enzymes such as superoxide dismutase, glutathione reductase, ceruloplasmin, lysosyme, lactoferrin or catalase, amylase, and lactate dehydrogenase have only a secondary antioxidant significance. Our group showed that both salivary amylase and lactate dehydrogenase can be inactivated as a result of direct exposure to CS.

One of the most studied biomarkers in correlation with OS in both systemic and local diseases are malondialdehyde (MDA) and 8-hydroxy-2-deoxyguanosine (8-OHdG). OS progression starts at the cellular level with membrane lipid peroxidation, MDA being a marker of this process. DNA damage is also a known effect of OS increase; 8-OHdG is formed through hydroxylation of DNA deoxyguanosine residues by hydroxyl radical and is therefore being used as a biomarker of DNA oxidation. Both markers, MDA and 8-OHdG, are increased in several chronic inflammatory diseases including periodontitis. In our studies the salivary levels of these compounds have been analyzed in relation with periodontal tissue initiation and development. 8-OHdG was significantly increased in chronic periodontal patients as compared to normal controls. However, the present reported data shows no statistical significance between 8-OHdG and bone resorption markers such as osteocalcin, C-terminal telopeptide of type I collagen, or matrix metalloproteinase 8. MDA results show that the compound was significantly elevated in saliva from chronic periodontal patients vs. controls. Similar to 8-OHdG, MDA could not be statistically correlated with bone resorption markers.

Our group has also analyzed various markers of periodontal development in saliva and their correlation with clinical parameters and molecules.

Matrix metalloproteinases (MMPs) are host proteinases with key roles in tissue degradation. MMP-8 is the most common MMP found to be increased in periodontal tissues. MMP-8 or collagenase-2 activity results in collagen type I, II, or III degradation, all of them found in alveolar bone. Some reports demonstrate that MMP-8 is significantly elevated in patients with periodontitis while others show that MMP-8 is increased in gingival crevicular fluid from patients with implants and severe bone loss. In our studies MMP-8 was significantly elevated in total saliva from chronic periodontitis patients. At the same time statistical analysis showed a negative correlation with uric acid, the most important antioxidant mechanism of saliva. MMP-8 was also positively correlated with clinical parameters such as probing depth. Osteocalcin is a noncollagenous protein produced by osteoblasts. It has been associated with rapid bone turnover in general conditions such as multiple myeloma or osteoporosis. High levels of osteocalcin in body fluids have also been linked to periodontal disease. Our results show that osteocalcin levels in total saliva from chronic periodontitis patients are significantly increased. Unlike MMP-8 no correlation could be found between osteocalcin and members of the salivary

antioxidant defense system. However, the marker showed an expected positive correlation with clinical parameters such as probing depth.

C-terminal telopeptide of type I collagen is a collagen related biomarker used for quantifying bone turnover rate. Although bone resorption is an important event in the development of periodontitis up to date there are very few studies that associate CTX I with periodontal disease. We have measured CTX I levels in saliva from patients with chronic periodontitis and found that they are markedly increased as compared to controls. At the same time CTX I could be positively correlated with antioxidant mechanisms such as uric acid. Also CTX I was negatively correlated with OS biomarker MDA. The results also show that CTX I was positively associated with probing depth.

Calcifediol or 25-hydroxycholecalciferol [25(OH)D] is another marker of bone resorption. Studies show that plasma levels of 25(OH)D are higher in aggressive periodontitis patients and that periodontal treatment decreases both local and systemic 25(OH)D levels. Data resulting from our experiments shows that 25(OH)D is significantly increased in saliva from patients with periodontitis as compared to controls. The marker, however, did not correlate with the analyzed salivary antioxidant systems or OS biomarkers but could be positively correlated with probing depth.

Taken together these results show that saliva contains several markers that are connected to tissue destruction in periodontitis and that functional associations can be found between these markers, salivary antioxidants and clinical parameters.

References

1. Tothova L, Celecova V, Celec P (2013) Salivary markers of oxidative stress and their relation to periodontal and dental status in children. Dis Markers 34(1):9–15
2. Yeh CK, Christodoulides NJ, Floriano PN et al (2010) Current development of saliva/oral fluid-based diagnostics. Tex Dent J 127(7):651–661
3. Malamud D (2011) Saliva as a diagnostic fluid. Dent Clin North Am 55(1):159–178
4. Castagnola M, Picciotti PM, Messana I et al (2011) Potential applications of human saliva as diagnostic fluid. Acta Otorhinolaryngol Ital 31(6):347–357
5. Baum BJ, Yates JR 3rd, Srivastava S, Wong DT, Melvin JE (2011) Scientific frontiers: emerging technologies for salivary diagnostics. Adv Dent Res 23(4):360–368
6. Miller CS, Foley JD, Bailey AL et al (2010) Current developments in salivary diagnostics. Biomark Med 4(1):171–189
7. Greabu M, Battino M, Mohora M et al (2009) Saliva—a diagnostic window to the body, both in health and in disease. J Med Life 2(2):124–132
8. Pendyala G, Thomas B, Joshi SR (2013) Evaluation of total antioxidant capacity of saliva in type 2 diabetic patients with and without periodontal disease: a case–control study. N Am J Med Sci 5(1):51–57
9. Kinney JS, Morelli T, Braun T et al (2011) Saliva/pathogen biomarker signatures and periodontal disease progression. J Dent Res 90(6):752–758
10. Petersen PE, Ogawa H (2012) The global burden of periodontal disease: towards integration with chronic disease prevention and control. Periodontol 2000 60(1):15–39
11. Nibali L, Donos N (2013) Periodontitis and redox status: a review. Curr Pharm Des 19(15): 2687–2697

12. Bullon P, Cordero MD, Quiles JL et al (2012) Autophagy in periodontitis patients and gingival fibroblasts: unraveling the link between chronic diseases and inflammation. BMC Med 10:122
13. Calenic B, Yaegaki K, Ishkitiev N, Kumazawa Y, Imai T, Tanaka T (2013) p53-pathway activity and apoptosis in hydrogen sulfide-exposed stem cells separated from human gingival epithelium. J Periodontal Res 48(3):322–330
14. Chapple IL, Matthews JB, Wright HJ, Scott AE, Griffiths HR, Grant MM (2013) Ascorbate and alpha-tocopherol differentially modulate reactive oxygen species generation by neutrophils in response to FcgammaR and TLR agonists. Innate Immun 19(2):152–159
15. Pradeep AR, Rao NS, Bajaj P, Agarwal E (2013) 8-Isoprostane: a lipid peroxidation product in gingival crevicular fluid in healthy, gingivitis and chronic periodontitis subjects. Arch Oral Biol 58(5):500–504
16. Calenic B, Yaegaki K, Kozhuharova A, Imai T (2010) Oral malodorous compound causes oxidative stress and p53-mediated programmed cell death in keratinocyte stem cells. J Periodontol 81(9):1317–1323
17. Greabu M, Battino M, Mohora M, Olinescu R, Totan A, Didilescu A (2008) Oxygen, a paradoxical element? Rom J Intern Med 46(2):125–135
18. Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB (2010) Oxidative stress, inflammation, and cancer: how are they linked? Free Radic Biol Med 49(11):1603–1616
19. Tran B, Oliver S, Rosa J, Galassetti P (2012) Aspects of inflammation and oxidative stress in pediatric obesity and type 1 diabetes: an overview of ten years of studies. Exp Diabetes Res 2012:683680
20. Kobulnik J, Delgado D (2012) Experimental biomarkers in heart failure: an update. Expert Rev Cardiovasc Ther 10(9):1119–1132
21. Bullon P, Morillo JM, Ramirez-Tortosa MC, Quiles JL, Newman HN, Battino M (2009) Metabolic syndrome and periodontitis: is oxidative stress a common link? J Dent Res 88(6):503–518
22. Bullon P, Cordero MD, Quiles JL, Morillo JM, Del Carmen Ramirez-Tortosa M, Battino M (2011) Mitochondrial dysfunction promoted by Porphyromonas gingivalis lipopolysaccharide as a possible link between cardiovascular disease and periodontitis. Free Radic Biol Med 50(10):1336–1343
23. Ekuni D, Tomofuji T, Endo Y et al (2012) Hydrogen-rich water prevents lipid deposition in the descending aorta in a rat periodontitis model. Arch Oral Biol 57(12):1615–1622
24. Sanz M, D'Aiuto F, Deanfield J, Fernandez-Avilés F (2010) European workshop in periodontal health and cardiovascular disease—scientific evidence on the association between periodontal and cardiovascular diseases: a review of the literature. Eur Heart J Suppl 12(Suppl B):B3–B12
25. Sardi Jde C (2013) Oxidative stress in diabetes and periodontitis. N Am J Med Sci 5(1):58–59
26. Soory M (2010) Oxidative stress induced mechanisms in the progression of periodontal diseases and cancer: a common approach to redox homeostasis? Cancers 2(2):670–692
27. Miller CS, King CP Jr, Langub MC, Kryscio RJ, Thomas MV (2006) Salivary biomarkers of existing periodontal disease: a cross-sectional study. J Am Dent Assoc 137(3):322–329
28. Giannobile WV, Beikler T, Kinney JS, Ramseier CA, Morelli T, Wong DT (2009) Saliva as a diagnostic tool for periodontal disease: current state and future directions. Periodontol 2000 50:52–64
29. Lamster IB, Ahlo JK (2007) Analysis of gingival crevicular fluid as applied to the diagnosis of oral and systemic diseases. Ann N Y Acad Sci 1098:216–229
30. Segal A, Wong DT (2008) Salivary diagnostics: enhancing disease detection and making medicine better. Eur J Dent Educ 12(Suppl 1):22–29
31. Wu ZZ, Wang JG, Zhang XL (2009) Diagnostic model of saliva protein finger print analysis of patients with gastric cancer. World J Gastroenterol 15(7):865–870
32. Castagnola M, Inzitari R, Rossetti DV et al (2004) A cascade of 24 histatins (histatin 3 fragments) in human saliva. Suggestions for a pre-secretory sequential cleavage pathway. J Biol Chem 279(40):41436–41443
33. Messana I, Cabras T, Inzitari R et al (2004) Characterization of the human salivary basic proline-rich protein complex by a proteomic approach. J Proteome Res 3(4):792–800

34. Inzitari R, Cabras T, Rossetti DV et al (2006) Detection in human saliva of different statherin and P-B fragments and derivatives. Proteomics 6(23):6370–6379
35. Cabras T, Inzitari R, Fanali C et al (2006) HPLC-MS characterization of cyclo-statherin Q-37, a specific cyclization product of human salivary statherin generated by transglutaminase 2. J Sep Sci 29(17):2600–2608
36. Veerman EC, Van Den Keybus PA, Vissink A, Nieuw Amerongen AV (1996) Human glandular salivas: their separate collection and analysis. Eur J Oral Sci 104(4 Pt 1):346–352
37. De Almeida PV, Gregio AM, Machado MA, De Lima AA, Azevedo LR (2008) Saliva composition and functions: a comprehensive review. J Contemp Dent Pract 9(3):72–80
38. Hu S, Denny P, Xie Y et al (2004) Differentially expressed protein markers in human submandibular and sublingual secretions. Int J Oncol 25(5):1423–1430
39. Siqueira WL, Salih E, Wan DL, Helmerhorst EJ, Oppenheim FG (2008) Proteome of human minor salivary gland secretion. J Dent Res 87(5):445–450
40. Nemolato S, Messana I, Cabras T et al (2009) Thymosin beta(4) and beta(10) levels in preterm newborn oral cavity and foetal salivary glands evidence a switch of secretion during foetal development. PLoS ONE 4(4):e5109
41. Dowling P, Wormald R, Meleady P, Henry M, Curran A, Clynes M (2008) Analysis of the saliva proteome from patients with head and neck squamous cell carcinoma reveals differences in abundance levels of proteins associated with tumour progression and metastasis. J Proteomics 71(2):168–175
42. Wong DT (2006) Towards a simple, saliva-based test for the detection of oral cancer 'oral fluid (saliva), which is the mirror of the body, is a perfect medium to be explored for health and disease surveillance'. Expert Rev Mol Diagn 6(3):267–272
43. Xie H, Onsongo G, Popko J et al (2008) Proteomics analysis of cells in whole saliva from oral cancer patients via value-added three-dimensional peptide fractionation and tandem mass spectrometry. Mol Cell Proteomics 7(3):486–498
44. Nakashima D, Uzawa K, Kasamatsu A et al (2006) Protein expression profiling identifies maspin and stathmin as potential biomarkers of adenoid cystic carcinoma of the salivary glands. Int J Cancer 118(3):704–713
45. An J, Sun JY, Yuan Q et al (2004) Proteomics analysis of differentially expressed metastasis-associated proteins in adenoid cystic carcinoma cell lines of human salivary gland. Oral Oncol 40(4):400–408
46. Dayan D, Hirshberg A, Kaplan I, Rotem N, Bodner L (1997) Experimental tongue cancer in desalivated rats. Oral Oncol 33(2):105–109
47. Tomasik A, Tarnawski R, Wierzynski J (1994) Measurements of amylase isoenzymes in sera and saliva of patients after radiotherapy because of larynx carcinoma. Otolaryngol Pol 48(2):132–137
48. Wu Y, Shu R, Luo LJ, Ge LH, Xie YF (2009) Initial comparison of proteomic profiles of whole unstimulated saliva obtained from generalized aggressive periodontitis patients and healthy control subjects. J Periodontal Res 44(5):636–644
49. Ryu OH, Atkinson JC, Hoehn GT, Illei GG, Hart TC (2006) Identification of parotid salivary biomarkers in Sjogren's syndrome by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry and two-dimensional difference gel electrophoresis. Rheumatology (Oxford) 45(9):1077–1086
50. Giusti L, Baldini C, Bazzichi L et al (2007) Proteome analysis of whole saliva: a new tool for rheumatic diseases—the example of Sjogren's syndrome. Proteomics 7(10):1634–1643
51. Sayer R, Law E, Connelly PJ, Breen KC (2004) Association of a salivary acetylcholinesterase with Alzheimer's disease and response to cholinesterase inhibitors. Clin Biochem 37(2):98–104
52. Fabian TK, Fejerdy P, Csermely P (2008) Salivary genomics, transcriptomics and proteomics: the emerging concept of the oral ecosystem and their use in the early diagnosis of cancer and other diseases. Curr Genomics 9(1):11–21
53. Greabu M, Battino M, Mohora M et al (2007) Could constitute saliva the first line of defence against oxidative stress? Rom J Intern Med 45(2):209–213

54. Battino M, Ferreiro MS, Gallardo I, Newman HN, Bullon P (2002) The antioxidant capacity of saliva. J Clin Periodontol 29(3):189–194
55. Miricescu D, Totan A, Calenic B et al (2013) Salivary biomarkers: relationship between oxidative stress and alveolar bone loss in chronic periodontitis. Acta Odontol Scand [Epub ahead of print]
56. Miricescu D, Greabu M, Totan A, Didilescu A, Rădulescu R (2011) The antioxidant potential of saliva: clinical significance in oral diseases. Ther Pharmacol Clin Toxicol 2:139–143
57. Battino M, Greabu M, Totan A et al (2008) Oxidative stress markers in oral lichen planus. Biofactors 33(4):301–310
58. Greabu M, Battino M, Totan A et al (2007) Effect of gas phase and particulate phase of cigarette smoke on salivary antioxidants. What can be the role of vitamin C and pyridoxine? Pharmacol Rep 59(5):613
59. Greabu M, Totan A, Battino M et al (2008) Cigarette smoke effect on total salivary antioxidant capacity, salivary glutathione peroxidase and gamma-glutamyltransferase activity. Biofactors 33(2):129–136
60. Greabu M, Didilescu A, Puiu L, Miricescu D, Totan A (2012) Salivary antioxidant biomarkers in non-ferrous metals mine workers—a pilot study. J Oral Pathol Med 41(6):490–493
61. Henkin RI, Martin BM, Agarwal RP (1999) Decreased parotid saliva gustin/carbonic anhydrase VI secretion: an enzyme disorder manifested by gustatory and olfactory dysfunction. Am J Med Sci 318(6):380–391
62. Amerongen A, Veerman EC (2008) Saliva: properties and functions. In: Wong DT (ed) Salivary diagnostics. Wiley Blackwell, Ames, pp 27–36
63. Sarosiek J, Mccallum RW (2000) Mechanisms of oesophageal mucosal defence. Baillieres Best Pract Res Clin Gastroenterol 14(5):701–717
64. Pedersen A, Bardow A, Jensen SB, Nauntofte B (2002) Saliva and gastrointestinal functions of taste, mastication, swallowing and digestion. Oral Dis 8(3):117–129
65. Humphrey SP, Williamson RT (2001) A review of saliva: normal composition, flow, and function. J Prosthet Dent 85(2):162–169
66. Scannapieco FA (1994) Saliva-bacterium interactions in oral microbial ecology. Crit Rev Oral Biol Med 5(3–4):203–248
67. De Abreu Pereira D, Areias VR, Franco MF et al (2013) Measurement of HER2 in saliva of women in risk of breast cancer. Pathol Oncol Res 19(3):509–513
68. Streckfus C, Bigler L, Dellinger T, Dai X, Kingman A, Thigpen JT (2000) The presence of soluble c-erbB-2 in saliva and serum among women with breast carcinoma: a preliminary study. Clin Cancer Res 6(6):2363–2370
69. Zhang L, Farrell JJ, Zhou H et al (2010) Salivary transcriptomic biomarkers for detection of resectable pancreatic cancer. Gastroenterology 138(3):949–957, e1–7
70. Xiao H, Zhang L, Zhou H, Lee JM, Garon EB, Wong DT (2012) Proteomic analysis of human saliva from lung cancer patients using two-dimensional difference gel electrophoresis and mass spectrometry. Mol Cell Proteomics 11(2):M111.012112
71. Giusti L, Bazzichi L, Baldini C et al (2007) Specific proteins identified in whole saliva from patients with diffuse systemic sclerosis. J Rheumatol 34(10):2063–2069
72. Van Holland BJ, Frings-Dresen MH, Sluiter JK (2012) Measuring short-term and long-term physiological stress effects by cortisol reactivity in saliva and hair. Int Arch Occup Environ Health 85(8):849–852
73. Monteleone P, Scognamiglio P, Canestrelli B, Serino I, Monteleone AM, Maj M (2011) Asymmetry of salivary cortisol and alpha-amylase responses to psychosocial stress in anorexia nervosa but not in bulimia nervosa. Psychol Med 41(9):1963–1969
74. Dodds MW, Johnson DA, Yeh C-K (2005) Health benefits of saliva: a review. J Dent 33(3):223–233
75. Taylor GW (2001) Bidirectional interrelationships between diabetes and periodontal diseases: an epidemiologic perspective. Ann Periodontol 6(1):99–112
76. Balamane M, Winters MA, Dalai SC et al (2010) Detection of HIV-1 in saliva: implications for case-identification, clinical monitoring and surveillance for drug resistance. Open Virol J 4:88

77. Cruz H, Marques V, Villela-Nogueira C et al (2012) An evaluation of different saliva collection methods for detection of antibodies against hepatitis C virus (anti-HCV). J Oral Pathol Med 41(10):793–800
78. Srivastava S, Krueger K (2008) Diagnostics other than blood. In: Wong D (ed) Salivary diagnostics. Wiley Blackwell, Ames, pp 95–103
79. Sue Carter C, Al E (2007) Oxytocin behavioral associations and potential as salivary biomarker. In: Malamud D, Niedbala R (eds) Oral-based diagnostics. Annals of the New York Academy of Sciences, Boston, pp 312–323
80. Goncalves Lda R, Soares MR, Nogueira FC et al (2010) Comparative proteomic analysis of whole saliva from chronic periodontitis patients. J Proteomics 73(7):1334–1341
81. Ito T, Komiya-Ito A, Arataki T et al (2008) Relationship between antimicrobial protein levels in whole saliva and periodontitis. J Periodontol 79(2):316–322
82. Fabian TK, Fejerdy P, Nguyen MT, Soti C, Csermely P (2007) Potential immunological functions of salivary Hsp70 in mucosal and periodontal defense mechanisms. Arch Immunol Ther Exp (Warsz) 55(2):91–98
83. Kibayashi M, Tanaka M, Nishida N et al (2007) Longitudinal study of the association between smoking as a periodontitis risk and salivary biomarkers related to periodontitis. J Periodontol 78(5):859–867
84. Nishida N, Yamamoto Y, Tanaka M et al (2006) Association between passive smoking and salivary markers related to periodontitis. J Clin Periodontol 33(10):717–723
85. Totan A, Greabu M, Totan C, Spinu T (2006) Salivary aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase: possible markers in periodontal diseases? Clin Chem Lab Med 44(5):612–615
86. Preza D, Thiede B, Olsen I, Grinde B (2009) The proteome of the human parotid gland secretion in elderly with and without root caries. Acta Odontol Scand 67(3):161–169
87. Vitorino R, De Morais Guedes S, Ferreira R et al (2006) Two-dimensional electrophoresis study of in vitro pellicle formation and dental caries susceptibility. Eur J Oral Sci 114(2):147–153
88. Van Nieuw Amerongen A, Bolscher JG, Veerman EC (2004) Salivary proteins: protective and diagnostic value in cariology? Caries Res 38(3):247–253
89. Dowd FJ (1999) Saliva and dental caries. Dent Clin North Am 43(4):579–597
90. Yang LL, Liu XQ, Liu W, Cheng B, Li MT (2006) Comparative analysis of whole saliva proteomes for the screening of biomarkers for oral lichen planus. Inflamm Res 55(10):405–407
91. Mizukawa N, Sawaki K, Nagatsuka H et al (2001) Human alpha-and beta-defensin immunoreactivity in oral mucoepidermoid carcinomas. Anticancer Res 21(3C):2171–2174
92. Pickering V, Jordan RC, Schmidt BL (2007) Elevated salivary endothelin levels in oral cancer patients—a pilot study. Oral Oncol 43(1):37–41

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