ROLE OF OXIDATIVE STRESS IN ORAL DISEASES

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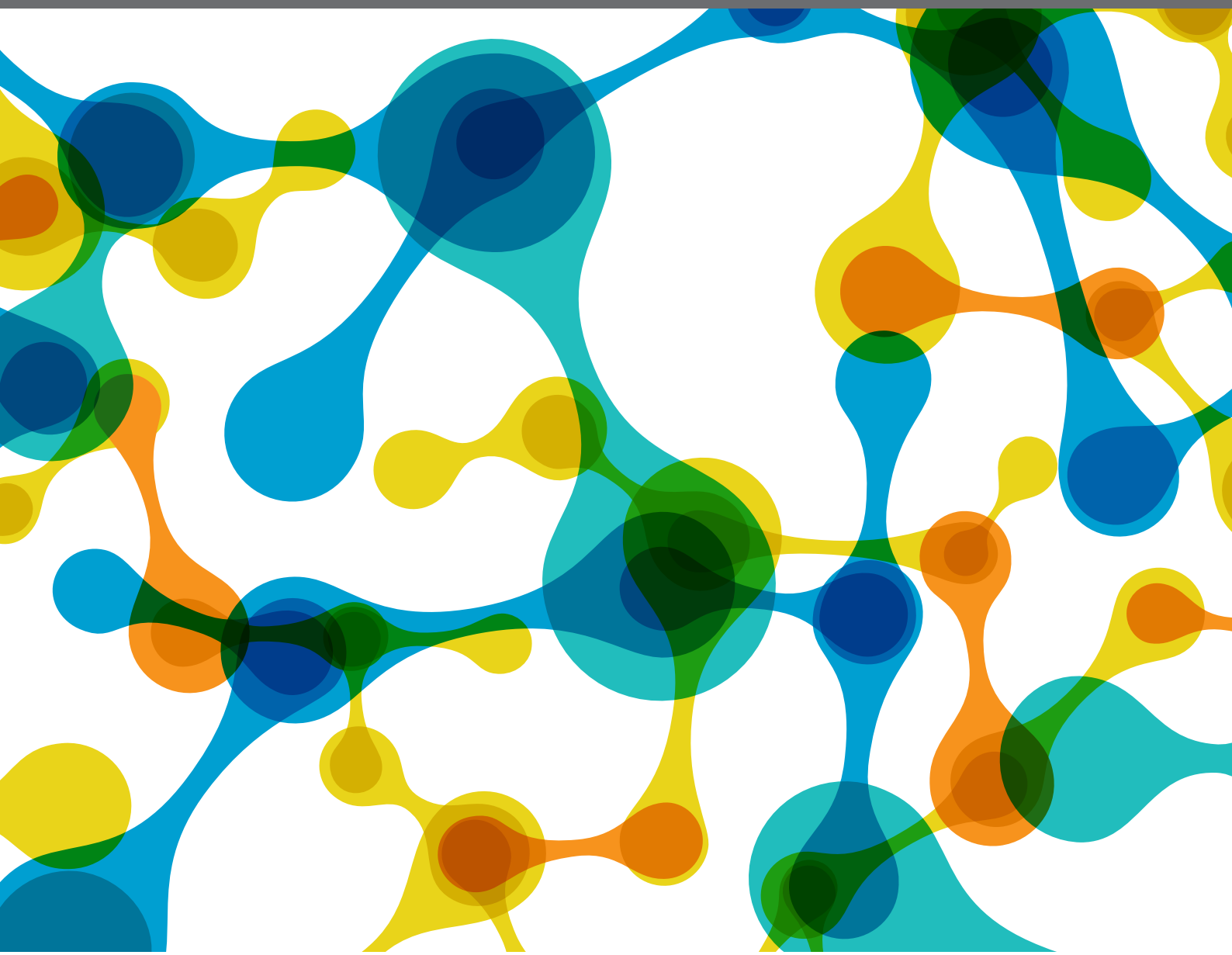

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ROLE OF OXIDATIVE STRESS IN ORAL DISEASES

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MINI REVIEW
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Oxidative Stress and Antioxidants in the Diagnosis and Therapy of Periodontitis

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Oxidative stress has been implicated in the pathogenesis of numerous diseases. However, large interventional studies with antioxidants failed to show benefits in the prevention or treatment of cardiovascular diseases, cancer, or diabetes mellitus. Numerous clinical studies have confirmed the association of oxidative stress markers and periodontitis. Technical and biological variability is high for most of the analyzed markers and none of them seems to be optimal for routine clinical use. In a research setting, analysis of a palette of oxidative stress markers is needed to cover lipid peroxidation, protein oxidation, and the antioxidant status. The source of reactive oxygen species and their role in the pathogenesis of periodontitis remains unclear. Interventional experiments indicate that oxidative stress might be more than just a simple consequence of the inflammation. Small studies have confirmed that some antioxidants could have therapeutic value at least as an addition to the standard non-surgical treatment of periodontitis. A clear evidence for the efficiency of antioxidant treatment in large patient cohorts is lacking. Potentially, because lowering of oxidative stress markers might be a secondary effect of anti-inflammatory or antibacterial agents. As the field of research of oxidative stress in periodontitis gains attraction and the number of relevant published papers is increasing a systematic overview of the conducted observational and interventional studies is needed. This review summarizes the currently available literature linking oxidative stress and periodontitis and points toward the potential of adjuvant antioxidant treatment, especially in cases where standard treatment fails to improve the periodontal status.

Keywords: reactive oxygen species, free radicals, antioxidative therapy, systematic review, oral diseases

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INTRODUCTION

Oxidative stress is both, a pathomechanism involved in numerous inflammatory diseases causing damage to lipids, nucleic acids and proteins—oxidative distress, as well as an important physiological process that enables the immune system to cope with microorganisms and intracellular cell signaling—oxidative eustress (Sies et al., 2017). Which edge of the sword is the dominant depends on the delicate balance between the production of reactive oxygen/nitrogen species and the antioxidant capacity of the tissue. The physiological functions of free radicals have

been neglected for years and so, much more is known about the pathological role of oxidative stress. A variety of free radicals is produced and interacts with a variety of substrates. This leads to a palette of biomarkers that can be used for the assessment of oxidative stress-induced damage (Frijhoff et al., 2015).

Oxidative stress is usually defined as a disbalance of the production of free radicals and antioxidant mechanisms (Kopáni et al., 2006). However, free radicals are not a simple negative byproduct of oxygen metabolism. They are involved in immune responses, liver metabolism, but also in intracellular signaling pathways (Espinosa-Diez et al., 2015). The mechanisms involved in the physiological intracellular role of free radicals include the modulation of cysteine residues of redox-sensitive enzymes and other regulatory proteins (Finkel, 2011; Russell and Cotter, 2015). It has been hypothesized that under physiological conditions even high concentrations of any one primary reactive oxygen or nitrogen species does not lead to oxidative damage, as the cell has preventive and reparative tools to cope with the radical. The reactions of superoxide, nitric oxide, and other primary reactive species are reversible and are ideal for intracellular signaling (Weidinger and Kozlov, 2015). Thus, measuring oxidative stress using any one marker can lead to wrong interpretations. This might include the hope that administration of antioxidants will effectively treat diseases associated with oxidative stress. As recently hypothesized, one of the prototypic oxidative stress diseases—diabetes mellitus might be actually a consequence of deficiency of reactive oxygen species rather than of oxidative damage (Watson, 2014). It should not escape our notice that this proposed mechanism might suggest that antioxidants could increase the risk rather than prevent metabolic diseases.

Periodontitis is an inflammatory disease affecting supporting structures of the teeth leading at the end to loss of alveolar bone and teeth (Kinane et al., 2017). The main causative factor are microorganisms that colonize the subgingival dental plaque inducing an inflammatory host response. The inflammation affects, however, also the surrounding healthy tissue ultimately leading to the destruction of the periodontium (Kinane et al., 2011). Although lipopolysaccharide and proteolytic enzymes are essential in periodontitis, exaggerated inflammatory response, genetic predisposition, smoking, bad oral hygiene, and malnutrition are important in pathogenesis of periodontitis as well (Laine et al., 2012).

The role of oxidative stress in periodontitis has been postulated already decades ago (Shapira et al., 1991; Chapple, 1997). However, the suggested involvement was not clear. Some studies showed that leukocytes from patients with periodontitis are exhausted and have a low oxidation activity (Loesche et al., 1988), other studies pointed toward higher production of free radicals by leukocytes from periodontitis patients (Kimura et al., 1993). The contradictory findings from these studies might be related to the dynamics of the mechanisms during the pathogenesis of the disease, but they might also be explained by the different forms of periodontitis (Biasi et al., 1999).

The term oxidative stress is vague, similarly, antioxidants might affect many processes not directly related to free radical generation or action (Niki, 2016). This makes the interpretation of studies focusing on oxidative stress in periodontitis difficult. A systematic review of these clinical studies and animal experiments might, thus, be needed.

BIOMARKERS OF OXIDATIVE STRESS AND PATHOGENESIS OF PERIODONTITIS

Surprisingly, many observational studies analyzing oxidative stress in patients with periodontitis had relatively consistent results with higher oxidative stress markers in either saliva or blood and/or decreased antioxidant status in comparison to controls. The summary table of the identified studies can be found in **Table 1**. One of the largest observational studies has shown that the antioxidant status in blood, analyzed as vitamin C, bilirubin, and calculated total antioxidant capacity was inversely associated with mild and severe periodontitis (Chapple et al., 2007b). The more severe periodontitis, the clearer the association. Additionally, in a subgroup of never-smokers the antioxidants seemed to protect against development of periodontitis. Others smaller studies confirmed these results. Total antioxidant capacity was lower in plasma or serum of patients with chronic periodontitis (Chapple et al., 2002; Brock et al., 2004; Konopka et al., 2007). Similarly, superoxide dismutase activity (Huang et al., 2014) along with catalase and glutathione peroxidase activity (Tonguç et al., 2011) as important contributor to the total antioxidant capacity was found to be lower in periodontitis. In line with these results from blood, in the majority of studies, the antioxidant status was lower also locally—in saliva. The total antioxidant status/potential/capacity is in general the ability of a tissue to resist artificially induced oxidative stress, but studies differ in the analytical approaches. Nevertheless, the local antioxidant capacity was found to be lower in saliva from patients with an aggressive form of periodontitis when compared to chronic periodontitis (Acquier et al., 2017). Patients with chronic periodontitis have lower antioxidant capacity than control patients (Zhang et al., 2015; Ahmadi-Motamayel et al., 2017). Although the total antioxidant capacity is not a specific marker of antioxidant power, uric acid, glutathione peroxidase (Miricescu et al., 2014), and reduced glutathione (Gumus et al., 2009) as specific antioxidants were reported to be significantly lower in saliva of patients with chronic or aggressive periodontitis. On contrary, activities of the major antioxidant enzymes were found to be higher in chronic periodontitis patients in all investigated samples, i.e., plasma, erythrocytes, and in the gingival tissue (Panjamurthy et al., 2005). Similarly, SOD2 and GPX1 genes were overexpressed in the gingiva of chronic periodontitis patients (Duarte et al., 2012). Lactoferrin, myeloperoxidase and interleukin 1 beta were all positively correlated with the clinical markers of periodontal damage (Wei et al., 2004). However, whether such associations of higher antioxidant and pro-inflammatory response are a consequence or cause of severe periodontitis cannot be judged only from observations.

Regarding the analyzed markers of oxidative stress, a comparison of the published studies is complicated, if not impossible due to the huge variability of measured markers.

TABLE 1 | Observational studies analyzing oxidative stress in periodontitis.

Study design	Sample size	Note	Sample type	Outcome	References
Cross-sectional study	20 AgP patients 20 ChP patients 20 controls	–	Saliva	↑ ROS, TBARS with AgP; TRAP ↓ with AgP compared to ChP	Acquier et al., 2017
Case-control study (cross-sectional)	55 ChP patients 55 healthy controls	–	Serum Saliva	↓ TAC in serum and saliva; ↑ MDA increased in serum and saliva vs. controls	Ahmadi-Motamayel et al., 2017
Prospective study	33 PW with ChP 18 PW with gingivitis 21 PW controls 27 non-PW ChP 25 non-PW controls	Pregnancy	Serum GCF	↓ TAC, SOD in PW vs. non-PW and in ChP group compared to controls; ↑ TAC, SOD in third trimester in PW with ChP vs. PW with ChP in the first trimester	Akalin et al., 2009
Comparative study (cross-sectional)	20 T2DM patients with periodontitis 20 T2DM patients PH 20 SH patients with periodontitis	T2DM	Plasma	↓ plasma small molecule antioxidant capacity ↑ PC in T2DM with periodontitis vs. PH T2DM patients	Allen et al., 2011
Cross-sectional study	33 ChP patients 16 patients with gingivitis 37 healthy controls	–	Saliva	↑ TAC, 8-OHdG, MDA and activity of SOD, GPx in ChP vs. controls	Almerich-Silla et al., 2015
Observational study (cross-sectional)	19 non-T2DM patients 24 T2DM patients with good metabolic control 27 T2DM patients with poor metabolic control T2DM	T2DM	Saliva	↓ GPx, glutathione reductase in poor metabolic control T2DM patients; ↑ GSSG/GSH ratio in poor metabolic control T2DM	Arana et al., 2017
Case-control study	15 PH + normal weight 15 gingivitis + normal weight 15 ChP + normal weight 15 PH + obese 18 gingivitis + obese 15 ChP + obese	Obesity	GCF	↑ MDA, PC and ↓ TAC in ChP + obese group	Atabay et al., 2017
Cross-sectional study	32 ChP post-menopausal women 31 ChP pre-menopausal 25 PH post-menopausal women 26 PH pre-menopausal women	Menopause	Serum GCF	↓ TAC, SOD activity in post-menopausal women with ChP in serum and GCF	Baltacioglu et al., 2006
Cross-sectional study	35 AgP patients 33 ChP patients 30 PH controls	–	Serum Saliva	↑ MDA, TOS, OSI in periodontitis groups; ↓ TAC in periodontitis groups vs. controls (except serum MDA)	Baltacioglu et al., 2014
Cross-sectional study	23 ChP patients 19 PH controls	–	Saliva	↑ TBARS in ChP (men) ↓ TAC in ChP (women); trend toward ↓ DNA integrity in ChP	Banasová et al., 2015
Cross-sectional study	9 patients with periodontitis 9 healthy controls	–	Gingival tissue	↑ activities of MPO, GPx, glutathione S-transferase in periodontitis group; ↑ TBARS and GSSG levels increased vs. controls	Borges et al., 2007
Cross-sectional study	20 ChP patients 20 healthy controls	–	Serum Saliva GCF	↓ TAC in GCF and plasma in ChP vs. control; ↓ TAC in saliva in ChP vs. control	Brock et al., 2004
Cross-sectional study	32 ChP patients 32 PH control	–	Saliva Gingival tissue	↑ 8-OHdG and mtDNA deletions in ChP group vs. control	Canakci et al., 2009
Cross-sectional study	30 ChP patients 30 PH control	–	Blood Gingival tissue	↑ mtDNA deletion in ChP group vs. control	Canakci et al., 2006
Cross-sectional study	10 preeclampsia ChP 10 preeclampsia PH 10 normotensive ChP 10 normotensive PH	Preeclampsia	Serum Saliva GCF	↓ TAC in ChP women with preeclampsia (GCF, serum, saliva); ↓ SOD and GPx activities decreased in ChP women with preeclampsia (GCF and serum); ↑ MDA in ChP preeclamptic women (GCF, serum)	Canakci et al., 2007
Cross-sectional study	31 pre-menopausal 31 peri-menopausal 31 post-menopausal ChP women	Menopause	GCF Gingival tissue	↑ 8-OHdG in ChP post-menopausal women in GCF and gingival tissue	Chandra et al., 2017
Cross-sectional study	10 ChP patients 10 PH control	–	Plasma GCF	↓ TAC in GCF and plasma in ChP vs. control; ↓ GSH and GSSG in GCF in ChP	Chapple et al., 2002

(Continued)

TABLE 1 | Continued

Study design	Sample size	Note	Sample type	Outcome	References
Observational correlational study	11,480 participants; 1,567 with mild periodontitis 609 with severe periodontitis	–	Serum	↓ vitamin C, bilirubin and TAC (calculated) in mild or severe periodontitis	Chapple et al., 2007b
Cross-sectional study	17 periodontitis patients 20 healthy controls	–	Saliva	↓ TAC in periodontitis	Diab-Ladki et al., 2003
Cross-sectional study	12 SH and PH controls 15 SH and ChP patients 8 well-controlled T2DM patients with ChP 14 poor-controlled T2DM patients with ChP	T2DM	Gingival tissue	Peroxiredoxin 1 and GPX1 overexpressed in ChP; Peroxiredoxin 2 and SOD2 up-regulated especially in poor—controlled T2DM with ChP	Duarte et al., 2012
Cross-sectional study	20 ChP patients with RA 20 PH patients with RA 20 ChP patients SH 20 SH and PH controls	Rheumatoid arthritis	Serum GCF	↑ TOS in GCF of ChP and RA ChP groups (no difference for TOS and OSI in serum)	Esen et al., 2012
Cross-sectional study	18 ChP patients with hyperlipidemia 18 PH patients with hyperlipidemia 19 ChP patients SH 19 PH SH controls	Hyperlipidemia	Serum	↑ MDA and 8-OHdG in patients with ChP and hyperlipidemia	Fentoglu Ö et al., 2015
Cross-sectional study	24 ChP with depression 23 ChP without depression	Depression	Plasma	↑ nitric oxide metabolites, lipid peroxides, AOPP and TRAP in ChP with depression	Gomes et al., 2017
Cross-sectional study	16 T1DM with periodontitis 25 T2DM with periodontitis 24 SH with periodontitis	T1DM T2DM	Saliva	↓ GSH and ↓GSSG in the patients with T1DM	Gumus et al., 2009
Case-control study	115 P women (6 month postpartum follow-up) 72 non-P women	Pregnancy	Saliva	↑ 8-OHdG in PW; ↓ GPx decreased in PW; ↓ TBARS postpartum vs. non-pregnant women	Gümüş et al., 2015
Prospective study	218 P women 459 P women with mild periodontitis 114 P women with moderate-severe periodontitis	Pregnancy	Serum	↑ 8-isoprostane in PW with moderate-severe periodontitis	Hickman et al., 2011
Prospective study	50 ChP patients 50 healthy controls	–	Serum Saliva	↓ SOD in saliva and serum; ↑ prostaglandin E2, D2, prostaglandin F2α and TXB2, 5- hydroxyeicosatetraenoic acid, F2-isoprostanes; ↑ prostacyclin I2 and 13- hydroxyoctadecadienoic acid, 9- hydroxyoctadecadienoic acid	Huang et al., 2014
Cross-sectional Study	55 patients with DS 74 patients with mental retardation 88 healthy controls	Down Syndrome Mental retardation	Whole blood	↑ oxidative burst activity of blood (monocytes and granulocytes) in DS patients with decreased periodontal health	Khocht et al., 2014
Cross-sectional study	26 AgP patients 30 ChP patients 25 healthy controls	–	Gingival blood Peripheral blood	↑ 8-OHdG in gingival blood of ChP and AgP patients; ↓ TAC in gingival blood of ChP patients; ↓ TAC in peripheral blood of both groups	Konopka et al., 2007
Cross-sectional study	1,258 old men	Old age	Serum	↓ beta-cryptoxanthin and beta-carotene with decreased periodontal health of old men	Linden et al., 2009
Cross-sectional study	356 periodontitis patients 207 PH controls	–	Plasma	↑ reactive oxygen metabolites and shorter leukocyte telomere length in ChP	Masi et al., 2011
Cross-sectional Correlational study	20 ChP patients 20 PH controls	–	Saliva	↑ 8-OHdG, MDA in ChP; ↓ uric acid, GPx activities and TAC in ChP (correlation with bone loss markers)	Miricescu et al., 2014
Cross-sectional study	10 T2DM patients PH 8 SH controls	T2DM	Periodontal tissue	↑ MDA, ↓ GSH in periodontal tissue of T2DM	Monea et al., 2014
Cross-sectional study	24 ChP patients with ACS 24 patients PH with ACS 24 ChP patients without ACS 24 controls PH without ACS	Acute coronary syndrome	Saliva	↑ 8-OHdG, MDA, and PC in patients (correlation with periodontal and cardiovascular markers)	Nguyen et al., 2016

(Continued)

TABLE 1 | Continued

Study design	Sample size	Note	Sample type	Outcome	References
Cross-sectional study	25 ChP patients 25 healthy controls	–	Plasma Gingival tissue Erythrocytes	↑ TBARS in ChP; ↑ SOD, CAT, GPx activities in ChP; ↓ vitamins E, C and GSH in ChP	Panjamurthy et al., 2005
Cross-sectional study	29 ChP patients 20 healthy controls	–	Saliva	↑ 8-OHdG in ChP (correlation with *P. gingivalis*)	Sawamoto et al., 2005
Cross-sectional cohort study	46 severe periodontitis patients 37 moderate periodontitis patients 46 mild periodontitis and healthy	–	Saliva	↑ PC increased in severe periodontitis; ↓ urate and FRAP in severe periodontitis	Sculley and Langley-Evans, 2003
Cross-sectional study	20 ChP patients with RA 20 PH patients with RA 20 ChP patients without RA 20 PH SH controls	Rheumatoid arthritis	Serum	↑ OSI and prolidase in ChP patients with RA	Sezer et al., 2013
Cross-sectional study	20 ChP patients with PS 20 PH patients with PS 20 ChP patients with PsA 20 PH patients with PsA 20 ChP SH patients 20 PH SH controls	Psoriasis Psoriatic arthritis	Serum	↑ OSI (irrespective of periodontitis) in patients groups; (PS and PsA showed no effect on clinical parameters in ChP patients)	Sezer et al., 2016
Cross-sectional study	4,717 participants	Diabetes mellitus Hypertension	Serum	Periodontitis with highest 8-isoprostane quartile associated with ↑ CRP	Singer et al., 2015
Cross-sectional correlational study	29 severe periodontitis 77 moderate periodontitis 96 mild periodontitis and healthy	Diabetes mellitus Hypertension	Serum	↑ ROM in patients with worst periodontal status	Tamaki et al., 2014b
Cross-sectional study	25 severe periodontitis 43 moderate periodontitis 92 mild periodontitis and healthy	Diabetes mellitus Hypertension	Saliva	Superoxide and hydroxyl radical scavenging activities associated with periodontitis	Tamaki et al., 2015
Cross-sectional study	39 patients with periodontitis	–	Saliva	↓ antioxidant concentrations related to periodontal status	Tartaglia et al., 2017
Cross-sectional study	23 smokers with ChP 23 former smokers with ChP 19 non-smokers with ChP 20 PH non-smokers controls	Smoking	Serum Gingival tissue	↑ MDA in serum and gingival tissue in smoking ChP patients groups; ↓ SOD, CAT and GPx in ChP groups	Tonguç et al., 2011
Cross-sectional study	82 children	–	Saliva	TBARS in correlation to periodontal status; TAC related to periodontal status and oral hygiene; AOPP related to caries in children	Tothova et al., 2013
Cross-sectional study	30 ChP patients with T2DM 30 ChP SH patients 30 PH patients with T2DM 30 PH SH controls	T2DM	Plasma Saliva Red blood cell lysate	↑ MDA in ChP patients irrespective of T2DM	Trivedi et al., 2014
Cross-sectional study	100 ChP patients 50 healthy controls	–	Saliva	↑ 8-OHdG and human neutrophil elastase/alpha1-proteinase inhibitor in ChP	Villa-Correa et al., 2015
Cross-sectional study	19 patients with periodontitis 8 healthy controls	-	GCF	↑ GPx, lactoferrin, myeloperoxidase and IL-1beta in periodontal tissues (correlation with clinical periodontal markers)	Wei et al., 2004
Cross-sectional study	31 smokers 90 non-smokers	Smoking	Saliva	↑ 8-epi-PGF(2alpha) with periodontal status and smoking; Smoking ↑ TXB(2) and PGF(2alphas) and ↓ 6-oxo-PGF(1alpha)	Wolfram et al., 2006
Cross-sectional study	58 ChP patients 42 AgP patients 60 healthy controls	–	Saliva Buccal mucosa	↑ micronuclei and nuclear abnormalities, as well as 8-OHdG in both periodontitis groups	Zamora-Perez et al., 2015
Cross-sectional study	45 severe periodontitis patients 37 healthy controls	–	Saliva	↓ TAC in periodontitis (TOS no difference)	Zhang et al., 2015

ChP, chronic periodontitis; AgP, aggressive periodontitis; SH, systematically healthy; PH, periodontal healthy; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; PS, psoriasis; PsA, psoriatic arthritis; DS, Down syndrome; PW, pregnant woman; RA, Rheumatoid arthritis; ACS, Acute coronary syndrome; GCF, gingival crevicular fluid; TAC, total antioxidant capacity; TBARS, thiobarbituric acid reacting substances; 8-HOdG, 8-hydroxydeoxyguanosine; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; PC, protein carbonyls; MDA, malondialdehyde; TOS, total oxidant status; SOD, superoxid dismutase; OSI, oxidative stress index; IL-1beta, interleukin 1beta; ROM, reactive oxygen metabolites; ROS, reactive oxygen species; FRAP, ferric reducing antioxidant power; 4-HNE, 4-hydroxy-2-nonenal; CAT, catalase; CRP, C-reactive protein; AOPP, advanced oxidation protein products; TRAP, total radical-trapping antioxidant potential; MPO, myeloperoxidase; mtDNA, mitochondrial DNA.

Malondialdehyde, 8-hydroxydeoxyguanosine (Konopka et al., 2007; Almerich-Silla et al., 2015), protein carbonyls (Nguyen et al., 2016; Atabay et al., 2017), thiobarbituric acid reacting substances (Borges et al., 2007), nitric oxide, advanced oxidation protein products, lipid peroxidation products (Gomes et al., 2017), 8-isprostanes (Hickman et al., 2011) were all higher in patients with periodontitis. The most commonly measured markers of oxidative stress seem to be malondialdehyde and thiobarbituric acid reacting substances pointing toward oxidative damage of lipids, especially, lipid membranes. Lipid peroxidation was higher in saliva (Tothova et al., 2013), serum (Tonguç et al., 2011), and in the gingival tissue (Panjamurthy et al., 2005) of patients with chronic or aggressive periodontitis. Correlational studies confirmed a positive association of these markers with periodontal status scores (Chapple et al., 2007b; Tamaki et al., 2014b). The less commonly measured markers related to oxidative damage as mitochondrial DNA (Canakci et al., 2006), micronuclei and nuclear abnormalities (Zamora-Perez et al., 2015), as well as a leukocyte telomere length shortening (Masi et al., 2011) were all higher in periodontitis as well.

Oxidative stress was found to be involved in the pathogenesis of many diseases besides periodontitis. Virtually, almost all inflammatory diseases lead to increased oxidative stress. This in turn can trigger more damage to the tissues, not excluding the gingival tissue and, thus, worsening periodontitis. There are several studies describing oxidative stress in systemic diseases with regard to the periodontal status. Nguyen et al. (2016) investigated patients with the acute coronary syndrome with or without chronic periodontitis, patients with periodontitis and healthy controls. Lipid, protein, and DNA oxidation markers were higher in periodontitis than in the control group (Nguyen et al., 2016). Another study with rheumatoid arthritis and chronic periodontitis confirmed higher oxidative stress markers in plasma and lower antioxidant capacity in both groups when compared to healthy control. However, if both comorbidities were present, there was no further enhancement of oxidative stress (Sezer et al., 2013).

Taken together, observational cross-sectional studies confirmed the association of oxidative stress and periodontitis. Higher oxidative stress and lower antioxidant status can be detected in plasma, saliva as well as in the gingival crevicular fluid of patients with various clinical forms of periodontitis. These findings support the use of body fluids, but especially the non-invasive diagnostic fluid saliva, as suitable sample types for diagnostics or monitoring the course of periodontitis. Current data do not support the use of a single oxidative stress marker. It is likely that a set of markers covering both, oxidative damage and antioxidative status, will be needed. The low specificity of oxidative stress markers calls for caution when interpreting the results even if more than one marker is used. The inter-individual and intra-individual variability of the analyzed markers is very high. This prevents their use at the level of individual diagnostics. The overview of the published observational studies shows the enormous heterogeneity of the patient populations as well as the used analytical tools. In a meta-analysis focusing on systematic oxidative stress, it was shown that higher malondialdehyde and nitric oxide as well as lower total antioxidant capacity of plasma/serum characterize patients with periodontitis in comparison to controls (Liu et al., 2014). Based on our overview, taken into account systemic and local oral biomarkers of oxidative stress, it is clear that there is a need for both, the use of a wider palette of markers to analyze oxidative stress and its causes in more detail, and the introduction of new biomarkers with a better sensitivity/specificity profile in specific subgroups of patients. Of special importance is the small sample size in most studies. A collaborative effort with a multi-center recruitment of patients and a standardized consensus protocol in the pre-analytical and analytical phase is highly needed.

OXIDATIVE STRESS AND TREATMENT OF PERIODONTITIS

The implication of oxidative stress in the pathogenesis of cardiovascular diseases and cancer as the major causes of death in the combination with the widespread availability of dietary antioxidants started a hype that was supported by the hypothesis that aging is caused by oxidative stress (Harman, 1956; Finkel and Holbrook, 2000). However, the hype was quickly over as large clinical studies revealed that antioxidants or at least antioxidant vitamins were not able to prevent any of the diseases of aging (Coulter et al., 2006; Sesso et al., 2008; Myung et al., 2013). Some studies even revealed a slight but increased risk in patients taking antioxidants in preeclampsia (Rumbold et al., 2006) and lung cancer (Alpha-Tocopherol, 1994). One meta-analysis showed that taking antioxidant may even increase the all-cause mortality by 5% (Bjelakovic et al., 2007). This might be related to the terminological and mechanistic confusion about antioxidants, which might have a very indirect effect on the production or effects of reactive oxygen species. In an era of evidence-based medicine the clear conclusion is that antioxidant dietary supplements should undergo clinical evaluation before marketing similarly to other medicinal drugs (Bjelakovic et al., 2012). Recent mechanistic experiments shed light on the details how antioxidants may stimulate tumor growth (Sayin et al., 2014) or increase the risk of metastasis (Piskounova et al., 2015). Antioxidants are highly variable in their mechanism and structure. Diseases and patients vary even more. So, negative results from oncology or cardiology should not be generalized to other diseases including periodontitis. It is very likely that the role of oxidative stress changes during disease progression and, thus, the potential antioxidant treatment affecting not only oxidative damage but also the inflammatory process might have different affects at various stages of the complex pathogenesis. The issue of antioxidants that mostly do not affect free radicals and their action *in vivo*, but rather interfere with variable cellular signaling pathways has been reviewed in a recently published paper (Azzi, 2017).

Many published studies have analyzed the effects of periodontitis treatment on oxidative stress (**Table 2**). Studies of special interest are those with covariates or comorbidities that were taken into account. A trial by Guentsch et al. (2008) examined healthy subjects and patients with periodontitis further

TABLE 2 | Interventional studies analyzing the effect of periodontitis on oxidative stress.

Treatment	Treatment duration	Sample size	Comorbidity	Sample type	Results	References
NST with vitamin C (2,000 mg/day)	1 month	30 ChP patients 30 healthy controls	None	Plasma	↓ TAC in ChP patients; NST lead to ↑ TAC in ChP patients; No effect of vitamin C	Abou Sulaiman and Shehadeh, 2010
NST with lycopene (8 mg/day)	2 month	20 ChP patients	None	Serum	↓ MDA and clinical parameters after therapy	Ambati et al., 2017
Oral hygiene education with insulin treatment for T1DM	3 months	32 T1DM patients at diagnosis 18 SH children with gingivitis 18 SH and PH children	T1DM	Serum Saliva GCF	↑ serum, salivary and GCF OSI in T1DM group; ↓ after treatment	Aral et al., 2017
NST with lycopene (8 mg/day)	–	42 ChP patients	None	Plasma Saliva	↑ IL-1ss, UA and clinical parameters after treatment	Arora et al., 2013
NST	14 days	13 ChP patients with FMF 15 ChP SH patients 14 PH patients with FMF 15 PH and SH controls	Familial Mediterranean fever	Serum GCF	↓ periodontal clinical markers after treatment; ↓ TOS in GCF in FMF patients with ChP; no difference in serum TOS and OSI	Bostanci et al., 2014
ST with antioxidant gel (2mg lycopene)	1 week	31 ChP patients	None	GCF	↓ 8-OHdG after treatment, as well as periodontal clinical markers	Chandra et al., 2013
NST	2 months	35 ChP patients 32 healthy controls	None	Plasma GCF	↓ TAC in GCF in ChP; ↑ after treatment; (plasma TAC no difference at baseline between groups, no change after treatment)	Chapple et al., 2007a
NST	4 h	145 ChP patients (14 with therapy) 56 healthy controls	None	Serum	Patients with severe periodontitis ↑ROM ↓ TAC; ↑ ROM after treatment	D'Aiuto et al., 2010
NST with fluorescence-controlled Er:YAG laser radiation	Laser therapy applied 1 day after NST	30 ChP patients	None	GCF	no difference between with/without laser treatment in TAC and clinical parameters; IL-1beta and ↑ TNF-alpha after NST only; ↓ after NST with laser	Dominguez et al., 2010
NST	–	30 ChP patients 30 healthy controls	Smokers/non-smokers	Serum Saliva	↑ MDA in smoking ChP patients; ↑ GPx in ChP groups; ↓ TAC in ChP groups; ↓ MDA and GPx after therapy	Guentsch et al., 2008
NST	14 days	47 (24 / 23) ChP patients 46 (23/23) healthy controls	Smokers/non-smokers	Serum Saliva GCF	↑ salivary 8-OHdG and GPx in ChP; ↑ 4-HNE in GCF in ChP smokers; ↓ 8-OHdG after therapy in saliva and GCF	Hendek et al., 2015
NST	–	7 ChP patients 7 healthy controls	None	Saliva	↑ TAC and ↓ SOD activity in ChP; ↓ TAC, SOD activity immediately after NST	Kim et al., 2010
NST	–	60 moderate to severe periodontitis patients with T2DM	T2DM	Serum	No effect of NST on d-8-iso, MMP-2, MMP-9 and hsCRP	Koromantzos et al., 2012
NST; NST with antioxidants (6 mg/day; lycopene, zinc, and selenium) or antioxidants only	3 doses in 2 weeks	30 ChP patients 30 gingivitis patients 10 healthy controls	None	Saliva	↓ UA in ChP patients; antioxidant treatment ↑ UA	Mathur et al., 2013
Tai Chi (5 days a week, 60 min)	6 months	71 sedentary patients with periodontitis	Old age	Saliva	↑ TAC, SOD after therapy	Mendoza-Núñez et al., 2014
NST and surgical treatment	Surgical treatment 6 weeks after NST	12 AgP patients	None	Serum Plasma	Periodontitis severity associated with LDL concentrations; No changes in lipid profile after treatment; No difference in GSH and lipid hyperoxide after therapy	Nibali et al., 2015

(Continued)

TABLE 2 | Continued

Treatment	Treatment duration	Sample size	Comorbidity	Sample type	Results	References
NST or oral hygiene instructions	2 visits within 7 days	42 ChP patients 21 healthy controls	None	Saliva	NST lead to ↑ TAC, ALB, UA, GPx and ↓ SOD; (no effect of oral hygiene instructed therapy was found)	Novakovic et al., 2014
NST	–	25 severe ChP patients 26 healthy controls	None	Serum Saliva	Salivary 8-OHdG ↑ before treatment in ChP group; ↓ after NST ↑ salivary MDA and serum 4-HNE in ChP patients; NST had no effect	Onder et al., 2017
NST with Coenzyme Q10; NST with tea tree oil gel or placebo	7 days	15 ChP patients (moderate to severe)	None	-	Both antioxidant treatment procedures effective in ↓ clinical markers of ChP (PI, GI, PPD and CAL)	Raut and Sethi, 2016
NST and oral hygiene instructions	2–4 months	29 ChP patients 20 healthy controls	None	Saliva	↑ 8-OHdG in ChP patients before therapy; ↓ 8-OHdG and *P. gingivalis* after treatment, as well as periodontal clinical markers	Sawamoto et al., 2005
NST	–	8 ChP patients 8 healthy controls	None	Platelet suspension	↓ periodontal clinical parameters and CRP after therapy, as well as ↑ cGMP and SOD activity	Siqueira et al., 2013
Surgical treatment with taurine (500 mg/day)	15 days	10 ChP patients	None	Plasma Gingival tissue	↓ TBARS, GPx in plasma and gingival tissue; ↑ GSH and ↓ periodontal clinical parameters after therapy	Sree and Sethupathy, 2014
NST	–	78 ChP patients 17 healthy controls	None	Saliva	↑ 8-OHdG in ChP patients before therapy; ↓ 8-OHdG and periodontal clinical markers after therapy	Takane et al., 2002
NST	–	22 ChP patients 22 healthy controls	None	Plasma	↑ oxidative index, oxidized LDL and CRP in ChP; ↓ of these parameters after treatment	Tamaki et al., 2011
NST	–	25 ChP patients 25 patients with gingivitis 25 healthy controls	None	Serum	↓ serum TAC and CAT in both groups of patients; TAC ↑ in ChP patients after therapy (no treatment effect on CAT)	Thomas et al., 2014
NST	4 visits within 14 days	30 (15/15) ChP patients 10 healthy controls	Smokers/non-smokers	GCF	↑ IL-1beta in ChP patients; ↓ IL-1beta after NST irrespective of smoking; (no difference in TAC and TOS before or after treatment between groups)	Toker et al., 2012
NST	2 weeks	25 ChP patients with MS 25 ChP SH patients	Metabolic syndrome	Serum Saliva	TOS and OSI showed no difference between groups in serum after therapy; ↑ TAC of MS ChP patients before treatment, but ↓ after therapy in serum; ↓ OSI and ↑ TAC in both groups after treatment in saliva	Torumtay et al., 2016
NST	Once per week/1 month	22 ChP patients	None	Saliva	↑ SOD in patients with low dental visits after NST; ↑ TAC in patients with regular dental visits after therapy	Yang et al., 2014
NST with dietary intervention	3 visits/6 months	37 ChP patients (19 without intervention; 18 with intervention)	None	Plasma Saliva	↑ TAC after dietary intervention in plasma; no differences in periodontal clinical parameters after dietary intervention	Zare Javid et al., 2014

ChP, chronic periodontitis; SH, systematically healthy; PH, periodontal healthy; NST, non-surgical treatment; ST, surgical treatment; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; FMF, familial Mediterranean fever; MS, metabolic syndrome; GCF, gingival crevicular fluid; TAC, total antioxidant capacity; TBARS, thiobarbituric acid reacting substances; 8-HOdG, 8-hydroxydeoxyguanosine; GPx, glutathione peroxidase; GSH, reduced glutathione; MDA, malondialdehyde; TOS, total oxidant status; SOD, superoxid dismutase; OSI, oxidative stress index; IL-1ss, salivary interleukin 1beta; ROM, reactive oxygen metabolites; 4-HNE, 4-hydroxy-2-nonenal; hsCRP, high-sensitivity C-reactive protein; d-8-iso, d—8-iso prostaglandin F2a; MMP-2, matrix metalloproteinase 2; MMP-9, matrix metalloproteinase 9; LDL, low density lipoprotein; ALB, albumin; UA, uric acid; CAT, catalase; CRP, C-reactive protein; PI, plaque index; GI, gingival bleeding index; PPD, probing pocket depth; CAL, clinical attachment level; TNF-alpha, tumor necrosis factor alpha; cGMP, L-arginine-nitric oxide (NO)-cyclic guanosine monophosphate.

divided into smokers and non-smokers. While smokers with periodontitis displayed highest malondialdehyde concentration and highest glutathione peroxidase activity along with lowest total antioxidant capacity in saliva. The non-surxgical treatment helped to normalize the values regardless of the smoking status (Guentsch et al., 2008). Several other trials confirmed the consistency of these findings (Chapple et al., 2007a; Abou Sulaiman and Shehadeh, 2010; Hendek et al., 2015). On the other hand, a severe systemic condition such as type 2 diabetes may lead to inefficiency of non-surgical therapies to improve the periodontal status or oxidative stress (Koromantzos et al., 2012). In general, most of the studies clearly show an improvement of oxidative damage after standard treatment of periodontitis. The studies, however, vary greatly regarding sample types, markers measured, and most cohorts were very small and highly variable. Thus, making relevant conclusions or recommendations for the clinical dentistry is difficult, if not impossible. Future research efforts should focus on the lack of uniformity and standardization as well as on the issue of the low informative value of small patient cohorts.

Although the causality of the association between oxidative stress and periodontitis is everything but clear, some clinical studies already tested antioxidants in periodontitis. One small, placebo controlled, randomized, and double-blind study showed, that single application of lycopene gel to periodontitis patients improved clinical attachment and decreased oxidative stress in gingival crevicular fluid (Chandra et al., 2013). Nevertheless, a published systematic review on antioxidant treatment of periodontitis revealed that a consistent effect in randomized clinical trials was found only for lipophile antioxidants such as lycopene and vitamin E, but not for hydrophile antioxidants such as vitamin C (Muniz et al., 2015). This might be related to the vulnerability of lipids to oxidative damage, but also to mitochondria as the site of effect of some antioxidants. Interestingly, antioxidants targeting directly mitochondria have been shown to be effective in decreasing inflammatory activity and organ damage in animal model of sepsis (Lowes et al., 2013).

ANIMAL EXPERIMENTS

The high number of observational and interventional studies analyzing the association between oxidative stress and periodontitis indicates that there are many open questions that cannot be answered by more and more clinical studies. Many of the questions need controlled conditions in experiments. The number of animal experiments analyzing the role oxidative stress in periodontitis is small, but it increases. Periodontitis is mostly induced by ligature placement around the first molars of the animals or local injection of periodontal pathogens or their toxins (Genco et al., 1998; Fine, 2009; Oz and Puleo, 2011). In such a rat model, it was shown that periodontitis leads to an increased production of reactive oxygen species and markers of oxidative damage (Ekuni et al., 2010). In addition, oxidative stress induced by periodontitis seems to be associated with the dynamics and severity of the periodontal inflammation (Bosca et al., 2016).

Some animal experiments focus on the distant effects of periodontitis that seem to be mediated by oxidative stress. It was shown that mitochondrial DNA is oxidatively modified in the liver, kidney, heart, and brain of rats with induced periodontitis (Tomofuji et al., 2011). Another similar experiment by the same group has shown that periodontitis might worsen ethanol-induced liver damage (Tomofuji et al., 2008). The oxidative damage to the heart, but also endothelial dysfunction and resulting atherosclerosis induced by experimental periodontitis can be prevented by antioxidant treatment (Ekuni et al., 2009; Ozdem et al., 2017; Saito et al., 2017). A majority of the published experiments focus on the use of antioxidants such as vitamin C (Tomofuji et al., 2009b), N-acetylcysteine (Toker et al., 2009), or resveratrol (Tamaki et al., 2014a), but also drugs with an antioxidant activity beyond their main mechanism of action (de Araujo Junior et al., 2013; Culic et al., 2014; Oktay et al., 2015). Dietary interventions have also been investigated—high cholesterol diet seems to worsen periodontitis (Tomofuji et al., 2006). On contrary, the phytoestrogen genistein and cacao-enriched diet was shown to be protective against periodontal damage and oxidative stress induced by periodontitis in mice and rats, respectively (Tomofuji et al., 2009a; Bhattarai et al., 2017). The most promising candidate drug at least according to animal experiments is melatonin. This amphiphile molecule has an optimal distribution in the tissues and can, thus, reach the periodontal tissues even after systemic administration (Köse et al., 2017). Of special clinical relevance is the induction of periodontitis in diabetic rats since periodontitis is a common complication of diabetes in humans. Melatonin was able to prevent alveolar bone loss also in this experimental model (Kose et al., 2016). However, regarding the mechanism of action, it is not clear whether the protective effect of melatonin is due to its direct antioxidant characteristics or due to its immunomodulatory effects that might reduce oxidative stress indirectly (Kara et al., 2013). This uncertainty is not specific for melatonin. Any antioxidant might affect the immune response and, thus, have anti-inflammatory properties. Beyond established systemic antioxidants novel approaches with a local periodontal application are tested (Saita et al., 2016). Experimental tools such as genetically engineered mice that produce luciferase under the regulation of transcription factors related to oxidative stress and antioxidant response have been developed and might greatly improve our understanding of the role of oxidative stress in periodontitis (Kataoka et al., 2016). The new treatment options together with new and improved models could be very helpful in the fight against this widespread and serious disease.

CONCLUSION

The role of oxidative stress in periodontitis is not clear despite decades of research. Numerous studies have been published showing the potential of oxidative stress markers for screening, diagnosis or monitoring of the disease, but none is in routine clinical use. Similarly, animal experiments, as well as most of

the interventional studies in patients, indicate that antioxidant treatment should be effective in the therapy of periodontitis, but no such treatment has been approved. The lack of translation could be either due to the lack of strong evidence for the clinical usefulness or due to obstacles in the application of the results including the low or absent commercial interest from major stakeholders. From a research perspective, an important issue is the lack of specificity—both, in diagnostics and treatment. Not even the source of free radical production is clear. While some studies point toward neutrophils (Katsuragi et al., 2003), others show that bacteria actively producing reactive oxygen species might contribute to oxidative stress in periodontitis (Huycke et al., 2002; Vlkova and Celec, 2009). It is of crucial importance that the number of conducted animal experiments in this field is increasing, especially of those focusing on the dynamics of oxidative stress during disease progression. The antioxidant treatment might be effective only in a subset of patients during a specific stage of periodontitis. The shift from pure observations to interventions and animal experiments that can be followed in the published literature in the recent years is highly positive and should bring this field of research closer to true clinical applications despite chronic lack of financial support and human resources.

AUTHOR CONTRIBUTIONS

Ľ'T has analyzed the data from the literature, prepared tables, and drafted the manuscript; PC has designed the review, conducted the literature search, and drafted the manuscript.

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REFERENCES

Abou Sulaiman, A. E., and Shehadeh, R. M. (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

Acquier, A. B., De Couto Pita, A. K., Busch, L., and Sánchez, G. A. (2017). Parameters of oxidative stress in saliva from patients with aggressive and chronic periodontitis. *Redox Rep.* 22, 119–126. doi: 10.1080/13510002.2016.1198104

Ahmadi-Motamayel, F., Goodarzi, M. T., Jamshidi, Z., and Kebriaei, R. (2017). Evaluation of salivary and serum antioxidant and oxidative stress statuses in patients with chronic periodontitis: a case-control study. *Front. Physiol.* 8:189. doi: 10.3389/fphys.2017.00189

Akalin, F. A., Baltacioglu, E., Alver, A., and 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, 457–467. doi: 10.1902/jop.2009.080218

Allen, E. M., Matthews, J. B., O' Halloran, D. J., Griffiths, H. R., and Chapple, I. L. (2011). Oxidative and inflammatory status in Type 2 diabetes patients with periodontitis. *J. Clin. Periodontol.* 38, 894–901. doi: 10.1111/j.1600-051X.2011.01764.x

Almerich-Silla, J. M., Montiel-Company, J. M., Pastor, S., Serrano, F., Puig-Silla, M., and Dasí, F. (2015). Oxidative stress parameters in saliva and its association with periodontal disease and types of bacteria. *Dis. Markers* 2015:653537. doi: 10.1155/2015/653537

Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group. (1994). The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. *N. Engl. J. Med.* 330, 1029–1035. doi: 10.1056/NEJM199404143301501

Ambati, M., Rani, K. R., Reddy, P. V., Suryaprasanna, J., Dasari, R., and Gireddy, H. (2017). Evaluation of oxidative stress in chronic periodontitis patients following systemic antioxidant supplementation: a clinical and biochemical study. *J. Nat. Sci. Biol. Med.* 8, 99–103. doi: 10.4103/0976-9668.198366

Aral, C. A., Nalbantoglu Ö, O., Nur, B. G., Altunsoy, M., and Aral, K. (2017). Metabolic control and periodontal treatment decreases elevated oxidative stress in the early phases of type 1 diabetes onset. *Arch. Oral Biol.* 82, 115–120. doi: 10.1016/j.archoralbio.2017.06.009

Arana, C., Moreno-Fernández, A. M., Gómez-Moreno, G., Morales-Portillo, C., Serrano-Olmedo, I., de la Cuesta Mayor, M. C., et al. (2017). Increased salivary oxidative stress parameters in patients with type 2 diabetes: relation with periodontal disease. *Endocrinol. Diabetes Nutr.* 64, 258–264. doi: 10.1016/j.endinu.2017.03.005

Arora, N., Avula, H., and Avula, J. K. (2013). The adjunctive use of systemic antioxidant therapy (lycopene) in nonsurgical treatment of chronic periodontitis: a short-term evaluation. *Quintessence Int.* 44, 395–405. doi: 10.3290/j.qi.a29188

Atabay, V. E., Lutfioglu, M., Avci, B., Sakallioglu, E. E., and Aydogdu, A. (2017). Obesity and oxidative stress in patients with different periodontal status: a case-control study. *J. Periodont. Res.* 52, 51–60. doi: 10.1111/jre.12368

Azzi, A. (2017). Antioxidants: wonder drugs or quackery? *Biofactors*. doi: 10.1002/biof.1388. [Epub ahead of print].

Baltacioglu, E., Akalin, F. A., Alver, A., Balaban, F., Unsal, M., and 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, 385–392. doi: 10.1111/j.1600-051X.2006.00923.x

Baltacioglu, E., Yuva, P., Aydin, G., Alver, A., Kahraman, C., Karabulut, E., et al. (2014). Lipid peroxidation levels and total oxidant/antioxidant status in serum and saliva from patients with chronic and aggressive periodontitis. oxidative stress index: a new biomarker for periodontal disease? *J. Periodontol.* 85, 1432–1441. doi: 10.1902/jop.2014.130654

Banasová, L., Kamodyová, N., Janšáková, K., Tóthová, L., Stanko, P., Turna, J., et al. (2015). Salivary DNA and markers of oxidative stress in patients with chronic periodontitis. *Clin. Oral Investig.* 19, 201–207. doi: 10.1007/s00784-014-1236-z

Bhattarai, G., Poudel, S. B., Kook, S. H., and Lee, J. C. (2017). Anti-inflammatory, anti-osteoclastic, and antioxidant activities of genistein protect against alveolar bone loss and periodontal tissue degradation in a mouse model of periodontitis. *J. Biomed. Mater. Res. A* 105, 2510–2521. doi: 10.1002/jbm.a.36109

Biasi, D., Bambara, L. M., Carletto, A., Caramaschi, P., Andrioli, G., Urbani, G., et al. (1999). Neutrophil migration, oxidative metabolism and adhesion in early onset periodontitis. *J. Clin. Periodontol.* 26, 563–568. doi: 10.1034/j.1600-051X.1999.260901.x

Bjelakovic, G., Nikolova, D., Gluud, L. L., Simonetti, R. G., and Gluud, C. (2007). Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis. *JAMA* 297, 842–857. doi: 10.1001/jama.297.8.842

Bjelakovic, G., Nikolova, D., Gluud, L. L., Simonetti, R. G., and Gluud, C. (2012). Antioxidant supplements for prevention of mortality in healthy participants and patients with various diseases. *Cochrane Database Syst. Rev.* Cd007176. doi: 10.1002/14651858.CD007176.pub2

Borges, I. Jr., Moreira, E. A., Filho, D. W., de Oliveira, T. B., da Silva, M. B., and Fröde, T. S. (2007). Proinflammatory and oxidative stress markers in patients with periodontal disease. *Mediators Inflamm.* 2007:45794. doi: 10.1155/2007/45794

Boşca, A. B., Miclăuş, V., Ilea, A., Câmpian, R. S., Rus, V., Ruxanda, F., et al. (2016). Role of nitro-oxidative stress in the pathogenesis of experimental rat periodontitis. *Clujul. Med.* 89, 150–159. doi: 10.15386/cjmed-529

Bostanci, V., Toker, H., Senel, S., Ozdemir, H., and Aydin, H. (2014). Effect of chronic periodontitis on serum and gingival crevicular fluid oxidant and antioxidant status in patients with familial mediterranean fever before and after periodontal treatment. *J. Periodontol.* 85, 706–712. doi: 10.1902/jop.2013.130230

Brock, G. R., Butterworth, C. J., Matthews, J. B., and Chapple, I. L. (2004). Local and systemic total antioxidant capacity in periodontitis and health. *J. Clin. Periodontol.* 31, 515–521. doi: 10.1111/j.1600-051X.2004.00509.x

Canakçi, C. F., Canakçi, V., Tatar, A., Eltas, A., Sezer, U., Ciçek, Y., et al. (2009). Increased salivary level of 8-hydroxydeoxyguanosine is a marker of premature oxidative mitochondrial DNA damage in gingival tissue of patients with periodontitis. *Arch. Immunol. Ther. Exp.* 57, 205–211. doi: 10.1007/s00005-009-0026-9

Canakçi, C. F., Tatar, A., Canakçi, V., Cicek, Y., Oztas, S., and Orbak, R. (2006). New evidence of premature oxidative DNA damage: mitochondrial DNA deletion in gingival tissue of patients with periodontitis. *J. Periodontol.* 77, 1894–1900. doi: 10.1902/jop.2006.060108

Canakci, V., Yildirim, A., Canakci, C. F., Eltas, A., Cicek, Y., and Canakci, H. (2007). Total antioxidant capacity and antioxidant enzymes in serum, saliva, and gingival crevicular fluid of preeclamptic women with and without periodontal disease. *J. Periodontol.* 78, 1602–1611. doi: 10.1902/jop.2007.060469

Chandra, R. V., Sailaja, S., and Reddy, A. A. (2017). Estimation of tissue and crevicular fluid oxidative stress marker in premenopausal, perimenopausal and postmenopausal women with chronic periodontitis. *Gerodontology* 34, 382–389. doi: 10.1111/ger.12279

Chandra, R. V., Srinivas, G., Reddy, A. A., Reddy, B. H., Reddy, C., Nagarajan, S., et al. (2013). Locally delivered antioxidant gel as an adjunct to nonsurgical therapy improves measures of oxidative stress and periodontal disease. *J. Periodontal Implant Sci.* 43, 121–129. doi: 10.5051/jpis.2013.43.3.121

Chapple, I. L. (1997). Reactive oxygen species and antioxidants in inflammatory diseases. *J. Clin. Periodontol.* 24, 287–296. doi: 10.1111/j.1600-051X.1997.tb00760.x

Chapple, I. L., Brock, G. R., Milward, M. R., Ling, N., and Matthews, J. B. (2007a). Compromised GCF total antioxidant capacity in periodontitis: cause or effect? *J. Clin. Periodontol.* 34, 103–110. doi: 10.1111/j.1600-051X.2006.01029.x

Chapple, I. L., Brock, G., Eftimiadi, C., and Matthews, J. B. (2002). Glutathione in gingival crevicular fluid and its relation to local antioxidant capacity in periodontal health and disease. *Mol. Pathol.* 55, 367–373. doi: 10.1136/mp.55.6.367

Chapple, I. L., Milward, M. R., and Dietrich, T. (2007b). The prevalence of inflammatory periodontitis is negatively associated with serum antioxidant concentrations. *J. Nutr.* 137, 657–664.

Coulter, I. D., Hardy, M. L., Morton, S. C., Hilton, L. G., Tu, W., Valentine, D., et al. (2006). Antioxidants vitamin C and vitamin e for the prevention and treatment of cancer. *J. Gen. Intern. Med.* 21, 735–744. doi: 10.1111/j.1525-1497.2006.00483.x

Culic, C., Parvu, A. E., Alb, S. F., Alb, C., and Pop, A. (2014). Effect of Cimetidine on nitro-oxidative stress in a rat model of periodontitis. *Clujul. Med.* 87, 177–181. doi: 10.15386/cjmed-273

D'Aiuto, F., Nibali, L., Parkar, M., Patel, K., Suvan, J., and Donos, N. (2010). Oxidative stress, systemic inflammation, and severe periodontitis. *J. Dent. Res.* 89, 1241–1246. doi: 10.1177/0022034510375830

de Araújo Júnior, R. F., Souza, T. O., de Moura, L. M., Torres, K. P., de Souza, L. B., Alves Mdo, S., et al. (2013). Atorvastatin decreases bone loss, inflammation and oxidative stress in experimental periodontitis. *PLoS ONE* 8:e75322. doi: 10.1371/journal.pone.0075322

Diab-Ladki, R., Pellat, B., and 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

Domínguez, A., Gómez, C., García-Kass, A. I., and García-Nuñez, J. A. (2010). IL-1beta, TNF-alpha, total antioxidative status and microbiological findings in chronic periodontitis treated with fluorescence-controlled Er:YAG laser radiation. *Lasers Surg. Med.* 42, 24–31. doi: 10.1002/lsm.20873

Duarte, P. M., Napimoga, M. H., Fagnani, E. C., Santos, V. R., Bastos, M. F., Ribeiro, F. V., et al. (2012). The expression of antioxidant enzymes in the gingivae of type 2 diabetics with chronic periodontitis. *Arch. Oral Biol.* 57, 161–168. doi: 10.1016/j.archoralbio.2011.08.007

Ekuni, D., Endo, Y., Irie, K., Azuma, T., Tamaki, N., Tomofuji, T., et al. (2010). Imbalance of oxidative/anti-oxidative status induced by periodontitis is involved in apoptosis of rat submandibular glands. *Arch. Oral Biol.* 55, 170–176. doi: 10.1016/j.archoralbio.2009.11.013

Ekuni, D., Tomofuji, T., Sanbe, T., Irie, K., Azuma, T., Maruyama, 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. doi: 10.1016/j.archoralbio.2009.02.006

Esen, C., Alkan, B. A., Kirnap, M., Akgül, O., Işikoglu, S., and 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. doi: 10.1902/jop.2011.110420

Espinosa-Diez, C., Miguel, V., Mennerich, D., Kietzmann, T., Sánchez-Pérez, P., Cadenas, S., et al. (2015). Antioxidant responses and cellular adjustments to oxidative stress. *Redox Biol.* 6, 183–197. doi: 10.1016/j.redox.2015.07.008

Fentoglu Ö, O., Kirzioglu, F. Y., Bulut, M. T., Kumbul Doguç, D., Kulaç, E., Önder, C., et al. (2015). Evaluation of lipid peroxidation and oxidative DNA damage in patients with periodontitis and hyperlipidemia. *J. Periodontol.* 86, 682–688. doi: 10.1902/jop.2015.140561

Fine, D. H. (2009). Of mice and men: animal models of human periodontal disease. *J. Clin. Periodontol.* 36, 913–914. doi: 10.1111/j.1600-051X.2009.01456.x

Finkel, T. (2011). Signal transduction by reactive oxygen species. *J. Cell Biol.* 194, 7–15. doi: 10.1083/jcb.201102095

Finkel, T., and Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature* 408, 239–247. doi: 10.1038/35041687

Frijhoff, J., Winyard, P. G., Zarkovic, N., Davies, S. S., Stocker, R., Cheng, D., et al. (2015). Clinical relevance of biomarkers of oxidative stress. *Antioxid. Redox Signal.* 23, 1144–1170. doi: 10.1089/ars.2015.6317

Genco, C. A., Van Dyke, T., and Amar, S. (1998). Animal models for *Porphyromonas gingivalis*-mediated periodontal disease. *Trends Microbiol.* 6, 444–449. doi: 10.1016/S0966-842X(98)01363-8

Gomes, C., Martinho, F. C., Barbosa, D. S., Antunes, L. S., Póvoa, H. C. C., Baltus, T. H. L., et al. (2017). Increased root canal endotoxin levels are associated with chronic apical periodontitis, increased oxidative and nitrosative stress, major depression, severity of depression, and a lowered quality of life. *Mol. Neurobiol.* doi: 10.1007/s12035-017-0545-z. [Epub ahead of print].

Guentsch, A., Preshaw, P., Bremer-Streck, S., Klinger, G., Glockmann, E., and Sigusch, B. (2008). Lipid peroxidation and antioxidant activity in saliva of periodontitis patients: effect of smoking and periodontal treatment. *Clin. Oral Investig.* 12, 345–352. doi: 10.1007/s00784-008-0202-z

Gümüş, P., Buduneli, N., Cetinkalp, S., Hawkins, S. I., Renaud, D., Kinane, D. F., et al. (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

Gümüş, P., Emingil, G., Öztürk, V. O., Belibasakis, G. N., and Bostanci, N. (2015). Oxidative stress markers in saliva and periodontal disease status: modulation during pregnancy and postpartum. *BMC Infect. Dis.* 15:261. doi: 10.1186/s12879-015-1003-z

Harman, D. (1956). Aging: a theory based on free radical and radiation chemistry. *J. Gerontol.* 11, 298–300. doi: 10.1093/geronj/11.3.298

Hendek, M. K., Erdemir, E. O., Kisa, U., and Ozcan, G. (2015). Effect of initial periodontal therapy on oxidative stress markers in gingival crevicular fluid, saliva, and serum in smokers and non-smokers with chronic periodontitis. *J. Periodontol.* 86, 273–282. doi: 10.1902/jop.2014.140338

Hickman, M. A., Boggess, K. A., Moss, K. L., Beck, J. D., and Offenbacher, S. (2011). Maternal periodontal disease is associated with oxidative stress during pregnancy. *Am. J. Perinatol.* 28, 247–252. doi: 10.1055/s-0030-1268706

Huang, Y., Zhu, M., Li, Z., Sa, R., Chu, Q., Zhang, Q., et al. (2014). Mass spectrometry-based metabolomic profiling identifies alterations in salivary redox status and fatty acid metabolism in response to inflammation and oxidative stress in periodontal disease. *Free Radic. Biol. Med.* 70, 223–232. doi: 10.1016/j.freeradbiomed.2014.02.024

Huycke, M. M., Abrams, V., and Moore, D. R. (2002). Enterococcus faecalis produces extracellular superoxide and hydrogen peroxide that damages colonic epithelial cell DNA. *Carcinogenesis* 23, 529–536. doi: 10.1093/carcin/23.3.529

Kara, A., Akman, S., Ozkanlar, S., Tozoglu, U., Kalkan, Y., Canakci, C. F., et al. (2013). Immune modulatory and antioxidant effects of melatonin

in experimental periodontitis in rats. *Free Radic. Biol. Med.* 55, 21–26. doi: 10.1016/j.freeradbiomed.2012.11.002

Kataoka, K., Ekuni, D., Tomofuji, T., Irie, K., Kunitomo, M., Uchida, Y., et al. (2016). Visualization of oxidative stress induced by experimental periodontitis in keap1-dependent oxidative stress detector-luciferase mice. *Int. J. Mol. Sci.* 17:1907. doi: 10.3390/ijms17111907

Katsuragi, H., Ohtake, M., Kurasawa, I., and Saito, K. (2003). Intracellular production and extracellular release of oxygen radicals by PMNs and oxidative stress on PMNs during phagocytosis of periodontopathic bacteria. *Odontology* 91, 13–18. doi: 10.1007/s10266-003-0022-1

Khocht, A., Russell, B., Cannon, J. G., Turner, B., and Janal, M. (2014). Oxidative burst intensity of peripheral phagocytic cells and periodontitis in down syndrome. *J. Periodont. Res.* 49, 29–35. doi: 10.1111/jre.12075

Kim, S. C., Kim, O. S., Kim, O. J., Kim, Y. J., and Chung, H. J. (2010). Antioxidant profile of whole saliva after scaling and root planing in periodontal disease. *J. Periodont. Implant Sci.* 40, 164–171. doi: 10.5051/jpis.2010.40.4.164

Kimura, S., Yonemura, T., and Kaya, H. (1993). Increased oxidative product formation by peripheral blood polymorphonuclear leukocytes in human periodontal diseases. *J. Periodontal Res.* 28, 197–203. doi: 10.1111/j.1600-0765.1993.tb01069.x

Kinane, D. F., Preshaw, P. M., Loos, B. G., and Working Group 2 of Seventh European Workshop on Periodontology. (2011). Host-response: understanding the cellular and molecular mechanisms of host-microbial interactions–consensus of the seventh European workshop on periodontology. *J. Clin. Periodontol.* 38(Suppl. 11), 44–48. doi: 10.1111/j.1600-051X.2010.01682.x

Kinane, D. F., Stathopoulou, P. G., and Papapanou, P. N. (2017). Periodontal diseases. *Nat. Rev. Dis. Primers* 3:17038. doi: 10.1038/nrdp.2017.38

Konopka, T., Król, K., Kopeć, W., and Gerber, H. (2007). Total antioxidant status and 8-hydroxy-2'-deoxyguanosine levels in gingival and peripheral blood of periodontitis patients. *Arch. Immunol. Ther. Exp.* 55, 417–422. doi: 10.1007/s00005-007-0047-1

Kopáni, M., Celec, P., Danisovic, L., Michalka, P., and Biró, C. (2006). Oxidative stress and electron spin resonance. *Clin. Chim. Acta* 364, 61–66. doi: 10.1016/j.cca.2005.05.016

Koromantzos, P. A., Makrilakis, K., Dereka, X., Offenbacher, S., Katsilambros, N., Vrotsos, I. A., et al. (2012). Effect of non-surgical periodontal therapy on C-reactive protein, oxidative stress, and matrix metalloproteinase (MMP)-9 and MMP-2 levels in patients with type 2 diabetes: a randomized controlled study. *J. Periodontol.* 83, 3–10. doi: 10.1902/jop.2011.110148

Kose, O., Arabaci, T., Kara, A., Yemenoglu, H., Kermen, E., Kizildag, A., et al. (2016). Effects of melatonin on oxidative stress index and alveolar bone loss in diabetic rats with periodontitis. *J. Periodontol.* 87, e82–e90. doi: 10.1902/jop.2016.150541

Köse, O., Arabaci, T., Kizildag, A., Erdemci, B., Özkal Eminoglu, D., Gedikli, S., et al. (2017). Melatonin prevents radiation-induced oxidative stress and periodontal tissue breakdown in irradiated rats with experimental periodontitis. *J. Periodont. Res.* 52, 438–446. doi: 10.1111/jre.12409

Laine, M. L., Crielaard, W., and Loos, B. G. (2012). Genetic susceptibility to periodontitis. *Periodontol 2000* 58, 37–68. doi: 10.1111/j.1600-0757.2011.00415.x

Linden, G. J., McClean, K. M., Woodside, J. V., Patterson, C. C., Evans, A., Young, I. S., et al. (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

Liu, Z., Liu, Y., Song, Y., Zhang, X., Wang, S., and Wang, Z. (2014). Systemic oxidative stress biomarkers in chronic periodontitis: a meta-analysis. *Dis. Markers* 2014:931083. doi: 10.1155/2014/931083

Loesche, W. J., Robinson, J. P., Flynn, M., Hudson, J. L., and Duque, R. E. (1988). Reduced oxidative function in gingival crevicular neutrophils in periodontal disease. *Infect. Immun.* 56, 156–160.

Lowes, D. A., Webster, N. R., Murphy, M. P., and Galley, H. F. (2013). Antioxidants that protect mitochondria reduce interleukin-6 and oxidative stress, improve mitochondrial function, and reduce biochemical markers of organ dysfunction in a rat model of acute sepsis. *Br. J. Anaesth.* 110, 472–480. doi: 10.1093/bja/aes577

Masi, S., Salpea, K. D., Li, K., 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, 730–735. doi: 10.1016/j.freeradbiomed.2010.12.031

Mathur, A., Mathur, L., Manohar, B., Mathur, H., Shankarapillai, R., Shetty, N., et al. (2013). Antioxidant therapy as monotherapy or as an adjunct to treatment of periodontal diseases. *J. Indian Soc. Periodontol.* 17, 21–24. doi: 10.4103/0972-124X.107469

Mendoza-Núñez, V. M., Hernández-Monjaraz, B., Santiago-Osorio, E., Betancourt-Rule, J. M., and Ruiz-Ramos, M. (2014). Tai Chi exercise increases SOD activity and total antioxidant status in saliva and is linked to an improvement of periodontal disease in the elderly. *Oxid. Med. Cell. Longev.* 2014:603853. doi: 10.1155/2014/603853

Miricescu, D., Totan, A., Calenic, B., Mocanu, B., Didilescu, A., Mohora, M., et al. (2014). Salivary biomarkers: relationship between oxidative stress and alveolar bone loss in chronic periodontitis. *Acta Odontol. Scand.* 72, 42–47. doi: 10.3109/00016357.2013.795659

Monea, A., Mezei, T., Popsor, S., and Monea, M. (2014). Oxidative stress: a link between diabetes mellitus and periodontal disease. *Int. J. Endocrinol.* 2014:917631. doi: 10.1155/2014/917631

Muniz, F. W., Nogueira, S. B., Mendes, F. L., Rosing, C. K., Moreira, M. M., de Andrade, G. M., et al. (2015). The impact of antioxidant agents complimentary to periodontal therapy on oxidative stress and periodontal outcomes: a systematic review. *Arch. Oral Biol.* 60, 1203–1214. doi: 10.1016/j.archoralbio.2015.05.007

Myung, S. K., Ju, W., Cho, B., Oh, S. W., Park, S. M., Koo, B. K., et al. (2013). Efficacy of vitamin and antioxidant supplements in prevention of cardiovascular disease: systematic review and meta-analysis of randomised controlled trials. *BMJ* 346:f10. doi: 10.1136/bmj.f10

Nguyen, T. T., Ngo, L. Q., Promsudthi, A., and Surarit, R. (2016). Salivary oxidative stress biomarkers in chronic periodontitis and acute coronary syndrome. *Clin. Oral Investig.* 21, 2345–2353. doi: 10.1007/s00784-016-2029-3

Nibali, L., Rizzo, M., Li Volti, G., D'Aiuto, F., Giglio, R. V., Barbagallo, I., et al. (2015). Lipid subclasses profiles and oxidative stress in aggressive periodontitis before and after treatment. *J. Periodont. Res.* 50, 890–896. doi: 10.1111/jre.12283

Niki, E. (2016). Oxidative stress and antioxidants: distress or eustress? *Arch. Biochem. Biophys.* 595, 19–24. doi: 10.1016/j.abb.2015.11.017

Novakovic, N., Todorovic, T., Rakic, M., Milinkovic, I., Dozic, I., Jankovic, S., et al. (2014). Salivary antioxidants as periodontal biomarkers in evaluation of tissue status and treatment outcome. *J. Periodont. Res.* 49, 129–136. doi: 10.1111/jre.12088

Oktay, S., Chukkapalli, S. S., Rivera-Kweh, M. F., Velsko, I. M., Holliday, L. S., and Kesavalu, L. (2015). Periodontitis in rats induces systemic oxidative stress that is controlled by bone-targeted antiresorptives. *J. Periodontol.* 86, 137–145. doi: 10.1902/jop.2014.140302

Önder, C., Kurgan, S., Altingöz, S. M., Bagiş, N., Uyanik, M., Serdar, M. A., et al. (2017). Impact of non-surgical periodontal therapy on saliva and serum levels of markers of oxidative stress. *Clin. Oral Investig.* 21, 1961–1969. doi: 10.1007/s00784-016-1984-z

Oz, H. S., and Puleo, D. A. (2011). Animal models for periodontal disease. *J. Biomed. Biotechnol.* 2011:754857. doi: 10.1155/2011/754857

Özdem, M., Kirzioglu, F. Y., Yilmaz, H. R., Vural, H., Fentoglu, Ö., Uz, E., et al. (2017). Antioxidant effects of melatonin in heart tissue after induction of experimental periodontitis in rats. *J. Oral Sci.* 59, 23–29. doi: 10.2334/josnusd.16-0034

Panjamurthy, K., Manoharan, S., and Ramachandran, C. R. (2005). Lipid peroxidation and antioxidant status in patients with periodontitis. *Cell. Mol. Biol. Lett.* 10, 255–264.

Piskounova, E., Agathocleous, M., Murphy, M. M., Hu, Z., Huddlestun, S. E., Zhao, Z., et al. (2015). Oxidative stress inhibits distant metastasis by human melanoma cells. *Nature* 527, 186–191. doi: 10.1038/nature15726

Raut, C. P., and Sethi, K. S. (2016). Comparative evaluation of co-enzyme Q10 and *Melaleuca alternifolia* as antioxidant gels in treatment of chronic periodontitis: a clinical study. *Contemp. Clin. Dent.* 7, 377–381. doi: 10.4103/0976-237X.188572

Rumbold, A. R., Crowther, C. A., Haslam, R. R., Dekker, G. A., and Robinson, J. S. (2006). Vitamins C and E and the risks of preeclampsia and perinatal complications. *N. Engl. J. Med.* 354, 1796–1806. doi: 10.1056/NEJMoa054186

Russell, E. G., and Cotter, T. G. (2015). New insight into the role of reactive oxygen species (ROS) in cellular signal-transduction processes. *Int. Rev. Cell Mol. Biol.* 319, 221–254. doi: 10.1016/bs.ircmb.2015.07.004

Saita, M., Kaneko, J., Sato, T., Takahashi, S. S., Wada-Takahashi, S., Kawamata, R., et al. (2016). Novel antioxidative nanotherapeutics in a rat periodontitis model: Reactive oxygen species scavenging by redox injectable gel suppresses alveolar bone resorption. *Biomaterials* 76, 292–301. doi: 10.1016/j.biomaterials.2015.10.077

Saito, T., Yamamoto, Y., Feng, G. G., Kazaoka, Y., Fujiwara, Y., and Kinoshita, H. (2017). Lidocaine prevents oxidative stress-induced endothelial dysfunction of the systemic artery in rats with intermittent periodontal inflammation. *Anesth. Analg.* 124, 2054–2062. doi: 10.1213/ANE.0000000000002102

Sawamoto, Y., Sugano, N., Tanaka, H., and Ito, K. (2005). Detection of periodontopathic bacteria and an oxidative stress marker in saliva from periodontitis patients. *Oral Microbiol. Immunol.* 20, 216–220. doi: 10.1111/j.1399-302X.2005.00215.x

Sayin, V. I., Ibrahim, M. X., Larsson, E., Nilsson, J. A., Lindahl, P., and Bergo, M. O. (2014). Antioxidants accelerate lung cancer progression in mice. *Sci. Transl. Med.* 6, 221ra215. doi: 10.1126/scitranslmed.3007653

Sculley, D. V., and Langley-Evans, S. C. (2003). Periodontal disease is associated with lower antioxidant capacity in whole saliva and evidence of increased protein oxidation. *Clin. Sci.* 105, 167–172. doi: 10.1042/CS20030031

Sesso, H. D., Buring, J. E., Christen, W. G., Kurth, T., Belanger, C., MacFadyen, J., et al. (2008). Vitamins E and C in the prevention of cardiovascular disease in men: the physicians' health study II randomized controlled trial. *JAMA* 300, 2123–2133. doi: 10.1001/jama.2008.600

Sezer, U., Erciyas, K., Ustün, K., Pehlivan, Y., Senyurt, S. Z., Aksoy, N., et al. (2013). Effect of chronic periodontitis on oxidative status in patients with rheumatoid arthritis. *J. Periodontol.* 84, 785–792. doi: 10.1902/jop.2012.120179

Sezer, U., Senyurt, S. Z., Gündogar, H., Erciyas, K., Ustun, K., Kimyon, G., et al. (2016). Effect of chronic periodontitis on oxidative status in patients with psoriasis and psoriatic arthritis. *J. Periodontol.* 87, 557–565. doi: 10.1902/jop.2015.150337

Shapira, L., Borinski, R., Sela, M. N., and Soskolne, A. (1991). Superoxide formation and chemiluminescence of peripheral polymorphonuclear leukocytes in rapidly progressive periodontitis patients. *J. Clin. Periodontol.* 18, 44–48. doi: 10.1111/j.1600-051X.1991.tb01118.x

Sies, H., Berndt, C., and Jones, D. P. (2017). Oxidative stress. *Annu. Rev. Biochem.* 86, 715–748. doi: 10.1146/annurev-biochem-061516-045037

Singer, R. E., Moss, K., Kim, S. J., Beck, J. D., and Offenbacher, S. (2015). Oxidative stress and IgG antibody modify periodontitis-CRP association. *J. Dent. Res.* 94, 1698–1705. doi: 10.1177/0022034515602693

Siqueira, M. A., Fischer, R. G., Pereira, N. R., Martins, M. A., Moss, M. B., Mendes-Ribeiro, A. C., et al. (2013). Effects of non-surgical periodontal treatment on the L-arginine-nitric oxide pathway and oxidative status in platelets. *Exp. Biol. Med.* 238, 713–722. doi: 10.1177/1535370213480690

Sree, S. L., and Sethupathy, S. (2014). Evaluation of the efficacy of taurine as an antioxidant in the management of patients with chronic periodontitis. *Dent. Res. J.* 11, 228–233.

Takane, M., Sugano, N., Iwasaki, H., Iwano, Y., Shimizu, N., and 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. doi: 10.1902/jop.2002.73.5.551

Tamaki, N., Cristina Orihuela-Campos, R., Inagaki, Y., Fukui, M., Nagata, T., and Ito, H. O. (2014a). Resveratrol improves oxidative stress and prevents the progression of periodontitis via the activation of the Sirt1/AMPK and the Nrf2/antioxidant defense pathways in a rat periodontitis model. *Free Radic. Biol. Med.* 75, 222–229. doi: 10.1016/j.freeradbiomed.2014.07.034

Tamaki, N., Hayashida, H., Fukui, M., Kitamura, M., Kawasaki, K., Nakazato, M., et al. (2014b). Oxidative stress and antibody levels to periodontal bacteria in adults: the Nagasaki Islands study. *Oral Dis.* 20, e49–e56. doi: 10.1111/odi.12127

Tamaki, N., Tomofuji, T., Ekuni, D., Yamanaka, R., and Morita, M. (2011). Periodontal treatment decreases plasma oxidized LDL level and oxidative stress. *Clin. Oral Investig.* 15, 953–958. doi: 10.1007/s00784-010-0458-y

Tamaki, N., Yoshino, F., Fukui, M., Hayashida, H., Yoshida, A., Kitamura, M., et al. (2015). Relationship among salivary antioxidant activity, cytokines, and periodontitis: the Nagasaki Island study. *J. Clin. Periodontol.* 42, 711–718. doi: 10.1111/jcpe.12438

Tartaglia, G. M., Gagliano, N., Zarbin, L., Tolomeo, G., and Sforza, C. (2017). Antioxidant capacity of human saliva and periodontal screening assessment in healthy adults. *Arch. Oral Biol.* 78, 34–38. doi: 10.1016/j.archoralbio.2017.02.003

Thomas, B., Madani, S. M., Prasad, B. R., and Kumari, S. (2014). Comparative evaluation of serum antioxidant levels in periodontally diseased patients: an interventional study. *Contemp. Clin. Dent.* 5, 340–344. doi: 10.4103/0976-237X.137938

Toker, H., Akpinar, A., Aydin, H., and Poyraz, O. (2012). Influence of smoking on interleukin-1beta level, oxidant status and antioxidant status in gingival crevicular fluid from chronic periodontitis patients before and after periodontal treatment. *J. Periodont. Res.* 47, 572–577. doi: 10.1111/j.1600-0765.2012.01468.x

Toker, H., Ozdemir, H., Eren, K., Ozer, H., and 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

Tomofuji, T., Azuma, T., Kusano, H., Sanbe, T., Ekuni, D., Tamaki, N., et al. (2006). Oxidative damage of periodontal tissue in the rat periodontitis model: effects of a high-cholesterol diet. *FEBS Lett.* 580, 3601–3604. doi: 10.1016/j.febslet.2006.05.041

Tomofuji, T., Ekuni, D., Irie, K., Azuma, T., Endo, Y., Tamaki, N., et al. (2009a). 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

Tomofuji, T., Ekuni, D., Irie, K., Azuma, T., Tamaki, N., Maruyama, T., et al. (2011). Relationships between periodontal inflammation, lipid peroxide and oxidative damage of multiple organs in rats. *Biomed. Res.* 32, 343–349. doi: 10.2220/biomedres.32.343

Tomofuji, T., Ekuni, D., Sanbe, T., Irie, K., Azuma, T., Maruyama, T., et al. (2009b). 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

Tomofuji, T., Sanbe, T., Ekuni, D., Azuma, T., Irie, K., Maruyama, T., et al. (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

Tonguç, M. O., Öztürk, O., Sutcu, R., Ceyhan, B. M., Kilinc, G., Sonmez, Y., et al. (2011). The impact of smoking status on antioxidant enzyme activity and malondialdehyde levels in chronic periodontitis. *J. Periodontol.* 82, 1320–1328. doi: 10.1902/jop.2011.100618

Torumtay, G., Kirzioglu, F. Y., Ozturk Tonguc, M., Kale, B., Calapoglu, M., and Orhan, H. (2016). Effects of periodontal treatment on inflammation and oxidative stress markers in patients with metabolic syndrome. *J. Periodont. Res.* 51, 489–498. doi: 10.1111/jre.12328

Tothova, L., Celecova, V., and Celec, P. (2013). Salivary markers of oxidative stress and their relation to periodontal and dental status in children. *Dis. Markers* 34, 9–15. doi: 10.1155/2013/591765

Trivedi, S., Lal, N., Mahdi, A. A., Mittal, M., Singh, B., and Pandey, S. (2014). Evaluation of antioxidant enzymes activity and malondialdehyde levels in patients with chronic periodontitis and diabetes mellitus. *J. Periodontol.* 85, 713–720. doi: 10.1902/jop.2013.130066

Villa-Correa, Y. A., Isaza-Guzman, D. M., and Tobon-Arroyave, S. I. (2015). Prognostic value of 8-Hydroxy-2'-Deoxyguanosine and human neutrophil elastase/alpha1-proteinase inhibitor complex as salivary biomarkers of oxidative stress in chronic periodontitis. *J. Periodontol.* 86, 1260–1267. doi: 10.1902/jop.2015.150293

Vlkova, B., and Celec, P. (2009). Does *Enterococcus faecalis* contribute to salivary thiobarbituric acid-reacting substances? *In Vivo* 23, 343–345.

Watson, J. D. (2014). Type 2 diabetes as a redox disease. *Lancet* 383, 841–843. doi: 10.1016/S0140-6736(13)62365-X

Wei, P. F., Ho, K. Y., Ho, Y. P., Wu, Y. M., Yang, Y. H., and Tsai, C. C. (2004). The investigation of glutathione peroxidase, lactoferrin, myeloperoxidase and interleukin-1beta in gingival crevicular fluid: implications for oxidative stress in human periodontal diseases.

J. Periodont. Res. 39, 287–293. doi: 10.1111/j.1600-0765.2004.00744.x

Weidinger, A., and Kozlov, A. V. (2015). Biological activities of reactive oxygen and nitrogen species: oxidative stress vs. signal transduction. *Biomolecules* 5, 472–484. doi: 10.3390/biom5020472

Wolfram, R. M., Budinsky, A. C., Eder, A., Presenhuber, C., Nell, A., Sperr, W., et al. (2006). Salivary isoprostanes indicate increased oxidation injury in periodontitis with additional tobacco abuse. *Biofactors* 28, 21–31. doi: 10.1002/biof.5520280103

Yang, P. S., Huang, W. C., Chen, S. Y., Chen, C. H., Lee, C. Y., Lin, C. T., et al. (2014). Scaling-stimulated salivary antioxidant changes and oral-health behavior in an evaluation of periodontal treatment outcomes. *Sci. World J.* 2014:814671. doi: 10.1155/2014/814671

Zamora-Perez, A. L., Ortiz-Garcia, Y. M., Lazalde-Ramos, B. P., Guerrero-Velazquez, C., Gomez-Meda, B. C., Ramirez-Aguilar, M. A., et al. (2015). Increased micronuclei and nuclear abnormalities in buccal mucosa and oxidative damage in saliva from patients with chronic and aggressive periodontal diseases. *J. Periodont. Res.* 50, 28–36. doi: 10.1111/jre.12175

Zare Javid, A., Seal, C. J., Heasman, P., and Moynihan, P. J. (2014). Impact of a customised dietary intervention on antioxidant status, dietary intakes and periodontal indices in patients with adult periodontitis. *J. Hum. Nutr. Diet.* 27, 523–532. doi: 10.1111/jhn.12184

Zhang, T., Andrukhov, O., Haririan, H., Muller-Kern, M., Liu, S., Liu, Z., et al. (2015). Total antioxidant capacity and total oxidant status in saliva of periodontitis patients in relation to bacterial load. *Front. Cell. Infect. Microbiol.* 5:97. doi: 10.3389/fcimb.2015.00097

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frontiers in Physiology

REVIEW
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Oxidative Stress and Antioxidant System in Periodontitis

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Periodontitis is a common inflammatory disease, which is initiated by bacterial infection and subsequently progressed by aberrant host response. It can result in the destruction of teeth supporting tissues and have an influence on systemic health. When periodontitis occurs, reactive oxygen species, which are overproduced mostly by hyperactive neutrophils, could not be balanced by antioxidant defense system and cause tissues damage. This is characterized by increased metabolites of lipid peroxidation, DNA damage and protein damage. Local and systemic activities of antioxidants can also be influenced by periodontitis. Total antioxidant capacity, total oxidant status and oxidative stress index have been used to evaluate the oxidative stress associated with periodontitis. Studies have confirmed that inflammatory response in periodontitis is associated with an increased local and systemic oxidative stress and compromised antioxidant capacity. Our review focuses on increased oxidative stress in periodontal disease, specifically, on the relationship between the local and systemic biomarkers of oxidative stress and periodontitis and their association with the pathogenesis of periodontitis. Also, the relationship between periodontitis and systemic inflammation, and the effects of periodontal therapy on oxidative stress parameters will be discussed.

Keywords: oxidative stress, reactive oxygen species, antioxidants, periodontitis, neutrophils

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INTRODUCTION

Periodontitis is a prevalent inflammatory disease, influencing at least 10% of people worldwide (Richards, 2014). It can result in the destruction of teeth supporting tissue and ends up with a loss of teeth. In addition, periodontitis has been suggested to have moderate association with several systemic diseases, e.g., cardiovascular disease, diabetes, and adverse pregnancy outcomes (Nazir, 2017). Current concept suggests that this inflammatory disease is initiated by bacterial infection and subsequently progressed by aberrant host response, which mainly contributes to periodontal tissue destruction (Bartold and Van Dyke, 2013).

In recent years, reactive oxygen species (ROS) have gained more and more attention, because of their central role to the progression of many inflammatory diseases (Mittal et al., 2014). ROS are described as oxygen free radicals and other non-radical oxygen derivatives involved in oxygen radical production (Lushchak, 2014). They are involved in normal cellular metabolism and continuously generated by the cells in most tissues. Another category of substances called antioxidants exist in the cells and can effectively delay or inhibit ROS-induced oxidation (Sies, 1997). Under physiological conditions, ROS are effectively neutralized by antioxidants, which prevent ROS-mediated tissue damage. When inflammation happens, ROS production is drastically increased mainly due to cells of innate immune system, e.g., neutrophils and macrophages during

the process of phagocytosis via the metabolic pathway of the "respiratory burst" (Mittal et al., 2014). Subsequently, high levels or activities of ROS cannot be balanced by the antioxidant defense system, which leads to the oxidative stress and tissue damage (Sies, 1997). ROS can directly cause tissue damage, involving lipid peroxidation, DNA damage, protein damage, and oxidation of important enzymes; meanwhile, they can function as signaling molecules or mediators of inflammation (Chapple and Matthews, 2007).

Over the past few years, numerous clinical and basic experimental studies have shown a strong association between oxidative stress and periodontitis. Getting a better understanding of this association can give us a deeper insight into the pathogenesis of periodontitis, relationship between periodontitis and systemic inflammation, and therapeutic strategies. Therefore, the aim of this review is to summarize the current findings of the association between local and systemic oxidative stress and periodontitis.

OVERPRODUCTION OF ROS ASSOCIATED WITH PERIODONTITIS

Neutrophils are the most abundant blood white cells and belong to first defense line against bacterial infection. After initiation of the host response by pathogenic biofilm, neutrophils become the most common inflammatory cells gathering in periodontal tissue and gingival sulcus and they are believed to be the predominant source of ROS in periodontitis (Miyasaki, 1991). Following the stimulation by pathogens, neutrophils produce O_2^- via the metabolic pathway called "respiratory burst" catalyzed by NADPH oxidase during phagocytosis (Chapple and Matthews, 2007). O_2^- can be released into phagosomal and extracellular environment and then converted to different radical and non-radical derivatives, such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hydroxyl radical (OH•) and singlet oxygen ($^1O_2$). **Figure 1** shows the mechanisms of increased ROS production in periodontal disease.

Numerous studies focused on the peripheral blood neutrophils of periodontitis patients and showed that their activity of producing ROS is higher compared to neutrophils from healthy individuals (Gustafsson and Asman, 1996; Fredriksson et al., 1998, 2003; Gustafsson et al., 2006; Matthews et al., 2007a,b; Wright et al., 2008; Aboodi et al., 2011; White et al., 2014; Ling et al., 2016). Consistent results have shown that peripheral blood neutrophils of people with chronic periodontitis (CP) or aggressive periodontitis (AgP) generate significantly more ROS upon simulation with purified immunoglobulin opsonized *Staphylococcus aureus* compared with peripheral blood neutrophils of healthy controls suggesting that people with periodontitis have a hyper-reactive phenotype of neutrophils and these neutrophils can be stimulated by the Fc-gamma receptor (FcγR) pathway (Gustafsson and Asman, 1996; Fredriksson et al., 1998; Gustafsson et al., 2006; Matthews et al., 2007a). One study by Fredriksson et al confirmed that increased ROS production by neutrophils of periodontitis patients occurs via the stimulation of FcγR pathway and not via complement receptor CR3 or intracellular protein kinase C enzyme (Fredriksson et al., 2003). Hyper-reactivity of both periodontitis patients and control neutrophils was also shown upon the stimulation of unopsonized periodontal pathogen *Fusobacterium nucleatum* (Matthews et al., 2007a). It has been shown that even without any stimulation neutrophils of periodontitis patients release more extracellular ROS than neutrophils of healthy controls (Matthews et al., 2007a; Ling et al., 2016). One longitudinal study showed that periodontal therapy could reduce FcγR-stimulated (with/without priming with *Porphyromonas gingivalis* and *F. nucleatum*) ROS production, but had no effect on unstimulated extracellular ROS (Matthews et al., 2007b). The same study observed that unstimulated ROS production was higher in periodontitis patients than in healthy controls therefore it was concluded that both constitutive and reactive mechanisms contribute to the hyperreactivity of neutrophils in periodontitis (Matthews et al., 2007b). A recent study demonstrated that peripheral blood neutrophils of CP patients produced more extracellular superoxide with or without stimulation of unopsonized *F. nucleatum*, *P. gingivalis* and phorbol myristate acetate and this superoxide overproduction was reduced upon non-surgical therapy, indicating that the hyperactivity of neutrophil is related to both reactive and constitutional mechanisms (Ling et al., 2016). Additionally, the level of superoxide released by unstimulated pre-therapy neutrophils significantly positively correlated with the level of C-reactive protein in plasma (Ling et al., 2016). This correlation might be partially explained by the fact that CRP increases toll-like receptor(s) induced superoxide released by neutrophils thus increasing oxidative stress (Ling et al., 2014). There are also studies suggesting an association between NADPH oxidase and FcγR polymorphism and periodontitis (Nibali et al., 2006; Dimou et al., 2010). These studies support the idea that an increased ROS generation in periodontitis could be not only due to stimulation by pathogens but also is genetically predisposed (Giannopoulou et al., 2008).

In vitro studies show that not only neutrophils but also other phagocytes and cells of periodontal tissues, e.g., monocytes, gingival fibroblasts and periodontal ligament cells exhibit enhanced ROS production upon stimulation by periodontal pathogens and/or their components (Bullon et al., 2011; Chang et al., 2013; Golz et al., 2014). However, their contribution into oxidative stress in periodontitis still remains to be elucidated by future studies.

METABOLIC PRODUCTS OF ROS IN PERIODONTITIS

ROS are very active and their life time is extremely short. They can cause direct damage to the tissues resulting in a variety of metabolites of lipid peroxidation, DNA damage, and protein damage, which are usually used to evaluate the destruction of tissue by ROS (Chapple and Matthews, 2007).

Lipid Peroxidation

Lipid peroxidation products are the most investigated derivatives of ROS in periodontitis. Lipid peroxidation by free radicals

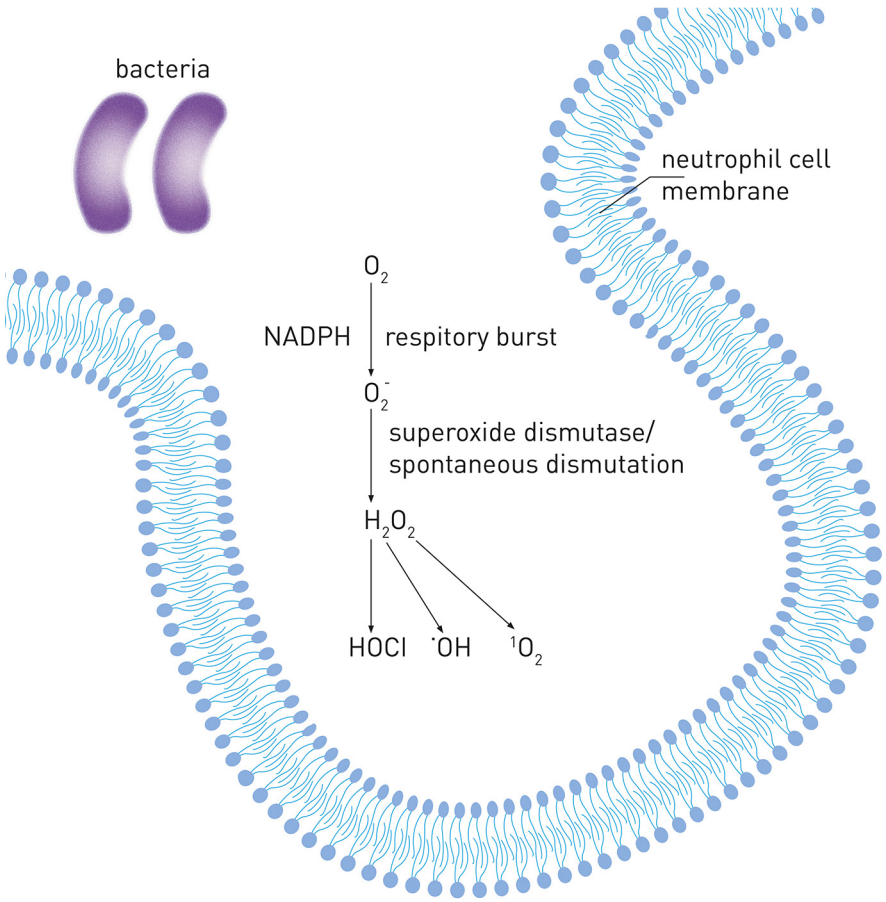

FIGURE 1 | Reactive oxygen species production in periodontal disease. Upon internalization of pathogens, neutrophils produce O_2^- via the metabolic pathway called "respiratory burst" by NADPH-oxidase. O_2^- can be converted to hydrogen peroxide (H_2O_2) by superoxide dismutase or spontaneous dismutation. H_2O_2 can be further converted to different derivatives, such as hypochlorous acid (HOCl), hydroxyl radical ($^{\bullet}OH$) and singlet oxygen ($^1O_2$).

results in the changes of structural integrity and function of cell membranes. Several products of lipid peroxidation such as malondialdehyde (MDA), 4-hydroxyl-2-115 nonenal (HNE), and isoprostane have been used to evaluate both local and systemic oxidative damages associated with periodontitis. **Table 1** summarizes the studies on the relationship between lipid peroxidation products and periodontitis.

MDA

MDA is a well-established lipid peroxidation product to evaluated oxidative stress, and it is also the most investigated lipid peroxidation product in periodontitis (Ahmadi-Motamayel et al., 2017).

Thiobarbituric acid reacting substances (TBARS) is a conventional method to detect MDA based on the reaction with thiobarbituric acid and measured by spectrophotometric assay (Yagi, 1976). It must be noted that this method is not specific for MDA and might also detect other aldehydes, which are also reactive with thiobarbituric acid and produce compound with similar absorption wavelengths as MDA (Halliwell and Whiteman, 2004). It has been shown that periodontitis is associated with higher levels of TBARS in blood plasma and erythrocytes systemically, as well as in gingival crevicular fluid (GCF) and gingival tissue locally (Panjamurthy et al., 2005). The association between increased TBARS levels and deteriorating periodontal status has been also shown in saliva of adults (Celec et al., 2005; Celecova et al., 2013; Gumus et al., 2015; Ahmadi-Motamayel et al., 2017), especially in men (Banasova et al., 2015), and children (Tothova et al., 2013).

Liquid chromatography and mass spectroscopy are more reliable and specific methods for the detection of MDA (Akalin et al., 2007). These methods were used to study MDA levels in serum, GCF, and saliva of periodontitis patients (Tsai et al., 2005; Akalin et al., 2007; Wei et al., 2010). Significantly higher levels of MDA were found in GCF and gingival tissue of periodontitis patients compared to periodontal healthy controls (Tsai et al., 2005; Wei et al., 2010; Tonguc et al., 2011). Moreover, a study by Ghallab et al. demonstrated that levels of MDA in GCF could discriminate between general AgP, CP, and periodontally healthy controls (Ghallab et al., 2016). Salivary MDA levels

TABLE 1 | Studies investigating the relationship between lipid peroxidation products and periodontitis.

Analyzed markers	Biological samples	Participants	Results	References
TBARS	Plasma; erythrocytes; erythrocyte membranes; gingival tissues	25 CP; 25 controls	↑TBARS in CP for all types of samples ($p < 0.001$)	Panjamurthy et al., 2005
TBARS	Saliva	217 dental patients	↑TBARS correlates with ↑BI ($p < 0.001$)	Celec et al., 2005
TBARS	Saliva	204 dental patients	↑TBARS correlates with ↑BI ($p < 0.026$) and ↑age ($p < 0.006$)	Celecova et al., 2013
TBARS	Saliva	115 pregnant women; 72 non-pregnant women	↓TBARS after giving birth than being pregnant; ↑TBARS correlates with ↑ probing depth ($p < 0.001$), ↑ clinical attachment level ($p < 0.003$), ↑ bleeding on probing ($p < 0.016$), ↑ plaque index ($p < 0.001$) in non-pregnant women	Gumus et al., 2015
TBARS	Saliva; blood	55 CP; 55 controls	↑TBARS in CP for all types of samples ($p = 0.0001$); no influence of gender	Ahmadi-Motamayel et al., 2017
TBARS	Saliva	23 CP; 19 controls	↑TBARS in male CP than male controls ($p < 0.01$)	Banasova et al., 2015
TBARS	Saliva	82 pediatric dental patients	↑TBARS correlates with ↑BI in children ($p < 0.05$)	Tothova et al., 2013
MDA	GCF; saliva	13 CP; 9 controls	↑MDA of GCF ($p < 0.005$) and saliva ($p < 0.05$) in CP than controls, and decreases after therapy ($p < 0.05$)	Tsai et al., 2005
MDA	GCF; saliva; serum	65 CP; 35 controls	↑MDA of GCF in CP than controls ($p < 0.05$), and decreases after therapy ($p < 0.05$)	Wei et al., 2010
MDA	Gingival tissue; serum	49 CP (23 smokers, 23 former smokers, 20 non-smokers); 20 controls (non-smokers)	↑MDA of both types of samples in CP than controls ($p < 0.01$); ↑MDA in CP (smokers) than CP (former smokers and non-smokers) ($p < 0.01$).	Tonguc et al., 2011
MDA	GCF	25 CP; 25 General AgP; 15 controls	Concentration of MDA: General AgP > CP > controls ($p < 0.001$).	Ghallab et al., 2016
MDA	GCF; saliva; serum	36 CP; 28 controls	↑MDA of GCF and saliva in CP than controls ($p < 0.05$).	Akalin et al., 2007
MDA	Saliva	30 CP (15 smokers, 15 non-smokers); 30 controls (15 smokers, 15 non-smokers)	↑MDA in CP (smokers) than controls (non-smokers) ($p < 0.05$), and decreases after therapy ($p < 0.05$).	Guentsch et al., 2008
MDA	Saliva	30 CP; 30 controls	↑MDA in CP than controls ($p < 0.001$).	Canakci et al., 2009
MDA	Saliva	20 CP; 20 controls	↑MDA in CP than controls ($p < 0.05$)	Miricescu et al., 2014
MDA	Saliva	33 CP; 16 gingivitis; 37 controls	↑MDA in CP than controls and gingivitis, and correlates with the percentage of bleeding on probing and presence of periodontal pathogens.	Almerich-Silla et al., 2015
MDA	Saliva; serum	30 CP; 35 general AgP; 30 controls	↑MDA of GCF in CP and general AgP than controls ($p < 0.05$), and correlates with clinical parameters.	Baltacioglu et al., 2014b
MDA	Saliva; plasma	60 CP (30 with type 2 diabetics, 30 systemically healthy); 60 controls (30 with type 2 diabetics, 30 systemically healthy)	↑MDA in CP ($p < 0.05$); no difference between CP with type 2 diabetics and systemically healthy CP	Trivedi et al., 2014
MDA	Blood	37 CP (18 with hyperlipidemia, 19 systemically healthy); 37 controls (18 with hyperlipidemia, 19 systemically healthy)	↑MDA in CP (with hyperlipidemia) than CP (systemically healthy) and controls (systemically healthy).	Fentoglu et al., 2015
MDA	Saliva	32 CP (16 with acute coronary syndrome, 16 systemically healthy); 32 controls (16 with acute coronary syndrome, 19 systemically healthy)	↑MDA in CP (with acute coronary syndrome) than CP (systemically healthy) and controls; MDA correlates with clinical parameters.	Nguyen et al., 2016
MDA	Saliva	217 dental patients	↑MDA of saliva in smokers than non-smokers ($p < 0.003$)	Celec et al., 2005
MDA	GCF; saliva; serum	25 CP; 26 controls	↑MDA of saliva in CP than controls ($p < 0.001$), No change at 3-weeks after therapy ($p < 0.05$)	Onder et al., 2017

(Continued)

TABLE 1 | Continued

Analyzed markers	Biological samples	Participants	Results	References
HNE	GCF; saliva; serum	47 CP (24 smokers, 23 non-smokers); 46 controls (23 smokers, 23 non-smokers)	↑HNE of GCF in CP(smokers) than controls (non-smokers)($p = 0.001$), No change at 3-months after therapy ($p < 0.05$)	Hendek et al., 2015
HNE	Saliva; serum	25 CP; 26 controls	↑HNE of serum in CP than controls ($p < 0.001$), No change at 6-weeks after therapy ($p < 0.05$)	Onder et al., 2017
HNE	Saliva; serum	30 CP (15 with type 2 diabetics, 15 systemically healthy); 10 controls (systemically healthy)	HNE concentration: CP with type 2 diabetics > systemically healthy CP >controls ($p < 0.05$)	Pradeep et al., 2013a
Isoprostane	GCF	26 CP; 26 gingivitis; 26 controls	8-Isoprostane concentration: CP >gingivitis >controls ($p < 0.001$)	Pradeep et al., 2013c
Isoprostane	Saliva	121 adults (31 smokers, 90 non-smokers)	↑8-epi-prostaglandin F2 alpha in smokers than non-smokers ($p < 0.0001$), and correlates with plaque index.	Wolfram et al., 2006
Isoprostane	Saliva	58 CP; 234 controls	↑8-epi-prostaglandin F2 alpha in CP than controls ($p < 0.0001$)	Su et al., 2009

TBARS, thiobarbituric acid reacting substances; MDA, malondialdehyde; HNE, 4-hydroxyl-2-nonenal; GCF, gingival crevicular fluid; CP, chronic periodontitis; AgP, aggressive periodontitis; BI, bleeding index.

in periodontitis were extensively investigated and most of the studies showed higher salivary MDA in periodontitis patients compared to periodontally healthy controls (Tsai et al., 2005; Akalin et al., 2007; Guentsch et al., 2008; Canakci et al., 2009; Wei et al., 2010; Baltacioglu et al., 2014b; Miricescu et al., 2014; Trivedi et al., 2014; Almerich-Silla et al., 2015; Onder et al., 2017). A study by Baltacioglu et al. compared the level of MDA in saliva between people with AgP, CP, and periodontally healthy controls and found that AgP and CP groups have significantly higher levels of MDA than control group, but no differences between AgP and CP groups were observed (Baltacioglu et al., 2014b). It has also been shown that the higher local levels of MDA in periodontitis patients can be diminished upon periodontal therapy (Tsai et al., 2005; Guentsch et al., 2008; Wei et al., 2010). There are also some studies investigating the level of MDA in serum of periodontitis patients; however, in contrast to the data on local MDA levels, their results are controversial (Akalin et al., 2007; Wei et al., 2010; Baltacioglu et al., 2014b; Trivedi et al., 2014; Fentoglu et al., 2015; Onder et al., 2017). Two studies, in which MDA levels were measured in GCF, saliva, and serum of CP patients, showed that periodontitis had no effect on systemic MDA levels, although local MDA levels were increased in periodontitis patients (Akalin et al., 2007; Wei et al., 2010). This finding suggests that the influence of periodontitis on systemic oxidative stress might be limited. However, a meta-analysis performed by Liu et al included 5 studies on systemic MDA in periodontitis and showed that periodontitis patients had higher level of circulating MDA than healthy controls (Liu et al., 2014). Recently, a study with rather large sample size (55 CP and 55 healthy controls) also confirmed the significant difference of MDA level in serum between CP and healthy controls (Ahmadi-Motamayel et al., 2017). Meanwhile, studies including patients with diabetes mellitus, hyperlipidemia and acute coronary syndrome indicated that periodontitis could also contribute to higher circulating level of MDA among people with these systemic diseases (Trivedi et al., 2014; Fentoglu et al., 2015; Nguyen et al., 2016). Smoking is one of the most important risk factors for periodontitis and several studies showed that systemic and local MDA levels were increased by smoking independently on the impact of periodontitis (Celec et al., 2005; Guentsch et al., 2008; Tonguc et al., 2011). All above data suggest that MDA may reflect increased local and systemic oxidative stress associated with periodontitis in combination with either systemic disease or smoking.

HNE

HNE is another major aldehydes end product associated with lipid peroxidation (Petersen and Doorn, 2004) but data on this biomarker in periodontitis are limited to date. A study by Hendek et al. investigated the impact of periodontitis, smoking and periodontal treatment on HNE levels in GCF, saliva, and serum, and found significant different GCF levels of HNE between smokers with periodontitis and periodontally healthy non-smokers (Hendek et al., 2015). In contrast to this study, Onder et al. showed that the levels of HNE are increased by periodontitis only in serum but not in saliva (Onder et al., 2017). Both of the above studies did not show the reduction of HNE level after periodontal treatment. A study detecting HNE modified histidine adducts showed that the level of HNE-Histidine adducts in both GCF and serum were significantly increased in periodontitis with or without diabetes mellitus (Pradeep et al., 2013a).

Isoprostane

Isoprostane is a product of arachidonic acid peroxidation and is often measured in urine, serum or plasma as a reliable marker of oxidative stress (Roberts and Morrow, 2002). There are few studies investigating isoprostane levels in periodontitis (Wolfram et al., 2006; Su et al., 2009; Pradeep et al., 2013c). Elevated salivary levels of 8-epi-prostaglandin F2 alpha, one of isoprostanes, were associated with periodontal disease severity and were significantly increased by smoking (Wolfram et al., 2006; Su et al., 2009). Another study by Pradeep et al. (2013c) showed that 8-isoprostane levels in GCF increased progressively from

healthy controls to gingivitis and periodontitis and correlated with gingival index, probing depth, and clinical attachment level (Pradeep et al., 2013c). All the above studies indicated that specific isoprostanes could be promising oxidative stress markers for periodontitis, and further longitudinal and prospective studies with a larger population are required.

Protein Damage

ROS can cause fragmentation of polypeptides or covalent crosslinking resulting in changes of protein functional activity (Shacter, 2000). Some protein damage by ROS was investigated in periodontitis. **Table 2** summarizes the studies investigating the relationship between protein damage products and periodontitis.

Protein Carbonyl Groups

Protein carbonyl (PC) groups are relatively stable end-products of protein oxidation generated by multiple forms of ROS. It is the most widely used biomarker for oxidative protein damage with earlier production and greater stability compared with lipid peroxidation products (Frijhoff et al., 2015). The association between periodontal status and PC groups has been investigated in GCF, saliva and serum and higher levels of PC groups were associated with worse periodontal status, as well as significant correlation between the level of PC groups and clinical periodontal parameters was observed within periodontitis patients (Sculley and Langley-Evans, 2003; Baltacioglu et al., 2008; Pradeep et al., 2013b; Nguyen et al., 2017). One study even showed that some specific salivary proteins such as transferrin, human IgG1 heavy chain fragment, and amylase exhibited higher oxidation levels in periodontitis compared to healthy controls (Su et al., 2009).

Advanced Oxidation Protein Products

Advanced oxidation protein products (AOPP) is also thought to be a sensitive biomarker of protein oxidation, especially related to the activation of neutrophil and the activity of myeloperoxidase (Witko-Sarsat et al., 1996). AOPP have been detected in saliva, however, no relationship was found between their levels and periodontal status among adults or children (Celecova et al., 2013; Tothova et al., 2013; Banasova et al., 2015).

DNA Damage

ROS can react with DNA and cause damage to purine and pyrimidine bases or the deoxyribose backbone (Halliwell, 2000). 8-Hydroxy-deoxyguanosine (8-OHdG) is most often used biomarker of oxidative stress-induced DNA damage, although it may not precisely reflect the whole DNA damage resulting from oxidative stress (Chapple and Matthews, 2007). **Table 3** summarizes the studies on the relationship between DNA damage products and periodontitis.

Numerous studies showed higher level of 8-OHdG in GCF and saliva of periodontitis patients compared with that of healthy controls as well as their significant association with clinical periodontal parameters (Takane et al., 2002; Canakci et al., 2009; Su et al., 2009; Sezer et al., 2012; Dede et al., 2013; Hendek et al., 2015; Kurgan et al., 2015; Zamora-Perez et al., 2015; Shin et al., 2016; Onder et al., 2017). 8-OHdG levels are significantly reduced by periodontal treatment (Takane et al., 2002; Dede et al., 2013; Hendek et al., 2015; Kurgan et al., 2015; Ongoz Dede et al., 2016; Yang et al., 2016; Onder et al., 2017). However, there is no difference in the local levels of 8-OHdG between individuals with gingivitis and periodontitis (Sezer et al., 2012), as well as between CP and AgP patients (Zamora-Perez et al., 2015). A recent study suggested that liquid chromatography tandem mass spectrometry is a more sensitive approach to evaluate the levels of 8-OHdG in saliva with reliability similar to the conventional enzyme linked immune sorbent assay (Kurgan et al., 2015). However, this method of 8-OHdG detection needs to be applied for other samples such as GCF and plasma. Several studies indicated that the level of 8-OHdG is associated with the presence and/or quantity of bacteria such as *P. gingivalis, Tannerella forsythia, Treponema denticola* and *Streptococcus anginosus* (Sugano et al., 2003; Sawamoto et al., 2005; Almerich-Silla et al., 2015; Yang et al., 2016). Recently, studies have shown that the levels of 8-OHdG in saliva is significantly elevated by pregnancy and smoking (Gumus et al., 2015; Kurgan et al., 2015). Moreover, studies investigating the serum levels of 8-OHdG showed that it could be influenced by several systemic conditions such as obesity and hyperlipidemia independently on periodontitis (Fentoglu et al., 2015; Hendek et al., 2015; Onder et al., 2017). Based on above mentioned studies, we can conclude

TABLE 2 | Studies investigating the relationship between protein damage products and periodontitis.

Analyzed markers	Biological samples	Participants	Results	References
PC	GCF	25 CP; 25 gingivitis; 25 controls	↑PC in CP than gingivitis and controls, and correlates with clinical parameters.	Pradeep et al., 2013b
PC	Saliva	48 CP (24 with acute coronary syndrome, 24 systemically healthy); 48 controls (24 with acute coronary syndrome, 24 systemically healthy)	↑PC in CP and controls with acute coronary syndrome than systemically healthy controls ($p < 0.05$).PC correlates with probing depth, plaque index ($p < 0.05$).	Nguyen et al., 2017
PC	Saliva	58 CP; 234 controls	↑PC and ↑specific oxidation of transferrin, human IgG1 heavy chain fragment, and amylase in CP than controls ($p < 0.0001$)	Su et al., 2009
AOPP	Saliva	204 dental patients	AOPP doesn't correlates with BI, and correlates with caries.	Celecova et al., 2013
AOPP	Saliva	82 pediatric dental patients	AOPP doesn't correlates with BI, and correlates with caries.	Tothova et al., 2013
AOPP	Saliva	23 CP; 19 controls	No difference of AOPP between groups	Banasova et al., 2015

PC, protein carbonyls; AOPP, advanced oxidation protein products; GCF, gingival crevicular fluid; CP, chronic periodontitis (CP); AgP, aggressive periodontitis; BI, bleeding index.

TABLE 3 | Studies investigating the relationship between DNA damage products and periodontitis.

Analyzed markers	Biological samples	Participants	Results	References
8-OHdG	saliva	30 CP; 30 controls	↑8-OHdG in CP ($p < 0.001$)	Canakci et al., 2009
8-OHdG	saliva	58 CP; 234 controls	↑8-OHdG in CP ($p = 0.0003$); 8-OHdG negatively correlates with Community Periodontal Index of Treatment Needs ($p = 0.004$)	Su et al., 2009
8-OHdG	saliva	20 CP; 20 gingivitis; 20 controls	↑8-OHdG in CP than gingivitis and controls ($p < 0.001$), correlates with age ($p < 0.05$), probing depth ($p < 0.001$) and CAL ($p < 0.001$)	Sezer et al., 2012
8-OHdG	GCF; saliva	24 CP; 24 controls	↑8-OHdG of GCF in CP ($p < 0.001$), decreases after therapy ($p < 0.001$); 8-OHdG of saliva doesn't differ between groups or after therapy.	Dede et al., 2013
8-OHdG	GCF; saliva; serum	47 CP (24 smokers, 23 non-smokers); 46 controls (23 smokers, 23 non-smokers)	↑8-OHdG of GCF in CP than controls ($p < 0.001$); ↑8-OHdG of saliva in CP than controls (non-smokers) ($p < 0.003$).	Hendek et al., 2015
8-OHdG	saliva	23 CP; 25 controls	↑8-OHdG correlates with clinical parameters ($p < 0.001$), and decreases after therapy ($p < 0.001$)	Kurgan et al., 2015
8-OHdG	saliva	58 CP; 42 AgP; 60 controls	↑8-OHdG in CP and AgP than controls ($p < 0.05$)	Zamora-Perez et al., 2015
8-OHdG	saliva; serum	25 CP; 26 controls	↑8-OHdG of saliva in CP than controls ($p < 0.001$), and decreases after therapy ($p < 0.001$).	Onder et al., 2017
8-OHdG	saliva	211 adults	↑8-OHdG correlates with periodontitis.	Shin et al., 2016
8-OHdG	GCF; saliva; plasma	45 obese individuals; 45 normal-weight individuals	↑8-OHdG of all types of samples in CP than controls ($p < 0.05$); ↑8-OHdG of plasma in obese individuals with periodontitis than normal-weight individuals ($p < 0.05$); 8-OHdG in CP and gingivitis decreases after therapy ($p < 0.01$).	Ongoz Dede et al., 2016
8-OHdG	saliva	45 CP; 47 controls	↑8-OHdG in CP than controls, correlates with clinical parameters and quantity of periodontal pathogens, and decreases after therapy.	Yang et al., 2016
8-OHdG	saliva	38 patients	↑8-OHdG in patients positive for S. anginosus, and decreases after therapy.	Sugano et al., 2003
8-OHdG	saliva	29 periodontitis; 20 controls	↑8-OHdG in CP than controls, correlates with quantity of P. gingivitis ($p < 0.01$), decreases after therapy ($p < 0.01$).	Sawamoto et al., 2005
8-OHdG	saliva	33 CP; 16 gingivitis; 37 controls	↑8-OHdG in CP than controls and gingivitis, and correlates with the percentage of bleeding on probing and presence of periodontal pathogens.	Almerich-Silla et al., 2015
8-OHdG	saliva	115 pregnant women; 72 non-pregnant women	↑8-OHdG in pregnant women than non-pregant women; ↑8-OHdG correlates with↑probing depth ($p < 0.001$), ↑CAL ($p < 0.001$) after partum.	Gumus et al., 2015
8-OHdG	blood	37 CP (18 with hyperlipidemia, 19 systemically healthy); 37 controls (18 with hyperlipidemia, 19 systemically healthy)	↑8-OHdG in CP with hyperlipidemia than systemically healthy CP and systemically healthy controls.	Fentoglu et al., 2015

8-OHdG, 8-Hydroxy-deoxyguanosine; GCF, gingival crevicular fluid; CP, chronic periodontitis (CP); AgP, aggressive periodontitis.

that local levels of 8-OHdG are closely related to periodontitis with some impact of systemic conditions, whereas the systemic levels of 8-OHdG depend more on systemic conditions than on periodontal status.

ANTIOXIDANT

Under normal physiological conditions, there is a balance between ROS and antioxidants. Oxidative stress happens only when the antioxidant defense system could not neutralize the elevated ROS production (Sies, 1997). Antioxidants can be classified as two categories based on their mode of function (Chapple and Matthews, 2007). First category comprises preventive antioxidants including enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase, and DNA repair enzymes, as well as some metal ion sequestrators such as albumin. Second category comprises scavenging antioxidants or chain breaking antioxidants such as ascorbic acid (vitamin C), carotenoids (including retinol-vitamin A), uric acid, α-tocopherol (vitamin E), reduced glutathione, and polyphenols (flavenoids). **Table 4** summarizes the studies investigating the relationship between antioxidants and periodontitis.

SOD and CAT activities were measured in human gingival tissue and these activities were found to be reduced with the increasing periodontal pocket depth (Ellis et al., 1998).

TABLE 4 | Studies investigating the relationship between antioxidants and periodontitis.

Analyzed markers	Biological samples	Participants	Results	References
SOD; CAT	Gingival tissue	< or =3 mm; 4–6 mm; >6 mm gingival tissues	↓SOD and ↓CAT in >6 mm than other groups	Ellis et al., 1998
SOD; GPx	Saliva	30 CP; 30 controls	↓SOD and ↓GPx in CP ($p < 0.05$); SOD and GPx negatively correlates with MDA and 8-OHdG ($p < 0.001$)	Canakci et al., 2009
SOD; CAT; glutathione reductase	Saliva	30 CP; 30 controls	↓SOD, ↓CAT, and ↓glutathione reductase in CP; SOD, CAT, and glutathione negatively correlates with clinical parameters.	Trivedi et al., 2015
SOD; CAT; GPx; glutathione reductase; vitamin C; vitamin E	Plasma; erythrocytes; erythrocyte membranes; gingival tissues	25 CP; 25 controls	↓SOD, ↓CAT, ↓GPx and glutathione reductase of all types of samples in CP; vitamin C and vitamin E of all types of samples in CP (except for reduced glutathione in the gingival tissues).	Panjamurthy et al., 2005
SOD; GPx; albumins; uric acid	Saliva	42 CP; 21 controls	↑SOD ($p = 0.021$) and ↑GPx ($p = 0.000$) in CP; ↓albumins in CP ($p = 0.039$); GPx, albumins and uric acid increases ($p < 0.001$), and SOD decreases ($p < 0.005$) after therapy.	Novakovic et al., 2014
GPx; uric acid	Saliva	20 CP; 20 controls	↓GPx and ↓uric acid in CP ($p < 0.05$); uric acid negatively correlates with C-terminal telopeptide of type I collagen and matrix metalloproteinases-8 ($p < 0.05$).	Miricescu et al., 2014
SOD; CAT; glutathione; total thiol	Gingival tissue; blood	35 CP (20 smokers, 10 non-smokers)	↑CAT and ↑total thiol of all types of samples in smokers; ↓glutathione of gingival tissue in smokers; ↓SOD of all types of samples in smokers.	Garg et al., 2006
Urate; vitamin A; vitamin C; vitamin E; thiols; bilirubin; cholesterol; thiglycerides; albumin	Serum	256 participants	Vitamin A ($p < 0.0001$), urate ($p < 0.0001$) and thiols ($p < 0.01$) are influenced by gender.	Maxwell et al., 2006
SOD	Gingival tissue	34 CP (17 with type 2 diabetics, 17 systemically healthy); 35 controls (18 with type 2 diabetics, 17 systemically healthy)	↓SOD in CP than controls ($p < 0.05$); ↑SOD in participants with diabetics than systemically healthy participants.	Akalin et al., 2008
SOD	GCF; saliva	60 smokers; 10 non-smokers	↓SOD of all types of samples in smokers than controls, and correlates with the extent of smoking.	Agnihotri et al., 2009
SOD	GCF; saliva	60 CP (33 pregnant, 27 non-pregnant); 18 gingivitis (pregnant); 46 controls (21 pregnant, 25 non-pregnant);	↑clinical parameters and ↓SOD in pregnancy, especially for the last phase of pregnancy	Akalin et al., 2009
SOD; CAT; GPx	Serum; gingival tissue	49 CP (23 smokers, 23 former smokers, 20 non-smokers); 20 controls (non-smokers)	↓SOD, ↓CAT, and ↓GPx of gingival tissue in CP than controls ($p < 0.01$); ↓SOD, ↓CAT, and ↓GPx of gingival tissue in CP (non-smokers) than CP (smokers and former smokers) ($p < 0.01$).	Tonguc et al., 2011

SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GCF, gingival crevicular fluid; CP, chronic periodontitis.

The activities of SOD and GPx in saliva were decreased in periodontitis patients (Canakci et al., 2009). Additionally, this study suggested a significant negative relationship between the level of 8-OHdG and MDA and the activities of SOD and GPx, whereas no correlation between clinical parameters and the enzymatic antioxidants activities was observed. Another study showed that the activities of antioxidant enzymes SOD, CAT, and glutathione reductase in saliva of periodontitis patients exhibited a significant negative correlation with periodontal parameters (Trivedi et al., 2015). In contrast to these studies, Panjamurthy et al. showed that activities of enzymatic antioxidants including SOD, CAT measured in plasma, erythrocytes and gingival tissues were elevated in periodontitis, whereas activities of non-enzymatic antioxidants including vitamins E, vitamin C, and reduced glutathione were decreased in periodontitis (Panjamurthy et al., 2005). Similarly, another study by Novakovic et al. showed higher activities of enzymatic antioxidants including SOD and GPx and lower activities of non-enzymatic antioxidants in saliva of periodontitis patients (Novakovic et al., 2014). Furthermore, periodontal treatment resulted in elevating the activities of albumins, uric acid, and GPx and decreasing the activity of SOD (Novakovic et al., 2014). The activity of uric acid was found to be lower in saliva of periodontitis patients and it was shown to be negatively correlated with bone resorption biomarkers such as C-terminal telopeptide of type I collagen and matrix metalloproteinases-8 (Miricescu et al., 2014). Therefore, current results on the relationship between periodontal status and enzymatic antioxidants activity are contradictory. Meta-analysis performed with 6 articles investigating the levels of circulating SOD found no significant difference in this parameter between periodontitis patients and healthy controls (Liu et al., 2014). In contrast, the results for non-enzymatic antioxidants are

rather consistent and they suggest that the decreased activities of non-enzymatic antioxidants are associated with periodontitis. Thus, more additional well-designed studies are still required to clarify the relationship between enzymatic antioxidant activities and periodontitis.

Similarly to ROS production, numerous studies indicate that the changes in the activity of antioxidants in periodontitis are influenced by systemic conditions (Garg et al., 2006; Maxwell et al., 2006; Akalin et al., 2008, 2009; Agnihotri et al., 2009; Tonguc et al., 2011; Duarte et al., 2012; Trivedi et al., 2014). One study showed that women have lower activity of vitamin A and urate in serum than men (Maxwell et al., 2006). Gingival activities of specific antioxidants like SOD, CAT, and GPx could be increased by smoking among people with periodontitis, and these changes were considered as a protective or adoptive mechanism (Tonguc et al., 2011). In contrast, another study indicated a compromised activity of gingival SOD and glutathione in smokers (Garg et al., 2006). Smoking is also associated with decreased levels of SOD in GCF and saliva in both periodontitis patients and healthy individuals (Agnihotri et al., 2009). Diabetes mellitus can increase the activity of SOD and gene expression of SOD1 in gingival tissue of periodontitis patients (Akalin et al., 2008; Duarte et al., 2012). However, higher activities of SOD, CAT and glutathione reductase were found in saliva and plasma of systemically and periodontally healthy individuals compared to those with CP and/or diabetes mellitus (Trivedi et al., 2014). Activities of SOD were also found to be decreased by pregnancy among periodontitis patients (Akalin et al., 2009).

Antioxidants present a strong defense function against ROS; therefore, numerous studies tried to examine the application of antioxidants in the treatment of periodontitis. It has been shown that supplemental periodontal treatments with antioxidants like vitamin E, taurine and lycopene result in improved clinical periodontal parameters, higher activities of local and systemic antioxidants, and lower levels of local and systemic ROS compared with conventional periodontal treatment (Arora et al., 2013; Singh et al., 2014; Sree and Sethupathy, 2014). A recent review concluded a useful effect of vitamin C on maintaining periodontal health for elderly people (Alagl and Bhat, 2015). Another recent review focused on the effects of the complimentary use of lycopene, vitamin C, vitamin E, capsules with fruits/vegetables/berry and dietary interventions to periodontal therapy (Muniz et al., 2015). It confirmed that only the use of lycopene and vitamin E is associated with improved clinical parameters (Muniz et al., 2015). These results indicate a promising use of antioxidants for periodontitis treatment, which could be beneficial for both periodontal status and systemic oxidative status.

TOTAL ANTIOXIDANT CAPACITY, TOTAL OXIDANT STATUS AND OXIDATIVE STRESS INDEX

Total Antioxidant Capacity

The antioxidant system is highly complex and therefore the measurement of total antioxidant capacity (TAOC) was developed as a cost-effective instrument to assess the activity of the whole antioxidant system (Chapple et al., 1997). Most of the related studies suggested that periodontitis is associated with compromised local TAOC (Brock et al., 2004; Chapple et al., 2007a; Guentsch et al., 2008; Baltacioglu et al., 2014b; Baser et al., 2015; Zhang et al., 2016; Ahmadi-Motamayel et al., 2017). Moreover, some studies also indicated that periodontitis could influence the circulating TAOC (Brock et al., 2004; Chapple et al., 2007b; Abou Sulaiman and Shehadeh, 2010; D'Aiuto et al., 2010; Baltacioglu et al., 2014b; Thomas et al., 2014; Baser et al., 2015; Ahmadi-Motamayel et al., 2017). TAOC in plasma and saliva was shown to correlate with periodontal parameters (Baser et al., 2015; Zhang et al., 2016). However, there are contradictory data on the question whether periodontal treatment can improve local and/or circulating compromised TAOC (Guentsch et al., 2008; D'Aiuto et al., 2010; Novakovic et al., 2014; Thomas et al., 2014). Therefore, additional controlled studies on the effect of periodontal therapy on local and systemic TAOC are required. A recent study showed no relationship between TAOC and bacterial load in periodontitis suggesting that the changes of TAOC could be related to the host immune response rather than to the bacterial load (Zhang et al., 2016).

TAOC associated with periodontitis can be affected by systemic conditions like gender, smoking, pregnancy, and systemic diseases (Brock et al., 2004; Buduneli et al., 2006; Maxwell et al., 2006; Chapple et al., 2007a; Akalin et al., 2009; Pendyala et al., 2013a,b; Bakhtiari et al., 2015; Ahmadi-Motamayel et al., 2017). Some studies suggested that men have higher serum TAOC than women (Brock et al., 2004; Maxwell et al., 2006; Chapple et al., 2007a). One study also showed similar difference in saliva TAOC (Maxwell et al., 2006). One study showed that lower TAOC in serum and GCF was also associated with pregnancy, especially in the last trimester, and within the pregnant women decreasing TAOC was correlated to deteriorating periodontal status (Akalin et al., 2009). There is one study indicating that salivary TAOC among smokers is significantly lower than that among non-smokers; however, this study did not consider the worse periodontal status of smokers (Bakhtiari et al., 2015). Another study found that neither the gingivitis nor smoking status have influence on salivary TAOC (Buduneli et al., 2006). Further studies showed that both periodontitis and diabetes mellitus could contribute to lower TAOC in saliva, and decreased TAOC in saliva was also associated with periodontal status among people with diabetes mellitus (Pendyala et al., 2013a,b).

TOS and OSI

In 2005, an assay based on the oxidation of ferrous ion to ferric ion in the presence of various oxidant species in acidic medium was introduced to measure the total oxidative status (Erel, 2005). Differently to previous methods focused on specific ROS or ROS products, this method could be used as a stable, cost-efficient and convenient measurement of the whole oxidant status. Another parameter called oxidative stress index (OSI), which is calculated as TOS/TAOC, was also introduced to show

the level of oxidative stress with the balance of antioxidants (Erel, 2005).

These two parameters have been widely used to measure whole oxidative stress associated with periodontitis. Studies have shown that periodontitis is associated with higher value of TOS and OSI in GCF, saliva and serum (Erel, 2005; Akalin et al., 2007; Baltacioglu et al., 2014a,b), and these levels can also be reduced by periodontal treatment (Wei et al., 2010; Akpinar et al., 2013). Aggressive periodontitis was shown to be associated with significantly higher values of TOS and OSI compared to chronic periodontitis (Baltacioglu et al., 2014a,b). One study had even proposed OSI as a new biomarker for periodontitis based on its significant correlation with clinical parameters of periodontitis (Baltacioglu et al., 2014b). However, in contrast to this observation, our previous study did not show any significant difference in salivary TOS between generalized severe periodontitis patients and healthy controls (Zhang et al., 2016). This discrepancy might be due to the less restricted selection of periodontitis patients and indicates the limited utilization of salivary TOS as a marker of periodontitis.

TOS and OSI were also used to show the interaction between periodontitis and systemic conditions. One study showed that rheumatoid arthritis had no significant impact on local and systemic OSI of people with periodontitis (Esen et al., 2012). In contrast, another study showed that although rheumatoid arthritis or periodontitis have limited effect on serum OSI, individuals with both rheumatoid arthritis and periodontitis showed significant higher serum OSI compared to systemic and periodontally healthy individuals (Sezer et al., 2013). Another systemic disease familial Mediterranean fever was also shown to affect the local OSI of periodontitis patients (Bostanci et al., 2014). One study suggested that people with obesity were more likely to have higher value of TOS and OSI in serum and GCF and were predisposed to periodontitis (Dursun et al., 2016). TOS or OSI can also be used as the measurements of the effectiveness of newly developed periodontal therapy. Particularly, studies on rats show that boric acid, sumac extract and low-dose doxycycline could significantly reduce the oxidative stress indicated by OSI or TOS (Balci Yuce et al., 2014; Yagan et al., 2014; Saglam et al., 2015; Kose et al., 2016).

Summarizing, TOS and OSI can show the association between increased local and systemic oxidative stress and deteriorated periodontal status; however, their sensitivity needs to be further tested. Furthermore, these two measurements have the potential to evaluate the interaction between periodontal and systemic status and the effectiveness of periodontal treatment.

CONCLUSIONS

It has been confirmed that periodontitis is associated with a hyperactivity of peripheral blood neutrophils, which are supposed to be the predominant source of ROS. Recent reports suggest that hyperactivity of neutrophils is likely to be a host-immune reaction to the inflammation of periodontitis, which might be also genetically predisposed. Numerous studies suggested that periodontitis could contribute to both local and systemic oxidative stress. Products of lipid peroxidation, protein damage and DNA damage can be used as the biomarkers of oxidative stress associated with periodontitis. Local and systemic activities of antioxidants can also be influenced by periodontitis. Some studies suggested decreased activities of enzymatic antioxidants, like SOD and CAT, are associated with periodontitis, whereas others showed increased activities of enzymatic antioxidants among people with periodontitis as a protective reaction. The results for non-enzymatic antioxidants as well as TAOC are consistent and indicate compromised antioxidant capacity in periodontitis patients. Different antioxidants have been applied as supplements to the conventional periodontal treatment and optimistic results were obtained, which provides new possibilities in the periodontal therapy. In recent years, TOS and OSI have been used more and more to evaluate total oxidative status or oxidative stress associated with periodontitis. Studies measuring these parameters also confirmed increased local and systemic oxidative stress was associated with the inflammation resulted from periodontitis, but their sensitivity to be used as biomarkers for oxidative stress associated with periodontitis needs to be further verified.

Our review focused on the presence of oxidative stress associated with periodontitis, especially on the relationship between the local and systemic biomarkers of oxidative stress and periodontitis, giving us an implication of pathogenesis of periodontitis through oxidative stress, close relationship between periodontitis and systemic conditions, and promising therapeutic strategies involving antioxidants.

AUTHOR CONTRIBUTIONS

Conception and design: XR-F, OA, and YW. Search references: YW. Drafted manuscript: YW. Critically revised the manuscript: YW, OA, and XR-F.

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REFERENCES

Aboodi, G. M., Goldberg, M. B., and Glogauer, M. (2011). Refractory periodontitis population characterized by a hyperactive oral neutrophil phenotype. *J. Periodontol.* 82, 726–733. doi: 10.1902/jop.2010.100508

Abou Sulaiman, A. E., and Shehadeh, R. M. (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

Agnihotri, R., Pandurang, P., Kamath, S. U., Goyal, R., Ballal, S., Shanbhogue, A. Y., et al. (2009). Association of cigarette smoking with superoxide dismutase

enzyme levels in subjects with chronic periodontitis. *J. Periodontol.* 80, 657–662. doi: 10.1902/jop.2009.080545

Ahmadi-Motamayel, F., Goodarzi, M. T., Jamshidi, Z., and Kebriaei, R. (2017). Evaluation of salivary and serum antioxidant and oxidative stress statuses in patients with chronic periodontitis: a case-control study. *Front. Physiol.* 8:189. doi: 10.3389/fphys.2017.00189

Akalin, F. A., Baltacioglu, E., Alver, A., and 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. doi: 10.1111/j.1600-051X.2007.01091.x

Akalin, F. A., Baltacioglu, E., Alver, A., and 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, 457–467. doi: 10.1902/jop.2009.080218

Akalin, F. A., Isiksal, E., Baltacioglu, E., Renda, N., and Karabulut, E. (2008). Superoxide dismutase activity in gingiva in type-2 diabetes mellitus patients with chronic periodontitis. *Arch. Oral Biol.* 53, 44–52. doi: 10.1016/j.archoralbio.2007.07.009

Akpinar, A., Toker, H., Ozdemir, H., Bostanci, V., and 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. doi: 10.1016/j.archoralbio.2012.11.009

Alagl, A. S., and Bhat, S. G. (2015). Ascorbic acid: new role of an age-old micronutrient in the management of periodontal disease in older adults. *Geriatr. Gerontol. Int.* 15, 241–254. doi: 10.1111/ggi.12408

Almerich-Silla, J. M., Montiel-Company, J. M., Pastor, S., Serrano, F., Puig-Silla, M., and Dasi, F. (2015). Oxidative stress parameters in saliva and its association with periodontal disease and types of bacteria. *Dis. Mark.* 2015:653537. doi: 10.1155/2015/653537

Arora, N., Avula, H., and Avula, J. K. (2013). The adjunctive use of systemic antioxidant therapy (lycopene) in nonsurgical treatment of chronic periodontitis: a short-term evaluation. *Quintessence Int.* 44, 395–405. doi: 10.3290/j.qi.a29188

Bakhtiari, S., Azimi, S., Mehdipour, M., Amini, S., Elmi, Z., and Namazi, Z. (2015). Effect of cigarette smoke on salivary total antioxidant capacity. *J. Dent. Res. Dent. Clin. Dent. Prospects* 9, 281–284. doi: 10.15171/joddd.2015.049

Balci Yuce, H., Toker, H., and Goze, F. (2014). The histopathological and morphometric investigation of the effects of systemically administered boric acid on alveolar bone loss in ligature-induced periodontitis in diabetic rats. *Acta Odontol. Scand.* 72, 729–736. doi: 10.3109/00016357.2014.898789

Baltacioglu, E., Akalin, F. A., Alver, A., Deger, O., and Karabulut, E. (2008). Protein carbonyl levels in serum and gingival crevicular fluid in patients with chronic periodontitis. *Arch. Oral Biol.* 53, 716–722. doi: 10.1016/j.archoralbio.2008.02.002

Baltacioglu, E., Kehribar, M. A., Yuva, P., Alver, A., Atagun, O. S., Karabulut, E., et al. (2014a). Total oxidant status and bone resorption biomarkers in serum and gingival crevicular fluid of patients with periodontitis. *J. Periodontol.* 85, 317–326. doi: 10.1902/jop.2013.130012

Baltacioglu, E., Yuva, P., Aydin, G., Alver, A., Kahraman, C., Karabulut, E., et al. (2014b). Lipid peroxidation levels and total oxidant/antioxidant status in serum and saliva from patients with chronic and aggressive periodontitis. Oxidative stress index: a new biomarker for periodontal disease? *J. Periodontol.* 85, 1432–1441. doi: 10.1902/jop.2014.130654

Banasova, L., Kamodyova, N., Jansakova, K., Tothova, L., Stanko, P., Turna, J., et al. (2015). Salivary DNA and markers of oxidative stress in patients with chronic periodontitis. *Clin. Oral. Investig.* 19, 201–207. doi: 10.1007/s00784-014-1236-z

Bartold, P. M., and Van Dyke, T. E. (2013). Periodontitis: a host-mediated disruption of microbial homeostasis. Unlearning learned concepts. *Periodontol* 62, 203–217. doi: 10.1111/j.1600-0757.2012.00450.x

Baser, U., Gamsiz-Isik, H., Cifcibasi, E., Ademoglu, E., and Yalcin, F. (2015). Plasma and salivary total antioxidant capacity in healthy controls compared with aggressive and chronic periodontitis patients. *Saudi Med. J.* 36, 856–861. doi: 10.15537/smj.2015.7.11954

Bostanci, V., Toker, H., Senel, S., Ozdemir, H., and Aydin, H. (2014). Effect of chronic periodontitis on serum and gingival crevicular fluid oxidant and antioxidant status in patients with familial Mediterranean fever before and after periodontal treatment. *J. Periodontol.* 85, 706–712. doi: 10.1902/jop.2013.130230

Brock, G. R., Butterworth, C. J., Matthews, J. B., and Chapple, I. L. (2004). Local and systemic total antioxidant capacity in periodontitis and health. *J. Clin. Periodontol.* 31, 515–521. doi: 10.1111/j.1600-051X.2004.00509.x

Buduneli, N., Kardesler, L., Isik, H., Willis, C. S. III., Hawkins, S. I., Kinane, D. F., et al. (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

Bullon, P., Cordero, M. D., Quiles, J. L., Morillo, J. M., del Carmen Ramirez-Tortosa, M., and 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

Canakci, C. F., Cicek, Y., Yildirim, A., Sezer, U., and 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.

Celec, P., Hodosy, J., Celecova, V., Vodrazka, J., Cervenka, T., Halcak, L., et al. (2005). Salivary thiobarbituric acid reacting substances and malondialdehyde–their relationship to reported smoking and to parodontal status described by the papillary bleeding index. *Dis. Markers* 21, 133–137. doi: 10.1155/2005/693437

Celecova, V., Kamodyova, N., Tothova, L., Kudela, M., and Celec, P. (2013). Salivary markers of oxidative stress are related to age and oral health in adult non-smokers. *J. Oral Pathol. Med.* 42, 263–266. doi: 10.1111/jop.12008

Chang, M. C., Tsai, Y. L., Chen, Y. W., Chan, C. P., Huang, C. F., Lan, W. C., et al. (2013). Butyrate induces reactive oxygen species production and affects cell cycle progression in human gingival fibroblasts. *J. Periodont. Res.* 48, 66–73. doi: 10.1111/j.1600-0765.2012.01504.x

Chapple, I. L., Brock, G. R., Milward, M. R., Ling, N., and Matthews, J. B. (2007a). Compromised GCF total antioxidant capacity in periodontitis: cause or effect? *J. Clin. Periodontol.* 34, 103–110. doi: 10.1111/j.1600-051X.2006.01029.x

Chapple, I. L., Mason, G. I., Garner, I., Matthews, J. B., Thorpe, G. H., Maxwell, S. R., et al. (1997). Enhanced chemiluminescent assay for measuring the total antioxidant capacity of serum, saliva and crevicular fluid. *Ann. Clin. Biochem.* 34 (Pt 4), 412–421. doi: 10.1177/000456329703400413

Chapple, I. L., and Matthews, J. B. (2007). The role of reactive oxygen and antioxidant species in periodontal tissue destruction. *Periodontol* 43, 160–232. doi: 10.1111/j.1600-0757.2006.00178.x

Chapple, I. L., Milward, M. R., and Dietrich, T. (2007b). The prevalence of inflammatory periodontitis is negatively associated with serum antioxidant concentrations. *J. Nutr.* 137, 657–664.

D'Aiuto, F., Nibali, L., Parkar, M., Patel, K., Suvan, J., and Donos, N. (2010). Oxidative stress, systemic inflammation, and severe periodontitis. *J. Dent. Res.* 89, 1241–1246. doi: 10.1177/0022034510375830

Dede, F. O., Ozden, F. O., and Avci, B. (2013). 8-hydroxy-deoxyguanosine levels in gingival crevicular fluid and saliva in patients with chronic periodontitis after initial periodontal treatment. *J. Periodontol.* 84, 821–828. doi: 10.1902/jop.2012.120195

Dimou, N. L., Nikolopoulos, G. K., Hamodrakas, S. J., and Bagos, P. G. (2010). Fcgamma receptor polymorphisms and their association with periodontal disease: a meta-analysis. *J. Clin. Periodontol.* 37, 255–265. doi: 10.1111/j.1600-051X.2009.01530.x

Duarte, P. M., Napimoga, M. H., Fagnani, E. C., Santos, V. R., Bastos, M. F., Ribeiro, F. V., et al. (2012). The expression of antioxidant enzymes in the gingivae of type 2 diabetics with chronic periodontitis. *Arch. Oral Biol.* 57, 161–168. doi: 10.1016/j.archoralbio.2011.08.007

Dursun, E., Akalin, F. A., Genc, T., Cinar, N., Erel, O., and Yildiz, B. O. (2016). Oxidative stress and periodontal disease in obesity. *Medicine (Baltimore)* 95, e3136. doi: 10.1097/MD.0000000000003136

Ellis, S. D., Tucci, M. A., Serio, F. G., and Johnson, R. B. (1998). Factors for progression of periodontal diseases. *J. Oral Pathol. Med.* 27, 101–105. doi: 10.1111/j.1600-0714.1998.tb01923.x

Erel, O. (2005). A new automated colorimetric method for measuring total oxidant status. *Clin. Biochem.* 38, 1103–1111. doi: 10.1016/j.clinbiochem.2005.08.008

Esen, C., Alkan, B. A., Kirnap, M., Akgul, O., Isikoglu, S., and 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. doi: 10.1902/jop.2011.110420

Fentoglu, O., Kirzioglu, F. Y., Bulut, M. T., Kumbul Doguc, D., Kulac, E., Onder, C., et al. (2015). Evaluation of lipid peroxidation and oxidative DNA damage in patients with periodontitis and hyperlipidemia. *J. Periodontol.* 86, 682–688. doi: 10.1902/jop.2015.140561

Fredriksson, M., Gustafsson, A., Asman, B., and Bergstrom, K. (1998). Hyper-reactive peripheral neutrophils in adult periodontitis: generation of chemiluminescence and intracellular hydrogen peroxide after *in vitro* priming and FcgammaR-stimulation. *J. Clin. Periodontol.* 25, 394–398. doi: 10.1111/j.1600-051X.1998.tb02461.x

Fredriksson, M. I., Gustafsson, A. K., Bergstrom, K. G., and Asman, B. E. (2003). Constitutionally hyperreactive neutrophils in periodontitis. *J. Periodontol.* 74, 219–224. doi: 10.1902/jop.2003.74.2.219

Frijhoff, J., Winyard, P. G., Zarkovic, N., Davies, S. S., Stocker, R., Cheng, D., et al. (2015). Clinical relevance of biomarkers of oxidative stress. *Antioxid. Redox Signal.* 23, 1144–1170. doi: 10.1089/ars.2015.6317

Garg, N., Singh, R., Dixit, J., Jain, A., and Tewari, V. (2006). Levels of lipid peroxides and antioxidants in smokers and nonsmokers. *J. Periodont. Res.* 41, 405–410. doi: 10.1111/j.1600-0765.2006.00889.x

Ghallab, N. A., Hamdy, E., and Shaker, O. G. (2016). Malondialdehyde, superoxide dismutase and melatonin levels in GCF of aggressive and chronic periodontitis patients. *Aust Dent J.* 61, 53–61. doi: 10.1111/adj.12294

Giannopoulou, C., Krause, K. H., and Muller, F. (2008). The NADPH oxidase NOX2 plays a role in periodontal pathologies. *Semin. Immunopathol.* 30, 273–278. doi: 10.1007/s00281-008-0128-1

Golz, L., Memmert, S., Rath-Deschner, B., Jager, A., Appel, T., Baumgarten, G., et al. (2014). LPS from *P.* gingivalis and hypoxia increases oxidative stress in periodontal ligament fibroblasts and contributes to periodontitis. *Med. Inflamm.* 2014:986264. doi: 10.1155/2014/986264

Guentsch, A., Preshaw, P. M., Bremer-Streck, S., Klinger, G., Glockmann, E., and Sigusch, B. W. (2008). Lipid peroxidation and antioxidant activity in saliva of periodontitis patients: effect of smoking and periodontal treatment. *Clin. Oral Investig.* 12, 345–352. doi: 10.1007/s00784-008-0202-z

Gumus, P., Emingil, G., Ozturk, V. O., Belibasakis, G. N., and Bostanci, N. (2015). Oxidative stress markers in saliva and periodontal disease status: modulation during pregnancy and postpartum. *BMC Infect. Dis.* 15:261. doi: 10.1186/s12879-015-1003-z

Gustafsson, A., and Asman, B. (1996). Increased release of free oxygen radicals from peripheral neutrophils in adult periodontitis after Fc delta-receptor stimulation. *J. Clin. Periodontol.* 23, 38–44. doi: 10.1111/j.1600-051X.1996.tb00502.x

Gustafsson, A., Ito, H., Asman, B., and Bergstrom, K. (2006). Hyper-reactive mononuclear cells and neutrophils in chronic periodontitis. *J. Clin. Periodontol.* 33, 126–129. doi: 10.1111/j.1600-051X.2005.00883.x

Halliwell, B. (2000). Why and how should we measure oxidative DNA damage in nutritional studies? How far have we come? *Am. J. Clin. Nutr.* 72, 1082–1087.

Halliwell, B., and 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. doi: 10.1038/sj.bjp.0705776

Hendek, M. K., Erdemir, E. O., Kisa, U., and Ozcan, G. (2015). Effect of initial periodontal therapy on oxidative stress markers in gingival crevicular fluid, saliva, and serum in smokers and non-smokers with chronic periodontitis. *J. Periodontol.* 86, 273–282. doi: 10.1902/jop.2014.140338

Kose, O., Arabaci, T., Kara, A., Yemenoglu, H., Kermen, E., Kizildag, A., et al. (2016). Effects of melatonin on oxidative stress index and alveolar bone loss in diabetic rats with periodontitis. *J. Periodontol.* 87, e82–e90. doi: 10.1902/jop.2016.150541

Kurgan, S., Onder, C., Altingoz, S. M., Bagis, N., Uyanik, M., Serdar, M. A., et al. (2015). High sensitivity detection of salivary 8-hydroxy deoxyguanosine levels in patients with chronic periodontitis. *J. Periodont. Res.* 50, 766–774. doi: 10.1111/jre.12263

Ling, M. R., Chapple, I. L., Creese, A. J., and Matthews, J. B. (2014). Effects of C-reactive protein on the neutrophil respiratory burst *in vitro*. *Innate Immun.* 20, 339–349. doi: 10.1177/1753425913493199

Ling, M. R., Chapple, I. L., and Matthews, J. B. (2016). Neutrophil superoxide release and plasma C-reactive protein levels pre- and post-periodontal therapy. *J. Clin. Periodontol.* 43, 652–658. doi: 10.1111/jcpe.12575

Liu, Z., Liu, Y., Song, Y., Zhang, X., Wang, S., and Wang, Z. (2014). Systemic oxidative stress biomarkers in chronic periodontitis: a meta-analysis. *Dis. Markers* 2014:931083. doi: 10.1155/2014/931083

Lushchak, V. I. (2014). Free radicals, reactive oxygen species, oxidative stress and its classification. *Chem. Biol. Interact.* 224, 164–175. doi: 10.1016/j.cbi.2014.10.016

Matthews, J. B., Wright, H. J., Roberts, A., Cooper, P. R., and Chapple, I. L. (2007a). Hyperactivity and reactivity of peripheral blood neutrophils in chronic periodontitis. *Clin. Exp. Immunol.* 147, 255–264. doi: 10.1111/j.1365-2249.2006.03276.x

Matthews, J. B., Wright, H. J., Roberts, A., Ling-Mountford, N., Cooper, P. R., and Chapple, I. L. (2007b). Neutrophil hyper-responsiveness in periodontitis. *J. Dent. Res.* 86, 718–722. doi: 10.1177/154405910708600806

Maxwell, S. R., Dietrich, T., and Chapple, I. L. (2006). Prediction of serum total antioxidant activity from the concentration of individual serum antioxidants. *Clin. Chim. Acta* 372, 188–194. doi: 10.1016/j.cca.2006.04.015

Miricescu, D., Totan, A., Calenic, B., Mocanu, B., Didilescu, A., Mohora, M., et al. (2014). Salivary biomarkers: relationship between oxidative stress and alveolar bone loss in chronic periodontitis. *Acta Odontol. Scand.* 72, 42–47. doi: 10.3109/00016357.2013.795659

Mittal, M., Siddiqui, M. R., Tran, K., Reddy, S. P., and Malik, A. B. (2014). Reactive oxygen species in inflammation and tissue injury. *Antioxid. Redox Sig.* 20, 1126–1167. doi: 10.1089/ars.2012.5149

Miyasaki, K. T. (1991). The neutrophil: mechanisms of controlling periodontal bacteria. *J. Periodontol.* 62, 761–774. doi: 10.1902/jop.1991.62.12.761

Muniz, F. W., Nogueira, S. B., Mendes, F. L., Rosing, C. K., Moreira, M. M., de Andrade, G. M., et al. (2015). The impact of antioxidant agents complimentary to periodontal therapy on oxidative stress and periodontal outcomes: a systematic review. *Arch. Oral Biol.* 60, 1203–1214. doi: 10.1016/j.archoralbio.2015.05.007

Nazir, M. A. (2017). Prevalence of periodontal disease, its association with systemic diseases and prevention. *Int. J. Health Sci. (Qassim).* 11, 72–80.

Nguyen, T. T., Ngo, L. Q., Promsudthi, A., and Surarit, R. (2016). Salivary lipid peroxidation in patients with generalized chronic periodontitis and acute coronary syndrome. *J. Periodontol.* 87, 134–141. doi: 10.1902/jop.2015.150353

Nguyen, T. T., Ngo, L. Q., Promsudthi, A., and Surarit, R. (2017). Salivary oxidative stress biomarkers in chronic periodontitis and acute coronary syndrome. *Clin. Oral. Investig.* 21, 2345–2353. doi: 10.1007/s00784-016-2029-3

Nibali, L., Parkar, M., Brett, P., Knight, J., Tonetti, M. S., and Griffiths, G. S. (2006). NADPH oxidase (CYBA) and FcgammaR polymorphisms as risk factors for aggressive periodontitis: a case-control association study. *J. Clin. Periodontol.* 33, 529–539. doi: 10.1111/j.1600-051X.2006.00952.x

Novakovic, N., Todorovic, T., Rakic, M., Milinkovic, I., Dozic, I., Jankovic, S., et al. (2014). Salivary antioxidants as periodontal biomarkers in evaluation of tissue status and treatment outcome. *J. Periodont. Res.* 49, 129–136. doi: 10.1111/jre.12088

Onder, C., Kurgan, S., Altingoz, S. M., Bagis, N., Uyanik, M., Serdar, M. A., et al. (2017). Impact of non-surgical periodontal therapy on saliva and serum levels of markers of oxidative stress. *Clin. Oral. Investig.* 21, 1961–1969. doi: 10.1007/s00784-016-1984-z

Ongoz Dede, F., Bozkurt Dogan, S., Balli, U., Avci, B., and Durmuslar, M. C. (2016). The effect of initial periodontal treatment on plasma, gingival crevicular fluid and salivary levels of 8-hydroxy-deoxyguanosine in obesity. *Arch. Oral Biol.* 62, 80–85. doi: 10.1016/j.archoralbio.2015.11.014

Panjamurthy, K., Manoharan, S., and Ramachandran, C. R. (2005). Lipid peroxidation and antioxidant status in patients with periodontitis. *Cell. Mol. Biol. Lett.* 10, 255–264.

Pendyala, G., Thomas, B., and Joshi, S. (2013a). Periodontitis, diabetes mellitus, and the lopsided redox balance: a unifying axis. *J. Indian Soc. Periodontol.* 17, 338–344. doi: 10.4103/0972-124X.115661

Pendyala, G., Thomas, B., and Joshi, S. R. (2013b). 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, 51–57. doi: 10.4103/1947-2714.106208

Petersen, D. R., and Doorn, J. A. (2004). Reactions of 4-hydroxynonenal with proteins and cellular targets. *Free Radic. Biol. Med.* 37, 937–945. doi: 10.1016/j.freeradbiomed.2004.06.012

Pradeep, A. R., Agarwal, E., Bajaj, P., and Rao, N. S. (2013a). 4-Hydroxy-2-nonenal, an oxidative stress marker in crevicular fluid and serum in type 2 diabetes with chronic periodontitis. *Contemp. Clin. Dent.* 4, 281–285. doi: 10.4103/0976-237X.118342

Pradeep, A. R., Ramchandraprasad, M. V., Bajaj, P., Rao, N. S., and Agarwal, E. (2013b). Protein carbonyl: an oxidative stress marker in gingival crevicular fluid in healthy, gingivitis, and chronic periodontitis subjects. *Contemp. Clin. Dent.* 4, 27–31. doi: 10.4103/0976-237X.111589

Pradeep, A. R., Rao, N. S., Bajaj, P., and Agarwal, E. (2013c). 8-Isoprostane: a lipid peroxidation product in gingival crevicular fluid in healthy, gingivitis and chronic periodontitis subjects. *Arch. Oral Biol.* 58, 500–504. doi: 10.1016/j.archoralbio.2013.01.011

Richards, D. (2014). Review finds that severe periodontitis affects 11% of the world population. *Evid. Based Dent.* 15, 70–71. doi: 10.1038/sj.ebd.6401037

Roberts, L. J. II., and Morrow, J. D. (2002). Products of the isoprostane pathway: unique bioactive compounds and markers of lipid peroxidation. *Cell. Mol. Life Sci.* 59, 808–820. doi: 10.1007/s00018-002-8469-8

Saglam, M., Koseoglu, S., Hatipoglu, M., Esen, H. H., and Koksal, E. (2015). Effect of sumac extract on serum oxidative status, RANKL/OPG system and alveolar bone loss in experimental periodontitis in rats. *J. Appl. Oral Sci.* 23, 33–41. doi: 10.1590/1678-775720140288

Sawamoto, Y., Sugano, N., Tanaka, H., and Ito, K. (2005). Detection of periodontopathic bacteria and an oxidative stress marker in saliva from periodontitis patients. *Oral Microbiol. Immunol.* 20, 216–220. doi: 10.1111/j.1399-302X.2005.00215.x

Sculley, D. V., and Langley-Evans, S. C. (2003). Periodontal disease is associated with lower antioxidant capacity in whole saliva and evidence of increased protein oxidation. *Clin. Sci.* 105, 167–172. doi: 10.1042/CS20030031

Sezer, U., Cicek, Y., and Canakci, C. F. (2012). Increased salivary levels of 8-hydroxydeoxyguanosine may be a marker for disease activity for periodontitis. *Dis. Markers* 32, 165–172. doi: 10.1155/2012/215430

Sezer, U., Erciyas, K., Ustun, K., Pehlivan, Y., Senyurt, S. Z., Aksoy, N., et al. (2013). Effect of chronic periodontitis on oxidative status in patients with rheumatoid arthritis. *J. Periodontol.* 84, 785–792. doi: 10.1902/jop.2012.120179

Shacter, E. (2000). Quantification and significance of protein oxidation in biological samples. *Drug Metab. Rev.* 32, 307–326. doi: 10.1081/DMR-100102336

Shin, M. S., Shin, H. S., Ahn, Y. B., and Kim, H. D. (2016). Association between periodontitis and salivary 8-hydroxydeoxyguanosine among Korean rural adults. *Commun. Dent. Oral Epidemiol.* 44, 381–389. doi: 10.1111/cdoe.12225

Sies, H. (1997). Oxidative stress: oxidants and antioxidants. *Exp. Physiol.* 82, 291–295. doi: 10.1113/expphysiol.1997.sp004024

Singh, N., Chander Narula, S., Kumar Sharma, R., Tewari, S., and Kumar Sehgal, P. (2014). Vitamin E supplementation, superoxide dismutase status, and outcome of scaling and root planing in patients with chronic periodontitis: a randomized clinical trial. *J. Periodontol.* 85, 242–249. doi: 10.1902/jop.2013.120727

Sree, S. L., and Sethupathy, S. (2014). Evaluation of the efficacy of taurine as an antioxidant in the management of patients with chronic periodontitis. *Dent. Res. J. (Isfahan).* 11, 228–233.

Su, H., Gornitsky, M., Velly, A. M., Yu, H., Benarroch, M., and Schipper, H. M. (2009). Salivary DNA, lipid, and protein oxidation in nonsmokers with periodontal disease. *Free Radic. Biol. Med.* 46, 914–921. doi: 10.1016/j.freeradbiomed.2009.01.008

Sugano, N., Yokoyama, K., Oshikawa, M., Kumagai, K., Takane, M., Tanaka, H., et al. (2003). Detection of Streptococcus anginosus and 8-hydroxydeoxyguanosine in saliva. *J. Oral Sci.* 45, 181–184. doi: 10.2334/josnusd.45.181

Takane, M., Sugano, N., Iwasaki, H., Iwano, Y., Shimizu, N., and 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. doi: 10.1902/jop.2002.73.5.551

Thomas, B., Rao, A., Prasad, B. R., and Kumari, S. (2014). Serum levels of antioxidants and superoxide dismutase in periodontitis patients with diabetes type 2. *J. Indian Soc. Periodontol.* 18, 451–455. doi: 10.4103/0972-124X.138686

Tonguc, M. O., Ozturk, O., Sutcu, R., Ceyhan, B. M., Kilinc, G., Sonmez, Y., et al. (2011). The impact of smoking status on antioxidant enzyme activity and malondialdehyde levels in chronic periodontitis. *J. Periodontol.* 82, 1320–1328. doi: 10.1902/jop.2011.100618

Tothova, L., Celecova, V., and Celec, P. (2013). Salivary markers of oxidative stress and their relation to periodontal and dental status in children. *Dis. Markers* 34, 9–15. doi: 10.1155/2013/591765

Trivedi, S., Lal, N., Mahdi, A. A., Mittal, M., Singh, B., and Pandey, S. (2014). Evaluation of antioxidant enzymes activity and malondialdehyde levels in patients with chronic periodontitis and diabetes mellitus. *J. Periodontol.* 85, 713–720. doi: 10.1902/jop.2013.130066

Trivedi, S., Lal, N., Mahdi, A. A., Singh, B., and Pandey, S. (2015). Association of salivary lipid peroxidation levels, antioxidant enzymes, and chronic periodontitis. *Int. J. Period. Restorat. Dent.* 35, e14–e19. doi: 10.11607/prd.2079

Tsai, C. C., Chen, H. S., Chen, S. L., Ho, Y. P., Ho, K. Y., Wu, Y. M., et al. (2005). Lipid peroxidation: a possible role in the induction and progression of chronic periodontitis. *J. Periodont. Res.* 40, 378–384. doi: 10.1111/j.1600-0765.2005.00818.x

Wei, D., Zhang, X. L., Wang, Y. Z., Yang, C. X., and 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. doi: 10.1111/j.1834-7819.2009.01123.x

White, P., Cooper, P., Milward, M., and Chapple, I. (2014). Differential activation of neutrophil extracellular traps by specific periodontal bacteria. *Free Radic. Biol. Med.* 75 (Suppl. 1):S53. doi: 10.1016/j.freeradbiomed.2014.10.827

Witko-Sarsat, V., Friedlander, M., Capeillere-Blandin, C., Nguyen-Khoa, T., Nguyen, A. T., Zingraff, J., et al. (1996). Advanced oxidation protein products as a novel marker of oxidative stress in uremia. *Kidney Int.* 49, 1304–1313. doi: 10.1038/ki.1996.186

Wolfram, R. M., Budinsky, A. C., Eder, A., Presenhuber, C., Nell, A., Sperr, W., et al. (2006). Salivary isoprostanes indicate increased oxidation injury in periodontitis with additional tobacco abuse. *Biofactors* 28, 21–31. doi: 10.1002/biof.5520280103

Wright, H. J., Matthews, J. B., Chapple, I. L. C., Ling-Mountford, N., and Cooper, P. R. (2008). Periodontitis associates with a type 1 IFN signature in peripheral blood neutrophils. *J. Immunol.* 181, 5775–5784. doi: 10.4049/jimmunol.181.8.5775

Yagan, A., Kesim, S., and Liman, N. (2014). Effect of low-dose doxycycline on serum oxidative status, gingival antioxidant levels, and alveolar bone loss in experimental periodontitis in rats. *J. Periodontol.* 85, 478–489. doi: 10.1902/jop.2013.130138

Yagi, K. (1976). A simple fluorometric assay for lipoperoxide in blood plasma. *Biochem. Med.* 15, 212–216. doi: 10.1016/0006-2944(76)90049-1

Yang, X., Li, C., and Pan, Y. (2016). The influences of periodontal status and periodontal pathogen quantity on salivary 8-hydroxydeoxyguanosine and interleukin-17 levels. *J. Periodontol.* 87, 591–600. doi: 10.1902/jop.2015.150390

Zamora-Perez, A. L., Ortiz-Garcia, Y. M., Lazalde-Ramos, B. P., Guerrero-Velazquez, C., Gomez-Meda, B. C., Ramirez-Aguilar, M. A., et al. (2015). Increased micronuclei and nuclear abnormalities in buccal mucosa and oxidative damage in saliva from patients with chronic and aggressive periodontal diseases. *J. Periodont. Res.* 50, 28–36. doi: 10.1111/jre.12175

Zhang, T., Andrukhov, O., Haririan, H., Muller-Kern, M., Liu, S., Liu, Z., et al. (2016). Total antioxidant capacity and total oxidant status in saliva of periodontitis patients in relation to bacterial load. *Front. Cell. Infect. Microbiol.* 5:97. doi: 10.3389/fcimb.2015.00097

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MINI REVIEW
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Oxidative Stress in the Local and Systemic Events of Apical Periodontitis

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Oxidative stress is involved in the pathogenesis of a variety of inflammatory disorders. Apical periodontitis (AP) usually results in the formation of an osteolytic apical lesion (AL) caused by the immune response to endodontic infection. Reactive oxygen species (ROS) produced by phagocytic cells in response to bacterial challenge represent an important host defense mechanism, but disturbed redox balance results in tissue injury. This mini review focuses on the role of oxidative stress in the local and associated systemic events in chronic apical periodontitis. During endodontic infection, ligation of Toll-like receptors (TLRs) on phagocytes' surface triggers activation, phagocytosis, synthesis of ROS, activation of humoral and cellular responses, and production of inflammatory mediators, such as, cytokines and matrix metalloproteinases (MMPs). The increment in ROS perturbs the normal redox balance and shifts cells into a state of oxidative stress. ROS induce molecular damage and disturbed redox signaling, that result in the loss of bone homeostasis, increased pro-inflammatory mediators, and MMP overexpression and activation, leading to apical tissue breakdown. On the other hand, oxidative stress has been strongly involved in the pathogenesis of atherosclerosis, where a chronic inflammatory process develops in the arterial wall. Chronic AP is associated with an increased risk of cardiovascular diseases (CVD) and especially atherogenesis. The potential mechanisms linking these diseases are also discussed.

Keywords: apical periodontitis, apical lesion, ROS, oxidative stress, atherosclerosis

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INTRODUCTION

Prevalent chronic inflammatory diseases that affect periodontal tissues include apical periodontitis (AP) and chronic marginal periodontitis (CP) (Gamonal et al., 2010; Graves et al., 2011). Both share similar etiological factors and pathogenesis—often Gram-negative anaerobic bacteria—and elicitation of a chronic immune-inflammatory host response. AP is initiated by endodontic infection and CP, by subgingival microbiota, both leading to periodontal tissue breakdown (Cotti et al., 2011a). In this review, we will focus on the role of oxidative stress in the local and associated systemic events in chronic apical periodontitis.

Apical periodontitis usually results from pulpal infection caused by anaerobic bacteria inside the root canal of the teeth where they organize in biofilms. The endodontic offenders and their products trigger the immune-inflammatory response. The host attempts to localize the infection and prevent further dissemination at the expense of apical periodontal tissue breakdown, involving periodontal ligament, radicular cementum, and alveolar bone. During the chronic phase a bone resorptive lesion results evident as an apical radiolucent area in a radiograph. Histologically, apical lesions (AL) consist of granulation tissue (apical granuloma) which can progress to form a radicular cyst, a pathological cavity lined by squamous epithelium. Both lesions seem to represent different stages from the same process (Nair, 2004).

Generation of reactive oxygen species (ROS) is an integral feature of normal cellular metabolism and takes part in cell signaling and metabolic processes, affecting cellular functions, which include gene expression, proliferation, cell death, migration, and inflammation (Xiang and Fan, 2010). Various ROS-producing catalytic pathways mediated by enzymes are differentially localized inside the cells, including NO synthases (NOS), enzymes of the respiratory chain, cytochrome P450 monoxygenases, xanthine oxidase, and NADPH oxidase (Shackelford et al., 2000). ROS include oxygen-derived free radicals and non-radicals. The former correspond to species that contain one or more unpaired electrons and are generally reactive with other species. They classically include superoxide (O_2^-) and hydroxyl anion ($^\bullet OH$). Non-radical species are oxidizing agents or are easily converted into radicals, or both, such as, hydrogen peroxide (H_2O_2), among others (Chapple, 1997; Trivedi and Lal, 2017). Additionally, other reactive species derived from nitrogen and chlorine can be of importance in disease, such as, nitric oxide ($NO^\bullet$) and hypochlorous acid (HOCl), among others (Biswas, 2016).

Antioxidants antagonize the effects of free radicals, and can be defined as "those substances which when present at low concentrations, compared to those of an oxidizable substrate, will significantly delay or inhibit oxidation of that substrate" (Halliwell and Gutteridge, 2015). Antioxidant mechanisms involve both, enzymatic and non-enzymatic reactions. Primary enzymes are superoxide dismutase (SOD), catalase (CAT), and thiol-dependent peroxidases, namely glutathione peroxidase (GSH-PX) and peroxiredoxins. In general, non-enzymatic antioxidants include either metabolic antioxidants, such as, thiol antioxidants, coenzyme Q10, uric acid, or bilirubin; or substances obtained exogenously from nutrients, which include both water and fat-soluble vitamins, polyphenolic compounds, and trace elements (carotenoids, ascorbic acid, tocopherols, polyphenols, folic acid, and cysteine) (Chapple and Matthews, 2007; Carocho and Ferreira, 2013; Flohe, 2016).

In general terms, ROS and antioxidant mechanisms interact in balance to maintain normal physiologic processes. Oxidative stress is "a disturbance in the pro-oxidant-antioxidant balance in favor of the former, leading to a disruption of redox signaling and/or molecular damage" (Halliwell and Whiteman, 2004). Oxidative stress induces local periapical tissue injury and also contributes to systemic diseases, including atherosclerosis, arthritis, and cancer (Akalin et al., 2007, 2008). Current epidemiologic evidence sustains that AL associate with increased risk of cardiovascular diseases (CVD) and especially atherogenesis in young adults, but the mechanisms are yet unclear (Paraskevas et al., 2008).

LOCAL EFFECTS OF OXIDATIVE STRESS IN APICAL PERIODONTITIS

During endodontic infection, ligation of Toll-like receptors (TLRs) on phagocytes' surface by bacterial motifs or dying cells (Chapple, 1997) triggers activation, phagocytosis, synthesis of ROS, activation of humoral and cellular responses, and production of inflammatory mediators, such as, cytokines and matrix metalloproteinases (MMPs) (Dezerega et al., 2012; Sima and Glogauer, 2013; Holden et al., 2014). ROS constitute an important host defense mechanism against invading pathogens. Hence, the combination of bacterial phagocytosis and secretion of proteolytic enzymes and immuno-modulatory compounds that assist in the killing and digestion of bacteria is accompanied by the "respiratory burst." The sudden increase in non-mitochondrial oxidative metabolism, results in the generation of superoxide radicals and a battery of other ROS, via the NADPH-oxidase complex (Babior, 1984). However, oxidants can also cause tissue injury through DNA and protein damage, involving enzymes and matrix constituents, lipid peroxidation, induction of pro-inflammatory cytokines, and hydrolytic enzymes, such as, MMPs, as well as inactivation of protease inhibitors (Chapple, 1997; Graves et al., 2011). Additionally, H_2O_2 overproduced extracellularly, can even pass through biologic membranes freely and act as intracellular second messengers, activating a variety of signal transduction pathways (Lamster and Novak, 1992; Mody et al., 2001; Canakci et al., 2005, 2006).

Given the close relation between inflammation and oxidative stress the role of ROS and antioxidant systems in the pathogenesis of periodontal tissue injury has regained attention in the last years. Malondialdehyde (MDA), a product of polyunsaturated fatty acid peroxidation, was reported to be significantly elevated and GSH-PX activity reduced in periapical granulomas compared to healthy gingival tissue, reflecting an oxidative imbalance (Marton et al., 1993). PMN obtained from apical granulomas showed increased production of hydrogen peroxide and superoxide anion, which tended to normalize after surgical treatment (Minczykowski et al., 2001). The imbalance between the generation and elimination of ROS in periapical lesions can also be assessed by the total oxidant status (TOS) and total antioxidant status (TAS) (Brock et al., 2004; Erel, 2005). In fact, TOS was reported to be significantly higher in ALs than in healthy periodontal ligament controls. Analysis of oral gingival crevicular fluid showed reduced TAS levels in asymptomatic AP teeth and were restored to normal levels after endodontic treatment (Dezerega et al., 2012). These reports evidence the existence of local oxidative stress in ALs, either at the expense of ROS increments and/or reduced antioxidant defense.

Bone homeostasis results from the balance between bone formation by osteoblasts and bone resorption by osteoclasts (Hofbauer and Heufelder, 2001; Crotti et al., 2003; Vernal et al., 2004; Hernandez et al., 2006; Kawai et al., 2006; Nagasawa et al., 2007; Gaffen and Hajishengallis, 2008; Ohyama et al., 2009). ROS suppress alveolar bone formation, by inhibiting osteoblastic differentiation and stimulate osteoclastogenesis (Mody et al., 2001; Jakovljevic et al., 2016). Direct exposure of periodontal ligament fibroblasts to hydrogen peroxide arrests cell viability, proliferation, and osteoblast differentiation. These effects seem to be mediated by the Wnt/β-catenin signaling and NFrf2 pathways (Kook et al., 2016). In contrast, osteoblastic differentiation accompanied by the induction of the transcription factors osterix and Runx2, was stimulated by continuous and low concentrations of hydrogen peroxide, whereas these effects were inhibited by CAT (Choe et al., 2012). These results suggest a dose-dependent dual role of ROS, and particularly of hydrogen peroxide, in bone formation.

Receptor activator of nuclear factor-kappa B ligand (RANKL) stimulates the differentiation, maturation, and survival of cells into osteoclasts from their monocyte-macrophage precursors, leading to bone loss. It has been established that RANKL is involved in ROS-mediated osteoclastogenesis. Superoxide and hydrogen peroxide-induced RANKL over expression with the participation of extracellular signal-regulated kinases (ERK) and nuclear factor erythroid-derived 2-related factor (Erf-2), among others, have been demonstrated in different human and mouse osteoblastic cell lines (Bai et al., 2005; Kanzaki et al., 2014). Experimentally-induced ALs in phagocyte oxidase (PHOX)—null mice resulted in the lack of identifiable tartrate resistant acid phosphatase (TRAP)—positive osteoclasts in ALs and healthy periodontal tissues, in contrast to wild type controls. Conversely, the same study demonstrated that inducible (i) NOS knockout mice overexpressed interleukin (IL)-β, tumor necrosis factor (TNF)-α, and RANKL and more severe ALs compared to PHOX null and wild-type mice (Silva et al., 2011). Similarly, targeting of p67*phox* or p22*phox* NADPH oxidase subunits through small interference RNA down regulated ROS generation and suppressed RANKL-stimulated differentiation of TRAP-positive cells in RAW264.7 cell lines (Sasaki et al., 2009). Altogether, these findings support that ROS might contribute to the development and/or progression of ALs by stimulating RANKL-mediated osteoclast differentiation and alveolar bone resorption, whereas iNOS seems to play a bone protective role.

Oxidative non-proteolytic MMP activation seems to be pivotal in periodontal inflammation. ROS are able to induce the activation of the key MMPs in periodontal tissues, such as, MMP-8 and MMP-9, through direct enzyme oxidation (Saari et al., 1990), although indirect mechanisms involving intracellular signaling cannot be precluded. MMP-8 and MMP-9 are both promising periodontal and apical disease biomarkers (Baeza et al., 2016), which cooperatively hydrolyze type I collagen, a key step in periodontal supporting tissue loss (Hernandez Rios et al., 2009; Hernandez et al., 2010). PMN-derived myeloperoxidase (MPO) catalyzes HOCl release and besides its antimicrobial effects, it has been reported to oxidatively activate latent proMMP-8 and -9 *in vitro* (Saari et al., 1990), and inactivate tissue inhibitor of metalloproteinase (TIMP)-1 (Wei et al., 2004; Hernandez et al., 2010; Marcaccini et al., 2010). *Ex vivo* studies suggest that oxidative activation of MMP-8 and MMP-9 represents the dominant mechanism in destructive periodontal lesions (Hernandez et al., 2010; Marcaccini et al., 2010). Additionally, our group demonstrated increased oxidative stress along with higher MMP-9 levels and activity in ALs (Dezerega et al., 2012) and gingival crevicular fluid (Belmar et al., 2008) from chronic AP teeth vs. healthy controls. Furthermore, a strong positive correlation was found between TOS, proMMP-9, and active MMP-9, suggesting that ROS might also be involved in MMP-9 synthesis and activation during progression of AP.

Experimental studies demonstrate that ROS-signaling is able to induce and/or activate MMPs and inflammatory mediators, particularly in periodontal tissues. MMP-2 and MMP-9 were activated by ROS in different cell systems (Yoon et al., 2002; Mori et al., 2004; Binker et al., 2011), including periodontal ligament fibroblasts exposed to non-toxic low concentrations of hydrogen peroxide (Cavalla et al., 2015; Osorio et al., 2015). In the same model, stromal-derived factor (SDF)-1/CXCL-12, IL-6, and vascular endothelial growth factor (VEGF) levels were enhanced by peroxide stimulation, effect that was modulated by MMPs (Cavalla et al., 2015). IL-1β and ROS were induced in *Aggregatibacter (A.) actinomycetencomitans*-infected RAW264 macrophages, whereas N-Acetyl-cysteine, a thiol-based antioxidant, prevented IL-1β production (Okinaga et al., 2015). Similarly, peroxide was also able to induce MAPK-mediated secretion of IL-8 in periodontal ligament fibroblasts, which was abolished in presence of high concentrations that associated to cell cytotoxicity (Lee et al., 2008). These results support that non-lethal concentrations of ROS enhance pro-inflammatory mediators and extracellular matrix enzymes contributing to destructive amplification loops in the apical tissues, leading to the development of an AL.

Periodontal ligament fibroblasts are key cells for periodontal soft and hard tissue homeostasis. Whereas concentrations higher than 10 μM of hydrogen peroxide are toxic for primary cultures of human periodontal ligament fibroblasts, lower concentrations ($\leq$5 μM) maintain cell viability, and morphology (Osorio et al., 2015), triggering CAT and SOD1 and two enzymatic anti-oxidant defense (Choe et al., 2012; Cavalla et al., 2015). Concomitantly, low concentrations of ROS can modify signal transduction pathways through the presence of redox-sensitive cysteines. It has been well established that NFκB transcription factor, among others, is redox sensitive (Yoon et al., 2002; Bai et al., 2005; Osorio et al., 2015). Accordingly, low peroxide stimulation in periodontal ligament fibroblasts induced intracellular calcium release along with NFκB activation. Moreover, NFκB activation was partly dependent on peroxide-induced calcium signals (Osorio et al., 2015). Overall, ROS can induce a plethora of signaling pathways and effects, depending on the cell target, concentration, and exposure patterns (Choe et al., 2012). The ROS-mediated mechanisms in apical tissue breakdown are summarized in **Figure 1**.

Molecular damage	Signaling
Protein oxidative modification: * ↑ MMP activation (MMP-8, -9). * ↓ Inhibition of proteinase inhibitors (TIMP-1). DNA, lipid and protein damage.	↓ Osteoblast differentiation. ↑ RANKL-mediated osteoclast differentiation and activation. ↑ MMP-2 and -9 expression and activity. ↑ Pro-inflammatory cytokine/ chemokine/ GF secretion (IL-8, CXCL12, IL-1β, IL-6, VEGF).

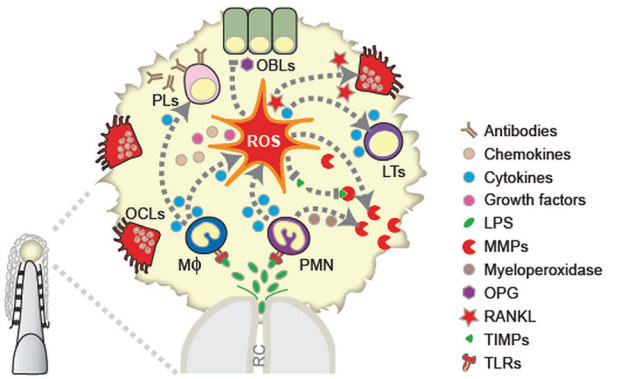

FIGURE 1 | ROS-mediated mechanisms in apical tissue breakdown. ROS induce apical tissue breakdown by causing molecular damage or disrupted cell signaling. Besides structural damage of cell and/or extracellular matrix components (DNA, protein, and lipids), the former mechanisms include the oxidative modification of matrix-degrading enzymes, such as, matrix metalloproteinase (MMP) and inhibition of their tissue inhibitors (TIMPs). Among the later mechanisms, ROS inhibit osteoblast differentiation, stimulate receptor activator nuclear factor κB (RANKL)-mediated osteoclast differentiation and activation, MMP expression and activity, and pro inflammatory response. OBLs, Osteoblats; LTs, lymphocytes; PMN, polymorphonuclear leukocytes; Mϕ, macrophages; OCls, osteoclasts; PLs, plasma cells.

OXIDATIVE STRESS IN AP-ASSOCIATED ATHEROGENESIS

The association between apical periodontitis and systemic diseases has regained the attention of researchers during the last years. Evidence sustains an epidemiologic link between chronic AP and CVD, such as, atherosclerosis (Petersen et al., 2014), coronary artery disease (Pasqualini et al., 2012; Liljestrand et al., 2016), and endothelial dysfunction (Cotti et al., 2011b) in an analogous fashion to marginal periodontal diseases, but the mechanisms involved are still unclear.

Although most mechanistic evidence still comes from chronic marginal periodontitis, few studies already support a role of ROS in systemic complications associated with AP. **Figure 2** shows an schematic representation linking oxidative stress, and oral infections and cardiovascular diseases. In fact, a hyper-reactive phagocyte phenotype characterized by higher ROS production was found in PMN from peripheral blood in chronic AP patients, in comparison to healthy controls, whereas superoxide levels significantly decreased after surgical removal of ALs (Minczykowski et al., 2001). A later study reported that chronic AP patients had higher levels of oxidants, measured as increased plasmatic reactive oxygen metabolites, and lower antioxidant potential compared to healthy individuals; while endodontic treatment tended to restore the systemic oxidative balance (Inchingolo et al., 2014). Accordingly, early endothelial dysfunction and overproduction of asymmetrical dimethylarginine, the endogenous inhibitor of NOS, were recently reported in serum from young women with chronic AP, compared to healthy volunteers (Cotti et al., 2011b).

Oxidative stress is strongly involved in the pathology of atherosclerosis, where a chronic inflammatory process develops in the arterial wall. In its early phases, the areas that are susceptible for lesion formation display diffuse intimal thickening that are sites for low density lipoprotein (LDL) particle retention. The retention predisposes LDL to oxidative modifications, especially during hyperlipidemia. On one hand, enzymes that can mediate the oxidation of LDL include lipoxygenase, MPO and peroxidase-like activity of hemoglobin (Tsimikas and Miller, 2011). On the other hand, free radicals that are generated in the presence of hydrogen peroxide, nitric oxide, and superoxide mediate the non-enzymatic oxidation of LDL (Tsimikas and Miller, 2011). Oxidized LDL (oxLDL) plays an essential role in atherogenesis as it represents a crucial pro-inflammatory stimulus and is recognized by various arms of the immune system (Matsuura et al., 2014). OxLDL is a ligand for cellular scavenger receptors, such as, CD36, and binding leads to accelerated LDL uptake by the arterial wall macrophages, foam cell formation, and generation of ROS, producing the vicious circle. It may also interact with the components of the complement cascade and C-reactive protein (CRP) forming proatherogenic oxLDL/CRP complexes (Miller et al., 2011). Oxidized structures also activate TLR further promoting inflammation in the atherosclerotic lesions (Miller et al., 2011). The signaling is essential for adaptive immune system to activate dendritic cells and macrophages, and subsequently T and B cells (Huang and Pope, 2010).

The concept of oxLDL refers to a wide range of reaction products in the particle: fatty acids, lipids, and apolipoprotein can be oxidized in various degrees (Jiang et al., 2011). OxLDL is immunogenic and induces a pro-inflammatory autoimmune response largely consisting of IgG1 and IgG3 subclasses in humans (Saad et al., 2006). The formed immunocomplexes promote phagocytosis by cells expressing Fcγ receptors (Schmidt and Gessner, 2005). They are also recognized by natural antibodies, mainly of IgM isotype, which are transcribed from the germ-line genes and do not require prior exposure to foreign antigens to be secreted (Baumgarth et al., 2005). They bind to oxidatively modified structures due to their specificity against highly conserved structures that are present on pathogen surfaces or endogenously generated by oxidative reactions. Collectively these structures are termed as pathogen-associated molecular patterns (PAMP) (Medzhitov and Janeway, 1997) and danger-associated molecular patterns (DAMP) (Matzinger, 2002), respectively.

Porphyromonas (P.) species, such as, *Porphyromonas endodontalis* and *Porphyromonas gingivalis,* are among the most commonly identified taxa in AP (Rocas and Siqueira, 2008,

2010; Siqueira et al., 2008; Ozbek and Ozbek, 2010). Sequence similarity or structural resemblance between self and non-self antigens leads to immune response called molecular mimicry (Cusick et al., 2012). This is also considered as a potential mechanism behind the association of periodontitis and CVD (Schenkein and Loos, 2013). Similar or closely related, conserved molecules among the self-antigens can be found in bacteria leading to production of autoantibodies (Leishman et al., 2012). Such antibodies described in periodontitis patients include anti-phosphorylcholine (Schenkein et al., 1999), anti-oxLDL (Monteiro et al., 2009), and anti-cardiolipin (Schenkein et al., 2003), suggesting that common epitopes between host and periodontal bacteria are diverse. Evidence supporting this was found recently, when natural IgM, specific for an oxLDL epitope on MDA-modified LDL (MDA-LDL) was shown to recognize antigens on *P. gingivalis* (Turunen et al., 2012). The epitope was identified as gingipain, one of the most important virulence factor and protease of the bacterium. To directly link this observation with atherosclerosis, immunization of mice with MDA-LDL was shown to reduce the aortic lipid deposition area after *P. gingivalis* challenge (Turunen et al., 2015).

In addition to oxLDL, other major molecules giving rise to molecular mimicry are members of the heat-shock protein (Hsp) families. Hsp are highly conserved stress molecules present both in humans and bacteria. The antibody response to them is implicated in atherosclerosis (Pockley et al., 2009) and marginal periodontitis (Sims et al., 2002; Buhlin et al., 2009). A natural IgM antibody binding to MDA-LDL but cross-reacting with *Aggregatibacter actinomycetemcomitans* HSP60 has recently been cloned and characterized (Wang et al., 2016), and *P. gingivalis* antibody levels were lately shown to correlate with each other and persist despite clinically successful periodontal treatment (Buhlin et al., 2015). After taking into account age, sex, smoking, and number of teeth, *A. actinomycetemcomitans* and *P. gingivalis IgG*, Hsp65-IgA, oxLDL-IgG, and -IgM antibody levels were directly associated, whereas Hsp60-IgG2 antibody levels were inversely associated with periodontitis. A recent study from our group also demonstrated an association between ALs, *P. endodontalis* and serum IgG-class antibodies against it. Importantly, a significant association between AL and risk of coronary artery disease was reported. The association was especially strong in subjects with untreated teeth with AL (Liljestrand et al., 2016). The role of *P. endodontalis* in atherogenesis is also supported experimentally, since it can directly invade endothelial and smooth muscle cells from human coronary artery and induce MMP expression *in vitro* (Dorn et al., 2002). Altogether, the immune responses in both chronic marginal periodontitis and AP are complex, including both disease- and health-associated antibodies.

Regarding antioxidants, there are only few studies available on the role of vitamin C in chronic periodontitis, since the lack of this vitamin is a rare condition in humans nowadays. In most populations presenting avitaminosis, the nutritional status is seasonal or other more serious nutritional problems are present. However, subclinical vitamin C deficiency is common in older individuals as the uptake declines with age (Michels et al., 2003). There is some evidence that vitamin C-deficient subjects have increased risk of periodontal diseases (Alagl and Bhat, 2015). The explanations include changes in the bone metabolism, lack of defense against oxidative stress, and susceptibility to quantitative and qualitative changes in the oral biofilm. In a study comprising two populations with different plasma vitamin C concentrations, systemic antibody levels against *P. gingivalis* showed an inverse correlation with the vitamin levels (Pussinen et al., 2003). Experimental models have indicated that vitamin E may have beneficial effects on periodontitis decreasing local inflammation and preventing alveolar bone loss (Zong et al., 2015). Human studies are scarce, but in the large NHANES study a nonlinear inverse association was found between serum α-tocopherol and severity of periodontitis in participants with relatively low vitamin levels. This suggests that normal α-tocopherol levels are needed for periodontal health but higher doses may not benefit further (Zong et al., 2015). Besides the risk of having periodontitis, the antioxidant status may have an effect on the treatment outcome. In a recent systemic review, it was concluded that use of some antioxidants may have the potential to improve periodontal healing, but only studies using lycopene and vitamin E demonstrated significant improvement compared to controls (Muniz et al., 2015).

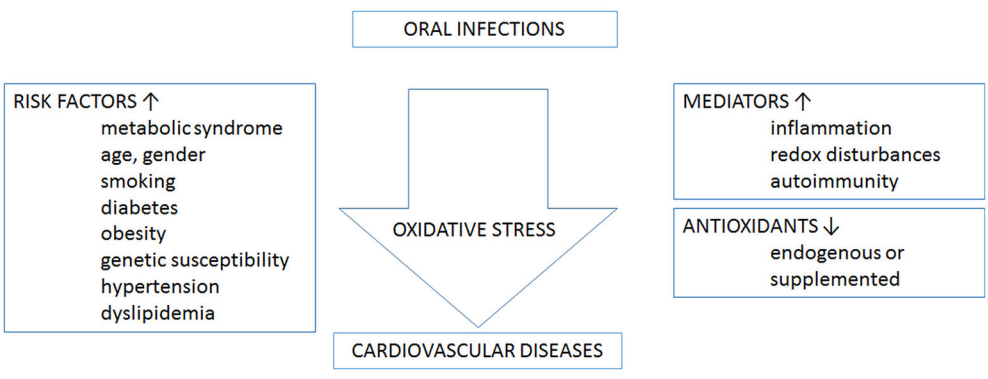

FIGURE 2 | Oxidative stress links oral infections and cardiovascular diseases. On one hand, oral infections and cardiovascular diseases share a number of risk factors. On the other hand, oral infections may contribute to the development of several cardiovascular risk factors. The processes that mediate the association between the two disease categories and involve oxidative stress are inflammation, redox disturbances, and autoimmunity. Categorically antioxidants, acquired either endogenous or supplemented, decrease oxidative stress.

In summary, oxidative stress plays a central role in the pathogenesis of AP. Although ROS represent an important host defense mechanism against endodontic bacterial challenge and modulate cell signaling, oxidant imbalance contributes locally to the formation and progression of AL, through direct molecular damage and redox-signaling. Altogether, these mechanisms result in impaired bone homeostasis, pro-inflammatory response, and the synthesis and activation of MMPs. Additionally, there is initial mechanistic evidence linking systemic oxidative stress and atherosclerosis during AP. Further studies are needed to unravel the complex effects of ROS in apical tissue breakdown and their associated systemic diseases, as well as the potential contributions of adjuvant antioxidant therapies.

AUTHOR CONTRIBUTIONS

All authors contributed to all the manuscript topics and revision. RV and PH worked specially in the local effects of ROS and developed the **Figure 1**. PP specially developed the topic of atherogenesis and **Figure 2**. The corresponding author wrote part of both topics, participated in design of the figures and edited the manuscript.

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REFERENCES

Akalin, F. A., Baltacioglu, E., Alver, A., and 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. doi: 10.1111/j.1600-051X.2007.01091.x

Akalin, F. A., Isiksal, E., Baltacioglu, E., Renda, N., and Karabulut, E. (2008). Superoxide dismutase activity in gingiva in type-2 diabetes mellitus patients with chronic periodontitis. *Arch. Oral Biol.* 53, 44–52. doi: 10.1016/j.archoralbio.2007.07.009

Alagl, A. S., and Bhat, S. G. (2015). Ascorbic acid: new role of an age-old micronutrient in the management of periodontal disease in older adults. *Geriatr. Gerontol. Int.* 15, 241–254. doi: 10.1111/ggi.12408

Babior, B. M. (1984). Oxidants from phagocytes: agents of defense and destruction. *Blood* 64, 959–966.

Baeza, M., Garrido, M., Hernandez-Rios, P., Dezerega, A., Garcia-Sesnich, J., Strauss, F., et al. (2016). Diagnostic accuracy for Apical and Chronic periodontitis biomarkers in gingival crevicular fluid: an exploratory study. *J. Clin. Periodontol.* 43, 34–45 doi: 10.1111/jcpe.12479

Bai, X. C., Lu, D., Liu, A. L., Zhang, Z. M., Li, X. M., Zou, Z. P., et al. (2005). Reactive oxygen species stimulates receptor activator of NF-kappaB ligand expression in osteoblast. *J. Biol. Chem.* 280, 17497–17506. doi: 10.1074/jbc.M409332200

Baumgarth, N., Tung, J. W., and Herzenberg, L. A. (2005). Inherent specificities in natural antibodies: a key to immune defense against pathogen invasion. *Springer Semin. Immunopathol.* 26, 347–362. doi: 10.1007/s00281-004-0182-2

Belmar, M. J., Pabst, C., Martinez, B., and Hernandez, M. (2008). Gelatinolytic activity in gingival crevicular fluid from teeth with periapical lesions. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* 105, 801–806. doi: 10.1016/j.tripleo.2007.12.002

Binker, M. G., Binker-Cosen, A. A., Gaisano, H. Y., de Cosen, R. H., and Cosen-Binker, L. I. (2011). TGF-β1 increases invasiveness of SW1990 cells through Rac1/ROS/NF-kappaB/IL-6/MMP-2. *Biochem. Biophys. Res. Commun.* 405, 140–145. doi: 10.1016/j.bbrc.2011.01.023

Biswas, S. K. (2016). Does the interdependence between oxidative stress and inflammation explain the antioxidant paradox? *Oxid. Med. Cell Longev.* 2016:5698931. doi: 10.1155/2016/5698931

Brock, G. R., Butterworth, C. J., Matthews, J. B., and Chapple, I. L. (2004). Local and systemic total antioxidant capacity in periodontitis and health. *J. Clin. Periodontol.* 31, 515–521. doi: 10.1111/j.1600-051X.2004.00509.x

Buhlin, K., Hultin, M., Norderyd, O., Persson, L., Pockley, A. G., Pussinen, P. J., et al. (2009). Periodontal treatment influences risk markers for atherosclerosis in patients with severe periodontitis. *Atherosclerosis* 206, 518–522. doi: 10.1016/j.atherosclerosis.2009.03.035

Buhlin, K., Holmer, J., Gustafsson, A., Horkko, S., Pockley, A. G., Johansson, A., et al. (2015). Association of periodontitis with persistent, pro-atherogenic antibody responses. *J. Clin. Periodontol.* 42, 1006–1014. doi: 10.1111/jcpe.12456

Canakci, C. F., Tatar, A., Canakci, V., Cicek, Y., Oztas, S., and Orbak, R. (2006). New evidence of premature oxidative DNA damage: mitochondrial DNA deletion in gingival tissue of patients with periodontitis. *J. Periodontol.* 77, 1894–1900. doi: 10.1902/jop.2006.060108

Canakci, C. F., Cicek, Y., and Canakci, V. (2005). Reactive oxygen species and human inflammatory periodontal diseases. *Biochemistry* 70, 619–628. doi: 10.1007/s10541-005-0161-9

Carocho, M., and Ferreira, I. C. (2013). A review on antioxidants, prooxidants and related controversy: natural and synthetic compounds, screening and analysis methodologies and future perspectives. *Food Chem. Toxicol.* 51, 15–25. doi: 10.1016/j.fct.2012.09.021

Cavalla, F., Osorio, C., Paredes, R., Valenzuela, M. A., Garcia-Sesnich, J., Sorsa, T., et al. (2015). Matrix metalloproteinases regulate extracellular levels of SDF-1/CXCL12, IL-6 and VEGF in hydrogen peroxide-stimulated human periodontal ligament fibroblasts. *Cytokine* 73, 114–121. doi: 10.1016/j.cyto.2015.02.001

Chapple, I. L., and Matthews, J. B. (2007). The role of reactive oxygen and antioxidant species in periodontal tissue destruction. *Periodontol 2000* 43, 160–232. doi: 10.1111/j.1600-0757.2006.00178.x

Chapple, I. L. (1997). Reactive oxygen species and antioxidants in inflammatory diseases. *J. Clin. Periodontol.* 24, 287–296. doi: 10.1111/j.1600-051X.1997.tb00760.x

Choe, Y., Yu, J. Y., Son, Y. O., Park, S. M., Kim, J. G., Shi, X., et al. (2012). Continuously generated H_2O_2 stimulates the proliferation and osteoblastic differentiation of human periodontal ligament fibroblasts. *J. Cell Biochem.* 113, 1426–1436. doi: 10.1002/jcb.24017

Cotti, E., Dessi, C., Piras, A., and Mercuro, G. (2011a). Can a chronic dental infection be considered a cause of cardiovascular disease? A review of the literature. *Int. J. Cardiol.* 148, 4–10. doi: 10.1016/j.ijcard.2010.08.011

Cotti, E., Dessi, C., Piras, A., Flore, G., Deidda, M., Madeddu, C., et al. (2011b). Association of endodontic infection with detection of an initial lesion to the cardiovascular system. *J. Endod.* 37, 1624–1629. doi: 10.1016/j.joen.2011.09.006

Crotti, T., Smith, M. D., Hirsch, R., Soukoulis, S., Weedon, H., Capone, M., et al. (2003). Receptor activator NF kappaB ligand (RANKL) and osteoprotegerin (OPG) protein expression in periodontitis. *J. Periodontal. Res.* 38, 380–387. doi: 10.1034/j.1600-0765.2003.00615.x

Cusick, M. F., Libbey, J. E., and Fujinami, R. S. (2012). Molecular mimicry as a mechanism of autoimmune disease. *Clin. Rev. Allergy Immunol.* 42, 102–111. doi: 10.1007/s12016-011-8294-7

Dezerega, A., Madrid, S., Mundi, V., Valenzuela, M. A., Garrido, M., Paredes, R., et al. (2012). Pro-oxidant status and matrix metalloproteinases in apical lesions and gingival crevicular fluid as potential biomarkers for asymptomatic apical periodontitis and endodontic treatment response. *J. Inflamm.* 9:8. doi: 10.1186/1476-9255-9-8

Dorn, B. R., Harris, L. J., Wujick, C. T., Vertucci, F. J., and Progulske-Fox, A. (2002). Invasion of vascular cells *in vitro* by *Porphyromonas endodontalis*. *Int. Endod. J.* 35, 366–371. doi: 10.1046/j.0143-2885.2001.00489.x

Erel, O. (2005). A new automated colorimetric method for measuring total oxidant status. *Clin. Biochem.* 38, 1103–1111. doi: 10.1016/j.clinbiochem.2005.08.008

Flohe, L. (2016). The impact of thiol peroxidases on redox regulation. *Free Radic. Res.* 50, 126–142. doi: 10.3109/10715762.2015.1046858

Gaffen, S. L., and Hajishengallis, G. (2008). A new inflammatory cytokine on the block: re-thinking periodontal disease and the Th1/Th2 paradigm in the context of Th17 cells and IL-17. *J. Dent. Res.* 87, 817–828. doi: 10.1177/154405910808700908

Gamonal, J., Mendoza, C., Espinoza, I., Munoz, A., Urzua, I., Aranda, W., et al. (2010). Clinical attachment loss in Chilean adult population: First Chilean National Dental Examination Survey. *J. Periodontol.* 81, 1403–1410. doi: 10.1902/jop.2010.100148

Graves, D. T., Oates, T., and Garlet, G. P. (2011). Review of osteoimmunology and the host response in endodontic and periodontal lesions. *J Oral Microbiol.* 3:5304. doi: 10.3402/jom.v3i0.5304

Halliwell, B., and Gutteridge, J. M. C. (eds.). (2015). *Free Radicals in Biology and Medicine, 5th Edn.* (Oxford University Press). doi: 10.1093/acprof:oso/9780198717478.001.0001

Halliwell, B., and 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. doi: 10.1038/sj.bjp.0705776

Hernandez, M., Valenzuela, M. A., Lopez-Otin, C., Alvarez, J., Lopez, J. M., Vernal, R., et al. (2006). Matrix metalloproteinase-13 is highly expressed in destructive periodontal disease activity. *J. Periodontol.* 77, 1863–1870. doi: 10.1902/jop.2006.050461

Hernandez, M., Gamonal, J., Tervahartiala, T., Mantyla, P., Rivera, O., Dezerega, A., et al. (2010). Associations between matrix metalloproteinase-8 and−14 and myeloperoxidase in gingival crevicular fluid from subjects with progressive chronic periodontitis: a longitudinal study. *J. Periodontol.* 81, 1644–1652. doi: 10.1902/jop.2010.100196

Hernandez Rios, M., Sorsa, T., Obregon, F., Tervahartiala, T., Valenzuela, M. A., Pozo, P., et al. (2009). Proteolytic roles of matrix metalloproteinase (MMP)-13 during progression of chronic periodontitis: initial evidence for MMP-13/MMP-9 activation cascade. *J. Clin. Periodontol.* 36, 1011–1017. doi: 10.1111/j.1600-051X.2009.01488.x

Hofbauer, L. C., and Heufelder, A. E. (2001). Role of receptor activator of nuclear factor-kappaB ligand and osteoprotegerin in bone cell biology. *J. Mol. Med.* 79, 243–253. doi: 10.1007/s001090100226

Holden, J. A., Attard, T. J., Laughton, K. M., Mansell, A., O'Brien-Simpson, N. M., and Reynolds, E. C. (2014). *Porphyromonas gingivalis* lipopolysaccharide weakly activates M1 and M2 polarized mouse macrophages but induces inflammatory cytokines. *Infect. Immun.* 82, 4190–4203. doi: 10.1128/IAI.02325-14

Huang, Q., and Pope, R. M. (2010). Toll-like receptor signaling: a potential link among rheumatoid arthritis, systemic lupus, and atherosclerosis. *J. Leukoc. Biol.* 88, 253–262. doi: 10.1189/jlb.0310126

Inchingolo, F., Marrelli, M., Annibali, S., Cristalli, M. P., Dipalma, G., Inchingolo, A. D., et al. (2014). Influence of endodontic treatment on systemic oxidative stress. *Int. J. Med. Sci.* 11, 1–6. doi: 10.7150/ijms.6663

Jakovljevic, A., Andric, M., Miletic, M., Beljic-Ivanovic, K., Knezevic, A., Mojsilovic, S., et al. (2016). Epstein-Barr virus infection induces bone resorption in apical periodontitis via increased production of reactive oxygen species. *Med. Hypotheses* 94, 40–42. doi: 10.1016/j.mehy.2016.06.020

Jiang, X., Yang, Z., Chandrakala, A. N., Pressley, D., and Parthasarathy, S. (2011). Oxidized low density lipoproteins–do we know enough about them? *Cardiovasc. Drugs Ther.* 25, 367–377. doi: 10.1007/s10557-011-6326-4

Kanzaki, H., Shinohara, F., Kajiya, M., Fukaya, S., Miyamoto, Y., and Nakamura, Y. (2014). Nuclear Nrf2 induction by protein transduction attenuates osteoclastogenesis. *Free Radic. Biol. Med.* 77, 239–248. doi: 10.1016/j.freeradbiomed.2014.09.006

Kawai, T., Matsuyama, T., Hosokawa, Y., Makihira, S., Seki, M., Karimbux, N. Y., et al. (2006). B and T lymphocytes are the primary sources of RANKL in the bone resorptive lesion of periodontal disease. *Am. J. Pathol.* 169, 987–998. doi: 10.2353/ajpath.2006.060180

Kook, S. H., Lee, D., Cho, E. S., Heo, J. S., Poudel, S. B., Ahn, Y. H., et al. (2016). Activation of canonical Wnt/β-catenin signaling inhibits H_2O_2-induced decreases in proliferation and differentiation of human periodontal ligament fibroblasts. *Mol. Cell Biochem.* 411, 83–94. doi: 10.1007/s11010-015-2570-4

Lamster, I. B., and Novak, M. J. (1992). Host mediators in gingival crevicular fluid: implications for the pathogenesis of periodontal disease. *Crit. Rev. Oral Biol. Med.* 3, 31–60. doi: 10.1177/10454411920030010501

Lee, Y. S., Bak, E. J., Kim, M., Park, W., Seo, J. T., and Yoo, Y. J. (2008). Induction of IL-8 in periodontal ligament cells by $H_{(2)}O_{(2)}$. *J. Microbiol.* 46, 579–584. doi: 10.1007/s12275-008-0182-3

Leishman, S. J., Ford, P. J., Do, H. L., Palmer, J. E., Heng, N. C., West, M. J., et al. (2012). Periodontal pathogen load and increased antibody response to heat shock protein 60 in patients with cardiovascular disease. *J. Clin. Periodontol.* 39, 923–930. doi: 10.1111/j.1600-051X.2012.01934.x

Liljestrand, J. M., Mantyla, P., Paju, S., Buhlin, K., Kopra, K. A., Persson, G. R., et al. (2016). Association of endodontic lesions with coronary artery disease. *J. Dent. Res.* 95, 1358–1365. doi: 10.1177/0022034516660509

Marcaccini, A. M., Meschiari, C. A., Zuardi, L. R., de Sousa, T. S., Taba, M. Jr., Teofilo, J. M., et al. (2010). Gingival crevicular fluid levels of MMP-8, MMP-9, TIMP-2, and MPO decrease after periodontal therapy. *J. Clin. Periodontol.* 37, 180–190. doi: 10.1111/j.1600-051X.2009.01512.x

Marton, I. J., Balla, G., Hegedus, C., Redi, P., Szilagyi, Z., Karmazsin, L., et al. (1993). The role of reactive oxygen intermediates in the pathogenesis of chronic apical periodontitis. *Oral Microbiol. Immunol.* 8, 254–257. doi: 10.1111/j.1399-302X.1993.tb00570.x

Matsuura, E., Atzeni, F., Sarzi-Puttini, P., Turiel, M., Lopez, L. R., and Nurmohamed, M. T. (2014). Is atherosclerosis an autoimmune disease? *BMC Med.* 12:47. doi: 10.1186/1741-7015-12-47

Matzinger, P. (2002). The danger model: a renewed sense of self. *Science* 296, 301–305. doi: 10.1126/science.1071059

Medzhitov, R., and Janeway, C. A. Jr. (1997). Innate immunity: the virtues of a nonclonal system of recognition. *Cell* 91, 295–298. doi: 10.1016/S0092-8674(00)80412-2

Michels, A. J., Joisher, N., and Hagen, T. M. (2003). Age-related decline of sodium-dependent ascorbic acid transport in isolated rat hepatocytes. *Arch. Biochem. Biophys.* 410, 112–120. doi: 10.1016/S0003-9861(02)00678-1

Miller, Y. I., Choi, S. H., Wiesner, P., Fang, L., Harkewicz, R., Hartvigsen, K., et al. (2011). Oxidation-specific epitopes are danger-associated molecular patterns recognized by pattern recognition receptors of innate immunity. *Circ. Res.* 108, 235–248. doi: 10.1161/CIRCRESAHA.110.223875

Minczykowski, A., Woszczyk, M., Szczepanik, A., Lewandowski, L., and Wysocki, H. (2001). Hydrogen peroxide and superoxide anion production by polymorphonuclear neutrophils in patients with chronic periapical granuloma, before and after surgical treatment. *Clin. Oral Investig.* 5, 6–10. doi: 10.1007/s007840000095

Mody, N., Parhami, F., Sarafian, T. A., and Demer, L. L. (2001). Oxidative stress modulates osteoblastic differentiation of vascular and bone cells. *Free Radic. Biol. Med.* 31, 509–519. doi: 10.1016/S0891-5849(01)00610-4

Monteiro, A. M., Jardini, M. A., Alves, S., Giampaoli, V., Aubin, E. C., Figueiredo Neto, A. M., et al. (2009). Cardiovascular disease parameters in periodontitis. *J. Periodontol.* 80, 378–388. doi: 10.1902/jop.2009.080431

Mori, K., Shibanuma, M., and Nose, K. (2004). Invasive potential induced under long-term oxidative stress in mammary epithelial cells. *Cancer Res.* 64, 7464–7472. doi: 10.1158/0008-5472.CAN-04-1725

Muniz, F. W., Nogueira, S. B., Mendes, F. L., Rosing, C. K., Moreira, M. M., de Andrade, G. M., et al. (2015). The impact of antioxidant agents complimentary to periodontal therapy on oxidative stress and periodontal outcomes: a systematic review. *Arch. Oral Biol.* 60, 1203–1214. doi: 10.1016/j.archoralbio.2015.05.007

Nagasawa, T., Kiji, M., Yashiro, R., Hormdee, D., Lu, H., Kunze, M., et al. (2007). Roles of receptor activator of nuclear factor-kappaB ligand (RANKL) and osteoprotegerin in periodontal health and disease. *Periodontol. 2000* 43, 65–84. doi: 10.1111/j.1600-0757.2006.00185.x

Nair, P. N. (2004). Pathogenesis of apical periodontitis and the causes of endodontic failures. *Crit. Rev. Oral Biol. Med.* 15, 348–381. doi: 10.1177/154411130401500604

Ohyama, H., Kato-Kogoe, N., Kuhara, A., Nishimura, F., Nakasho, K., Yamanegi, K., et al. (2009). The involvement of IL-23 and the Th17 pathway in periodontitis. *J. Dent. Res.* 88, 633–638. doi: 10.1177/0022034509339889

Okinaga, T., Ariyoshi, W., and Nishihara, T. (2015). *Aggregatibacter actinomycetemcomitans* invasion induces interleukin-1β production through reactive oxygen species and cathepsin, B. *J. Interferon Cytokine Res.* 35, 431–440. doi: 10.1089/jir.2014.0127

Osorio, C., Cavalla, F., Paula-Lima, A., Diaz-Araya, G., Vernal, R., Ahumada, P., et al. (2015). H_2O_2 activates matrix metalloproteinases through the nuclear

factor kappa B pathway and $Ca^{(2+)}$ signals in human periodontal fibroblasts. *J. Periodontal. Res.* 50, 798–806. doi: 10.1111/jre.12267

Ozbek, S. M., and Ozbek, A. (2010). Real-time polymerase chain reaction of "red complex" (*Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*) in periradicular abscesses. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod*. 110, 670–674. doi: 10.1016/j.tripleo.2010.07.001

Paraskevas, S., Huizinga, J. D., and Loos, B. G. (2008). A systematic review and meta-analyses on C-reactive protein in relation to periodontitis. *J. Clin. Periodontol*. 35, 277–290. doi: 10.1111/j.1600-051X.2007.01173.x

Pasqualini, D., Bergandi, L., Palumbo, L., Borraccino, A., Dambra, V., Alovisi, M., et al. (2012). Association among oral health, apical periodontitis, CD14 polymorphisms, and coronary heart disease in middle-aged adults. *J. Endod*. 38, 1570–1577. doi: 10.1016/j.joen.2012.08.013

Petersen, J., Glassl, E. M., Nasseri, P., Crismani, A., Luger, A. K., Schoenherr, E., et al. (2014). The association of chronic apical periodontitis and endodontic therapy with atherosclerosis. *Clin. Oral Invest*. 18, 1813–1823. doi: 10.1007/s00784-013-1156-3

Pockley, A. G., Calderwood, S. K., and Multhoff, G. (2009). The atheroprotective properties of Hsp70: a role for Hsp70-endothelial interactions? *Cell Stress Chaperones*. 14, 545–553. doi: 10.1007/s12192-009-0113-1

Pussinen, P. J., Laatikainen, T., Alfthan, G., Asikainen, S., and 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

Rocas, I. N., Siqueira, J. F. Jr. (2010). Distribution of *Porphyromonas gingivalis* fimA genotypes in primary endodontic infections. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod*. 109, 474–478. doi: 10.1016/j.tripleo.2009.11.009

Rocas, I. N., and Siqueira, J. F. Jr. (2008). Root canal microbiota of teeth with chronic apical periodontitis. *J. Clin. Microbiol*. 46, 3599–3606. doi: 10.1128/JCM.00431-08

Saad, A. F., Virella, G., Chassereau, C., Boackle, R. J., and Lopes-Virella, M. F. (2006). OxLDL immune complexes activate complement and induce cytokine production by MonoMac 6 cells and human macrophages. *J. Lipid Res*. 47, 1975–1983. doi: 10.1194/jlr.M600064-JLR200

Saari, H., Suomalainen, K., Lindy, O., Konttinen, Y. T., and Sorsa, T. (1990). Activation of latent human neutrophil collagenase by reactive oxygen species and serine proteases. *Biochem. Biophys. Res. Commun*. 171, 979–987. doi: 10.1016/0006-291X(90)90780-Q

Sasaki, H., Yamamoto, H., Tominaga, K., Masuda, K., Kawai, T., Teshima-Kondo, S., et al. (2009). Receptor activator of nuclear factor-kappaB ligand-induced mouse osteoclast differentiation is associated with switching between NADPH oxidase homologues. *Free Radic. Biol. Med*. 47, 189–199. doi: 10.1016/j.freeradbiomed.2009.04.025

Schenkein, H. A., and Loos, B. G. (2013). Inflammatory mechanisms linking periodontal diseases to cardiovascular diseases. *J. Clin. Periodontol*. 40(Suppl. 14), S51–S69. doi: 10.1111/jcpe.12060

Schenkein, H. A., Gunsolley, J. C., Best, A. M., Harrison, M. T., Hahn, C. L., Wu, J., et al. (1999). Antiphosphorylcholine antibody levels are elevated in humans with periodontal diseases. *Infect. Immun*. 67, 4814–4818.

Schenkein, H. A., Berry, C. R., Burmeister, J. A., Brooks, C. N., Barbour, S. E., Best, A. M., et al. (2003). Anti-cardiolipin antibodies in sera from patients with periodontitis. *J. Dent. Res*. 82, 919–922. doi: 10.1177/154405910308201114

Schmidt, R. E., and Gessner, J. E. (2005). Fc receptors and their interaction with complement in autoimmunity. *Immunol. Lett*. 100, 56–67. doi: 10.1016/j.imlet.2005.06.022

Shackelford, R. E., Kaufmann, W. K., and Paules, R. S. (2000). Oxidative stress and cell cycle checkpoint function. *Free Radic. Biol. Med*. 28, 1387–1404. doi: 10.1016/S0891-5849(00)00224-0

Silva, M. J., Sousa, L. M., Lara, V. P., Cardoso, F. P., Junior, G. M., Totola, A. H., et al. (2011). The role of iNOS and PHOX in periapical bone resorption. *J. Dent. Res*. 90, 495–500. doi: 10.1177/0022034510391792

Sima, C., and Glogauer, M. (2013). Macrophage subsets and osteoimmunology: tuning of the immunological recognition and effector systems that maintain alveolar bone. *Periodontol 2000* 63, 80–101. doi: 10.1111/prd.12032

Sims, T. J., Lernmark, A., Mancl, L. A., Schifferle, R. E., Page, R. C., and Persson, G. R. (2002). Serum IgG to heat shock proteins and *Porphyromonas gingivalis* antigens in diabetic patients with periodontitis. *J. Clin. Periodontol*. 29, 551–562. doi: 10.1034/j.1600-051X.2002.290612.x

Siqueira, J. F. Jr., Rocas, I. N., and Silva, M. G. (2008). Prevalence and clonal analysis of *Porphyromonas gingivalis* in primary endodontic infections. *J. Endod*. 34, 1332–1336. doi: 10.1016/j.joen.2008.08.021

Trivedi, S., and Lal, N. (2017). Antioxidant enzymes in periodontitis. *J. Oral Biol. Craniofac. Res*. 7, 54–57. doi: 10.1016/j.jobcr.2016.08.001

Tsimikas, S., and Miller, Y. I. (2011). Oxidative modification of lipoproteins: mechanisms, role in inflammation and potential clinical applications in cardiovascular disease. *Curr. Pharm. Design* 17, 27–37. doi: 10.2174/138161211795049831

Turunen, S. P., Kummu, O., Harila, K., Veneskoski, M., Soliymani, R., Baumann, M., et al. (2012). Recognition of *Porphyromonas gingivalis* gingipain epitopes by natural IgM binding to malondialdehyde modified low-density lipoprotein. *PLoS ONE* 7:e34910. doi: 10.1371/journal.pone.0034910

Turunen, S. P., Kummu, O., Wang, C., Harila, K., Mattila, R., Sahlman, M., et al. (2015). Immunization with malondialdehyde-modified low-density lipoprotein (LDL) reduces atherosclerosis in LDL receptor-deficient mice challenged with *Porphyromonas gingivalis*. *Innate Immun*. 21, 370–385. doi: 10.1177/1753425914542444

Vernal, R., Chaparro, A., Graumann, R., Puente, J., Valenzuela, M. A., and Gamonal, J. (2004). Levels of cytokine receptor activator of nuclear factor kappaB ligand in gingival crevicular fluid in untreated chronic periodontitis patients. *J. Periodontol*. 75, 1586–1591. doi: 10.1902/jop.2004.75.12.1586

Wang, C., Kankaanpaa, J., Kummu, O., Turunen, S. P., Akhi, R., Bergmann, U., et al. (2016). Characterization of a natural mouse monoclonal antibody recognizing epitopes shared by oxidized low-density lipoprotein and chaperonin 60 of *Aggregatibacter actinomycetemcomitans*. *Immunol. Res*. 64, 699–710. doi: 10.1007/s12026-015-8781-7

Wei, P. F., Ho, K. Y., Ho, Y. P., Wu, Y. M., Yang, Y. H., and Tsai, C. C. (2004). The investigation of glutathione peroxidase, lactoferrin, myeloperoxidase and interleukin-1β in gingival crevicular fluid: implications for oxidative stress in human periodontal diseases. *J. Periodontal. Res*. 39, 287–293. doi: 10.1111/j.1600-0765.2004.00744.x

Xiang, M., and Fan, J. (2010). Association of Toll-like receptor signaling and reactive oxygen species: a potential therapeutic target for posttrauma acute lung injury. *Mediators Inflamm*. 2010:916425. doi: 10.1155/2010/916425

Yoon, S. O., Park, S. J., Yoon, S. Y., Yun, C. H., and Chung, A. S. (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. doi: 10.1074/jbc.M202647200

Zong, G., Scott, A. E., Griffiths, H. R., Zock, P. L., Dietrich, T., and Newson, R. S. (2015). Serum α-tocopherol has a nonlinear inverse association with Periodontitis among US adults. *J. Nutr*. 145, 893–899. doi: 10.3945/jn.114.203703

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Activation of Casein Kinase II by Gallic Acid Induces BIK–BAX/BAK-Mediated ER Ca^{++}-ROS-Dependent Apoptosis of Human Oral Cancer Cells

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Induction of the generation of endoplasmic reticulum (ER) calcium (Ca^{++})-mediated reactive oxygen species (ROS) by gallic acid (GA) has been implicated in the mitochondrial apoptotic death of human oral cancer (OC) cells, but the molecular mechanism by which GA causes ER Ca^{++} release of OC cells to undergo cell death remains unclear. Here, we report that GA-induced phosphorylation of B-cell lymphoma 2 (BCL-2)-interacting killer (BIK) (threonine (Thr) 33/Serine (Ser) 35) and p53 (Ser 15 and Ser 392), Bcl-2-associated x protein (BAX)/BCL-2 antagonist killer 1 (BAK) oligomerization on the ER and mitochondria, rising of cytosolic Ca^{++} and ROS, cytochrome *c* (Cyt *c*) release from the mitochondria, Ψ_m loss, and apoptosis were suppressed in cells co-treated with a specific inhibitor of casein kinase II (CK II) (4,5,6,7-tetrabromobenzotriazole). Small interfering RNA (siRNA)-mediated suppression of BIK inhibited GA-induced oligomeric complex of BAX/BAK in the ER and mitochondria, increase of cytosolic Ca^{++} and ROS, and apoptosis, but did not attenuate the increase in the level of Ser 15-phosphated p53 induced by GA. Blockade of p53 expression by short hairpin RNA suppressed BAX/BAK oligomerization and ER Ca^{++}–ROS-associated apoptosis induced by GA but did not affect GA-induced phospho-BIK (Thr 33/Ser 35) levels. Induction of mitochondrial Cyt *c* release and ROS generation, increased cytosolic Ca^{++} level, and apoptosis by GA was attenuated by expression of the *BAX* or *BAK* siRNA. Over-expression of BCL-2 (but not BCL-X_L) inhibited formation of ER oligomeric BAX/BAK by GA. Our results demonstrated that activation of the CK II by GA is required for the BIK-mediated ROS-dependent apoptotic activity of ER-associated BAX/BAK.

Keywords: BAX/BAK, BIK, casein kinase II, ER Ca^{++}, gallic acid, ROS

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INTRODUCTION

Calcium (Ca^{++}) is an important second messenger responsible for a variety of the control of cellular processes, including cell growth and survival (Loughery et al., 2014). Transfer of Ca^{++} between the endoplasmic reticulum (ER) and mitochondria confers genotoxic damage-induced apoptosis (Rizzuto and Pozzan, 2006; Kroemer et al., 2007). The initiation of apoptotic process

is regulated by the B-cell lymphoma 2 (BCL-2) family of proteins, which can be identified as either pro-apoptotic BCL-2-associated x protein (BAX)/BCL-2 antagonist killer 1 (BAK) or anti-apoptotic BCL-2/BCL-X_L proteins (Unger et al., 1993; Cory et al., 2003). In response to apoptotic stimuli, BAX and BAK change their conformations to form oligomers that associate not only with the mitochondrial membrane but also with the ER (Sato and Seiki, 1993; Lu H. L. et al., 2016). ER targeting of oligomeric BAX/BAK causes ER Ca^{++} release, whereas mitochondria-targeted BAX/BAK selectively induces the release of cytochrome *c* (Cyt *c*) from mitochondria (Scorrano et al., 2003; Lu H. L. et al., 2016). The anti-apoptotic function of BCL-2 in the inhibition of BAX-mediated permeabilization of mitochondrial outer membrane was shown to interact with BAX, thereby attenuating the oligomerization and insertion of BAX into the outer mitochondrial membrane (Yin et al., 1994; Cheng et al., 2001; Ding et al., 2010). In addition to the anti-apoptotic function of BCL-2 in the mitochondria, this protein has been reported to modulate ER Ca^{++} homeostasis by the ER targeting (Pinton and Rizzuto, 2006). The protection of ER-targeted BCL-2 against BAX-induced apoptosis has suggested that BCL-2 exerts its anti-apoptotic function to BAX by targeting ER (Wang et al., 2001).

Bcl-2-interacting killer (BIK) is a pro-apoptotic BH3-only member of the BCL-2 family and is found complexed as a heterodimer with BCL-2 or BCL-X_L (Elangovan and Chinnadurai, 1997). The phosphorylation at the BIK residues threonine (Thr) 33 and serine (Ser) 35 has been linked to an increase its apoptotic activity (Verma et al., 2001). The kinase responsible for the phosphorylation of BIK Thr 33 and Ser 35 is probably a casein kinase II (CKII)-related enzyme (Verma et al., 2001). The pro-apoptotic activity of BIK is involved in ER-mitochondria Ca^{++} crosstalk by inducing the recruitment and oligomerization of BAX at the ER to confer the stress-induced cell apoptotic death (Mathai et al., 2005). Specific inhibition of *BIK* gene expression by small interfering RNA resulted in aborted p53-induced ER recruitment and oligomerization of BAX, and mitochondrial Cyt *c* release (Mathai et al., 2005).

The mechanisms by which p53 contribute to suppression of tumor growth by mediating apoptosis in response to genotoxic stress have been documented to occur transcription-dependent and transcription-independent pathways (Haupt et al., 2003; Moll et al., 2005). p53 exerts its transcription-independent pro-apoptotic functions through mitochondrial translocation (Moll et al., 2005). Interestingly, p53 lacking transactivation activity can localize to the mitochondrial surface of primary thymocytes undergoing γ-irradiation-induced apoptosis. The formation of the p53–BCL-2/BCL-X_L complexes is critical for the induction of permeabilization of the outer mitochondrial membrane by p53 (Mihara et al., 2003), suggesting the physiological relevance of cytoplasmic p53 in regulating the function and integrity of mitochondria *in vivo*. Mitochondrial localization of p53 allows it to induce the release of mitochondrial Cyt *c* by triggering the membrane permeabilization activity of BAX (Mihara et al., 2003; Chipuk et al., 2004). There is convincing evidence that ER-associated p53 can enhance the transfer of Ca^{++} from the ER lumen to the mitochondrial matrix triggering the mitochondrial apoptotic cascade. The findings indicate that apoptotic action of p53 on the ER by interacting with the carboxy-terminal portion of the sarco/ER Ca^{++}–ATPase pump enhances Ca^{++} loading resulting in a release of Ca^{++} from ER (Giorgi et al., 2015), indicating that p53 localization to the ER can regulate the response to genotoxic agent-induced apoptosis by modulating the Ca^{++} homeostasis.

The naturally-occurring phenolic compound gallic acid (3,4,5-trihydroxybenzoic acid, GA) exists in the seeds, fruits, and leaves of plants, such as grapes, berries, and tea (Heinonen et al., 1998; Zuo et al., 2002; Shi et al., 2003). It has been demonstrated to possess a variety of pharmacological activities such as antioxidant, anti-inflammatory, antimicrobial, antiviral, and anticancer activities in preclinical studies (Abdelwahed et al., 2007; Kim, 2007; Ozcelik et al., 2011). Experimental evidence supports the fact that GA can selectively induce apoptosis of a variety of human cancer cell lines (Inoue et al., 1995; Elangovan and Chinnadurai, 1997; Yoshioka et al., 2000; Agarwal et al., 2006). The apoptotic action of GA on human cancer cells was attributable to DNA-damage-induced ataxia telangiectasia mutated (ATM) activation (Elangovan and Chinnadurai, 1997; Agarwal et al., 2006), a membrane of the phosphatidylinositol 3-kinase (PI3K)-like family involving in the regulation of cell cycle progression and apoptosis (Guo et al., 2010a,b). We recently showed that GA-induced ER Ca^{++} efflux triggers apoptotic cell death in human oral cancer SSC-4 cells. ER Ca^{++}-mediated apoptosis, which occurs due to induction of ER-dependent Ca^{++}-mediated ROS generation, leads to activation of mitochondrial apoptotic and ATM-JNK signal pathways (Lu Y. C. et al., 2016). The finding promoted us to further clarify the effect of GA on the induction of ER Ca^{++} release. Toward this end, in this study we investigated the molecular mechanisms associated with GA-induced ER Ca^{++} release.

MATERIALS AND METHODS

Cell Culture

The human oral cancer SCC-4 cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cell line was cultured routinely in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) (both from Gibco BRL, Grand Island, NY, USA) and grown in 10-cm tissue culture dish at 37°C in a humidified incubator containing 5% CO_2.

Chemicals and Reagents

Bismaleimidohexane (BMH), gallic acid (GA), Tris-HCl, and Triton X-100 were obtained from Sigma-Aldrich (St. Louis, MO, USA). GA was dissolved in and diluted with methanol (Daneshfar et al., 2008), and then stored at −20°C as a 100 mM stock solution. Methanol and potassium phosphate were purchased from Merck (Darmstadt, Germany). 4,5,6,7-tetrabromobenzotriazole (TBB) was purchased from Calbiochem (San Diego, CA, USA). Lipofectamine 2000 was obtained from Invitrogen (Carlsbad, CA, USA). FBS, trypsin-EDTA, and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). BAX small interfering RNA (siRNA), BAK siRNA, BIK

siRNA, control siRNA, and Western blot luminol reagent were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) (Lin et al., 2014). The BAX siRNA, BAK siRNA, BIK siRNA, and control siRNA were dissolved in RNase-free water.

Antibodies

Anti-casein kinase II (CK II) antibody was provided by Santa Cruz Biotechnology. Antibodies against BAX, BAK, BCL-2, and BCL-X_L were purchased from BD Pharmingen (San Diego, CA, USA). Anti-BIK, phospho (p)-BIK (Thr 33), p-BIK (Ser 35), p-p53 (Ser 15), p-p53 (Ser 392), cytochrome *c* oxidase subunit II (COX2), calnexin, and cytochrome *c* (Cyt *c*) were purchased from Abcam (Cambridge, MA, USA). Antibody against β-actin was obtained from Sigma-Aldrich. Peroxidase-conjugated anti-mouse IgG, -goat IgG, and -rabbit IgG secondary antibodies were purchased from Jackson ImmunoResearch Laboratory (West Grove, PA, USA).

Plasmid and siRNA Transfection

Cells (at 60–70% confluence in a 12-well plate) were transfected with the FLAG-BCL-X_L or FLAG-BCL-2 expression plasmid or with BAX siRNA, BAK siRNA, BIK siRNA, or control siRNA using Lipofectamine 2000. The expression of FLAG-BCL-X_L, FLAG-BCL-2, BAX, BAK, and BIK in transfected cells was assessed by western blotting using antibodies specific to FLAG, BCL-X_L, BCL-2, BAX, BAK, and BIK.

Measurement of DNA Fragmentation

Histone-associated DNA fragments were determined using the Cell Death Detection enzyme-linked immunosorbent assay (ELISA) kit (Roche Applied Science, Mannheim, Germany). In the vehicle controls, methanol was diluted in culture medium to the same final concentration (0.01%, v/v) as in the medium with GA. Briefly, vehicle- or GA-treated cells were incubated in hypertonic buffer for 30 min at room temperature. After centrifugation, the cell lysates were transferred into an anti-histone-coated microplate to bind histone-associated DNA fragments. Plates were washed after 1.5 h of incubation, and non-specific binding sites were saturated with blocking buffer. Plates were then incubated with peroxidase-conjugated anti-DNA for 1.5 h at room temperature. To determine the amount of retained peroxidase, 2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonate) was added as a substrate, and a spectrophotometer (Thermo Labsystems Multiskan Spectrum, Frankin, MA, USA) was used to measure the absorbance at 405 nm (Lin et al., 2011).

Detection of ROS

Briefly, treated cells were then resuspended in 500 μl of 2,7-dichlorodihydrofluorescein diacetate (10 μM) and incubated for 30 min at 37°C. The level of ROS was determined using a FACSCount flow cytometer (Lin et al., 2011; Lu Y. C. et al., 2016).

Measurement of Cytosolic Ca^{++}

The Ca^{++} level was determined by measuring the retention of indo-1 acetomethoxy (Indo-1/AM) (Invitrogen, Carlsbad, CA, USA). Briefly, the treated cells were incubated with 3 μg/ml Indo-1/AM for 30 min at 37°C. The cells were then pelleted by centrifugation at 160 × g. The pellets were resuspended and washed twice with PBS. The level of Ca^{++} was evaluated as previously described (Lin et al., 2010).

Measurement of Mitochondrial Membrane Potential

Mitochondrial membrane potential (ψ_m) was determined by measuring the retention of the dye 3,3'-dihexyloxacarbocyanine ($DiOC_6$). Briefly, treated cells were incubated with 40 nM $DiOC_6$ for 30 min at 37°C. Cells were then pelleted by centrifugation at 160 × g. Pellets were resuspended and washed twice with PBS. The $\Delta\psi_m$ was determined with a FACSCount flow cytometer (Lin et al., 2011).

Western Blot Analysis

Treated or transfected cells were lysed in lysis buffer [50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 1 μg/ml aprotinin, 100 mM Na_3VO_4, 50 mM NaF, 0.5% NP-40]. Protein concentration was determined by the Bradford method (Bio-Rad, Hercules, CA, USA). Proteins were separated by electrophoresis on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis gel and then transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA). Membranes were blocked overnight with phosphate-buffered saline (PBS) containing 3% skim milk and then incubated with primary antibody against CA II, BAX, BAK, BCL-2, BCL-X_L, BIK, p-BIK (Thr 33), p-BIK (Ser 35), caspase-12, COX2, Cyt *c*, GRP78, p53, or -p-p53 (Ser 15). Proteins were detected with horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit, or donkey anti-goat antibodies and Western Blotting Luminol Reagent. To confirm equal protein loading, β-actin was measured (Lu Y. C. et al., 2016).

Establishment of Cell Clones Permanently Expressing p53 shRNA or GFP shRNA

To establish cells stably expressing p53 shRNA or GFP shRNA, cells were transfected using Lipofectamine 2000 with pPuro-p53 shRNA or pPuro-GFP shRNA plasmid. The transfected cells were selected and cloned in the presence of 2 μg/ml puromycin. The efficiency of p53 knockdown was confirmed by western blot analysis with anti-p53 antibody (Lin et al., 2011).

Subcellular Fractionation

Subcellular fractionation was performed according to the protocol of Zong et al. (2003). The treated cells were washed twice with ice-cold PBS and scraped into a 200 mM sucrose solution containing 25 mM HEPES (pH 7.5), 10 mM KCl, 15 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 1 μg/ml aprotinin. The cells were disrupted by passage through a 26-gauge hypodermic needle 30 times and then centrifuged for 10 min in an Eppendorf microcentrifuge (5804R) at 750 × g at 4°C to remove unlysed cells and nuclei. The supernatant was collected and then centrifuged for 20 min at 10,000 × g at 4°C to form a new supernatant and pellet. The resulting pellet was saved as the mitochondrial (Mt) fraction, and the supernatant was further centrifuged at 100,000 × g for 1 h at 4°C. The new supernatant was saved as the cytosolic (Cs) fraction, and the pellet was reserved as the ER/microsomal (Ms) fraction. The resulting

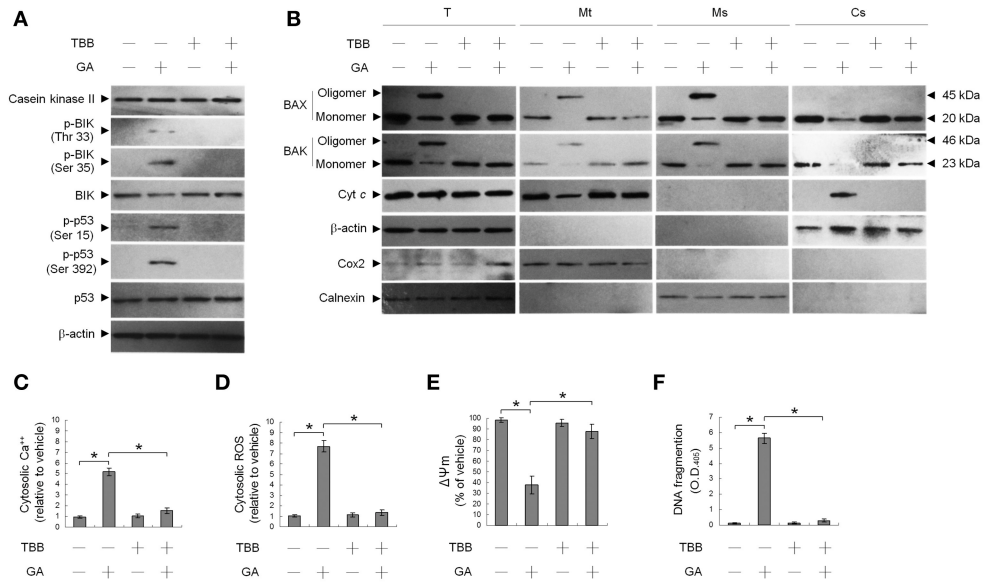

FIGURE 1 | Suppression of gallic acid (GA)-induced BCL-2-associated x protein (BAX)/BCL-2 antagonist killer 1 (BAK) oligomerization in the endoplasmic reticulum (ER), mitochondrial cytochrome c (Cyt c) release, Ψ_m loss, and apoptosis by 4,5,6,7-tetrabromobenzotriazole (TBB). **(A,B)** Cells were harvested 36 h after treatment with either vehicle, GA (300 μM), TBB (15 μM), or GA (300 μM) plus TBB (15 μM), and cell pellets were resuspended in hypotonic buffer. Crude homogenates were incubated with 5 mM bismaleimidohexane (BMH) in PBS for 30 min at room temperature and then subjected to subcellular fractionation to obtain the mitochondrial (Mt), ER/microsomal (Ms), and cytosolic (Cs) fractions. In total, 20 μg of total protein from the recovered fractions was analyzed by 10% SDS-PAGE and probed with specific antibodies, as indicated. **(C–F)** Cells were treated with either vehicle, GA (300 μM), TBB (15 μM), or GA (300 μM) plus TBB (15 μM) for for 36 h. The decrease in 3,3′-dihexyloxacarbocyanine fluorescence was measured by flow cytometry. The generation of cytosolic Ca^{++} level and ROS were monitored by measuring increased fluorescence of Indo-1 and 2,7-dichlorodihydrofluorescein by flow cytometry. DNA fragmentation was determined using a Cell Death Detection ELISA kit. The values presented are the mean standard errors from three independent experiments. *Significantly different at $p < 0.05$.

Mt and Ms fractions were lysed in RIPA buffer (1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 10 mM Tris-HCl [pH 8.0], and 0.14 M NaCl) for Western blot analysis. The purity of each subcellular fraction was confirmed by Western blotting using specific antibodies against the nuclear marker nucleolin, the mitochondrial marker Cox-2, and the ER marker calnexin.

Statistical Analysis of Data

Statistical calculations of the data were performed using the unpaired Student's *t*-test and ANOVA analysis. A value of $p < 0.05$ was considered statistically significant.

RESULTS

GA Induces OC Cell Apoptosis by Inducing the CK II-Mediated Phosphorylation of BIK

We first investigated the ability of GA to modulate CK II activity, which has been shown to play a key role in targeting of BAX/BAK to ER and the increase of ER Ca^{++} depletion (Verma et al., 2001; Mathai et al., 2005). Western blot analysis revealed that treatment of OC cells with GA resulted in increased in BIK (Thr 33/Ser 35) and p53 (Ser 15 and Ser 392) phosphorylation but had no effect on the expression level of CK II protein (**Figure 1A**). Activity of CK II appeared to be required for OC cell survival because abolishment of CK II activation by a CK II inhibitor (TBB) causes suppression of cells in the apoptotic induction by GA. Co-treatment of a TBB attenuated GA-induced phosphorylation of BIK (Thr 33/Ser 35) and p53 (Ser 15 and Ser 392), ER and mitochondrial oligomerization of BAX/BAK, increase of ROS, mitochondrial Cyt *c* release, and the alteration of Ψ_m (**Figures 1A,B,D,E**). Increase in cytosolic Ca^{++} level and DNA fragmentation induced by GA was also inhibited in cells co-treatment with TBB (**Figures 1C,F**). It has been demonstrated that p53 is a physiological substrate of CK II, which is phosphorylated on Ser 392 (corresponding to murine Ser 389) by CK II in response to DNA damage (Meek et al., 1990; Keller and Lu, 2002). These findings suggest that induction of CK II was involved in GA-induced phosphorylation of BIK and p53 and subsequent events of ER–mitochondrial apoptosis in OC cells.

This raised an interesting possibility that BIK may be a critical regulator of the ER targeting of BAX/BAK by GA in the OC cells. To confirm the role of BIK in BAX/BAK-mediated ER Ca^{++} homeostasis, we employed siRNA to knockdown BIK. siRNA-mediated targeting of *BIK* inhibited induction of BAX/BAK oligomerization in the ER, cytosolic Ca^{++}, and ROS elevation, and DNA fragmentation by GA, but there was no effect on the level of Ser-15-phosphated p53 (**Figure 2**). To address whether GA-induced BAX/BAK apoptotic function linked the induction of ER Ca^{++} release and mitochondrial death signal, cells were transfected with siRNA targeting *BAX* or *BAK*. Immunoblot analysis confirmed the specific knockdown of the expression of BAX or BAK (**Figure 3A**). **Figures 3B,C** show that silencing of *BAX* or *BAK* expression by siRNA blocked GA-induced elevation

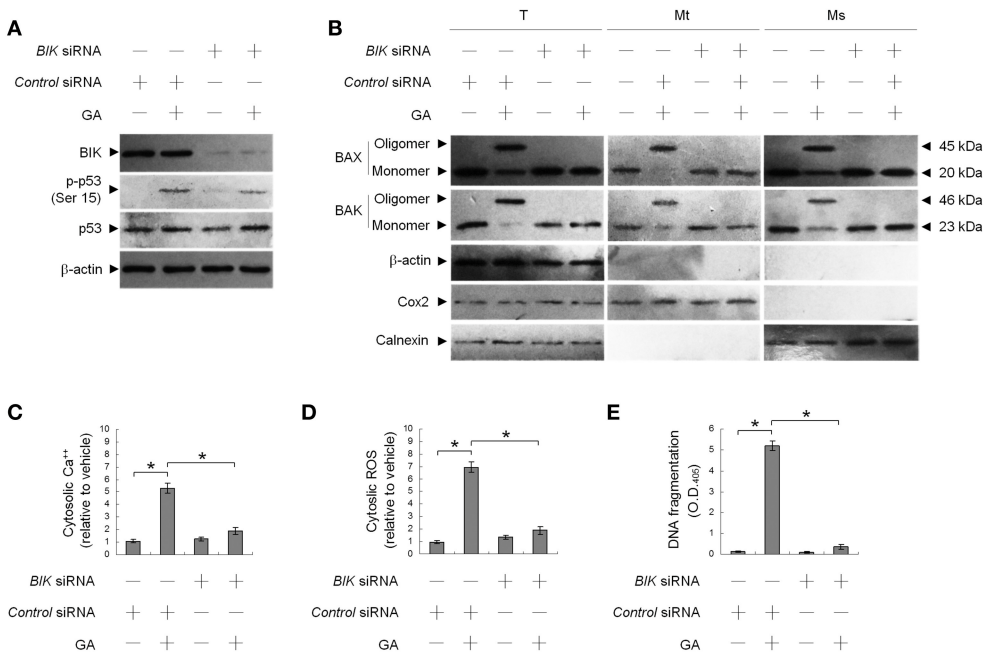

FIGURE 2 | GA-induced activity of Bcl-2-interacting killer (BIK) is responsible for gallic acid (GA)-induced BCL-2-associated x protein (BAX)/BCL-2 antagonist killer 1 (BAK) endoplasmic reticulum (ER) oligomerization, calcium (Ca^{++}) efflux from the ER, and cell apoptosis. At 12 h after transfection with control or *BIK* siRNA, the cells were treated with either vehicle or GA (300 μM) for an additional 36 h. **(A,B)** The levels of the indicated proteins in the lysates of the fractions of mitochondrial (Mt), ER/microsomal (Ms), and total cell (T) extracts were determined by Western blot analysis using specific antibodies. Cox-2, calnexin, and β-actin were used as internal controls for the mitochondria, ER, and cytosol, respectively. **(C–E)** The generation of cytosolic Ca^{++} level and ROS were monitored by measuring increased fluorescence of Indo-1 and 2,7-dichlorodihydrofluorescein by flow cytometry. DNA fragmentation was determined using a Cell Death Detection ELISA kit. The values presented are the mean standard errors from three independent experiments. *Significantly different at $p < 0.05$.

of the cytosolic Ca^{++} concentration, ROS production, release of Cyt *c* from mitochondria, and DNA fragmentation compared to cells transfected with control siRNA. These results indicate that GA-induced ER-associated apoptosis was dependent on the pro-apoptotic activity of CK II–BIK-mediated ER oligomeric BAX/BAK.

Ser 15 Phosphorylation of Mutant p53 (P151S) Protein Involves in BAX/BAK-Mediated Apoptosis Caused by GA

Previous work has shown that OC SCC-4 cells harbor p53 mutation (codon 151 proline to serine) (Kim et al., 1993). To ask whether mutant p53 modulated GA-induced BAX/BAK-mediated apoptotic death, we used cells stably expressing a shRNA to knock down p53 and examined the effect of GA on apoptosis induction. p53 protein level was reduced in cells expressing the p53 shRNA, demonstrating efficient and stable knockdown (**Figure 4A**). No change in DNA fragmentation was observed in vehicle-treated p53 shRNA–transfected cells compared to vehicle-treated non-specific GFP shRNA control cells. Expression of p53 shRNA in cells resulted in attenuation of GA-induced cytosolic Ca^{++} increase, BAX/BAK oligomer formation into the ER and mitochondria, and DNA fragmentation (**Figures 4B,C**). However, p53 shRNA expression had no detectable effect on the level of BIK Thr 33/Ser 35 phosphorylation (**Figure 4A**). These data indicate that Ser 15 phosphorylated mutant p53 (P151S) participates in the activation of GA-induced ER oligomeric BAX/BAK-mediated apoptosis.

Deregulated BCL-2 and BCL-X_L Involved in GA-Induced Oligomerization of BAX/BAK at the ER and Apoptosis

To address whether induction of BAX/BAK ER targeting and apoptosis by GA was associated with decreased BCL-2 protein levels (**Figure 5A**), transient ectopic FLAG-tagged BCL-2 or BCL-X_L was expressed in cells. Expression levels of BCL-2 and BCL-X_L were confirmed by western blotting using FLAG-, BAX-, and BAK-specific antibodies (**Figure 5B**). Ectopic expression of BCL-2, similar to that of BCL-X_L, suppressed BAX/BAK oligomerization in the mitochondria. In contrast to BCL-2, ectopic expression of BCL-X_L did not completely inhibit the increase in cytosolic Ca^{++}, ROS, and DNA fragmentation with GA (**Figure 5D**). BCL-2 (but not BCL-X_L) overexpression attenuated the GA-induced ER localization and oligomerization of BAX/ BAK (**Figure 5C**). These results demonstrate that a decrease in the deregulation of BCL-2 is associated with GA-induced apoptotic potency of oligomeric BAX/BAK in the ER of OC cells.

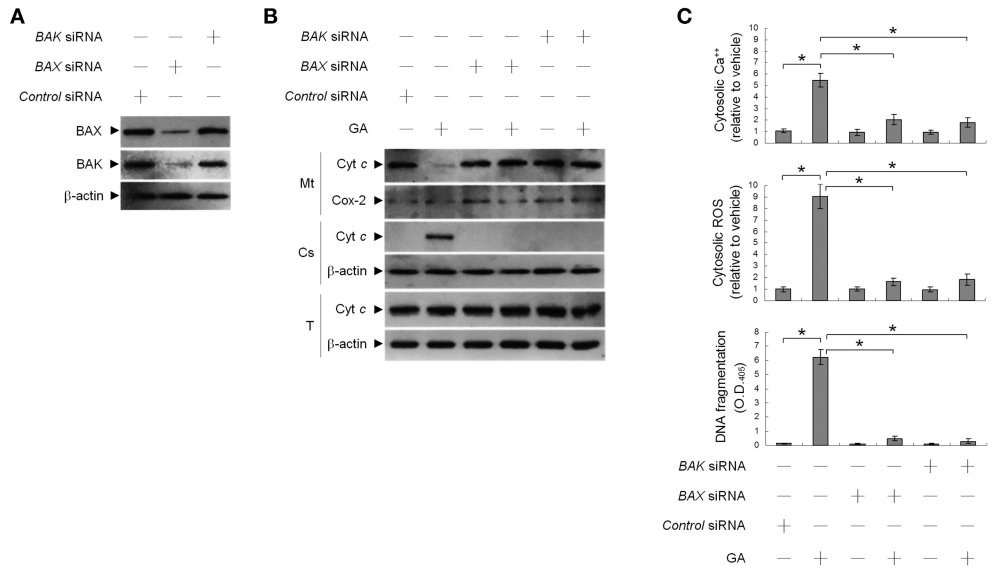

FIGURE 3 | Gallic acid (GA)-induced BCL-2-associated x protein (BAX)/BCL-2 antagonist killer 1 (BAK) apoptotic activity modulates the release of endoplasmic reticulum (ER)-associated calcium (Ca^{++}), mitochondrial Cyt c release, and apoptosis. At 12 h after transfection with control, *BAX*, or *BAK* siRNA, cells were treated with vehicle or GA (300 μM) for 36 h. **(A,B)** The levels of the indicated proteins in the lysates of the fractions of mitochondrial (Mt) and cytosolic (Cs) and total cell (T) extracts were determined by Western blot analysis using specific antibodies. Cox-2 and β-actin were used as internal controls for the mitochondria and cytosol, respectively. **(C)** The cytosolic levels of Ca^{++}, ROS, and DNA fragmentation were determined by measuring increased Indo-1 fluorescence and 2,7-dichlorodihydrofluorescein using flow cytometry and a Cell Death Detection ELISA kit, respectively. The values presented are the mean standard errors from three independent experiments. *Significantly different at $p < 0.05$.

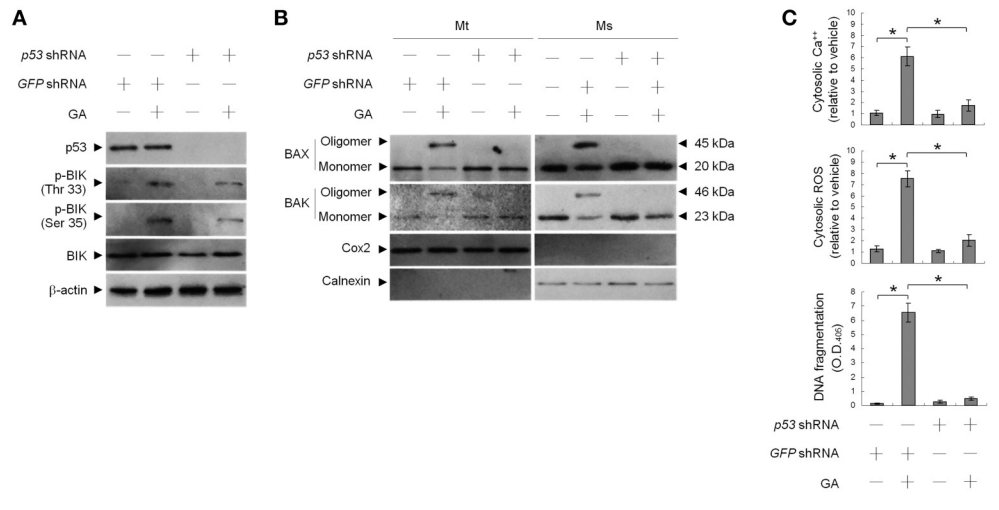

FIGURE 4 | Requirement of phospho-p53 (Ser 15) for targeting of BCL-2-associated x protein (BAX)/BCL-2 antagonist killer 1 (BAK) to the endoplasmic reticulum (ER) by gallic acid (GA). p53 shRNA cells were treated with vehicle or GA (300 μM) for 36 h. **(A,B)** The levels of the indicated proteins in the lysates of the fractions of mitochondrial (Mt) and ER/microsomal (Ms) extracts were determined by Western blot analysis using specific antibodies. Cox-2 and calnexin were used as internal controls for the mitochondria and ER, respectively. **(C)** The generation of cytosolic Ca^{++} level and ROS were monitored by measuring increased fluorescence of Indo-1 and 2,7-dichlorodihydrofluorescein by flow cytometry. DNA fragmentation was determined by using a Cell Death Detection ELISA kit. The values presented are the mean standard errors from three independent experiments. *Significantly different at $p < 0.05$.

DISCUSSION

Based on the present observations and data from our previous studies (Lu Y. C. et al., 2016) indicate that CK II-mediated Thr 33/Ser 35-phosphorylated forms of BIK appears to serve a modulator in initiating the ER Ca^{++}-mediated production of ROS through a oligomeric BAX/BAK-regulated mechanism in GA-treated OC cells. In view of observed suppression of

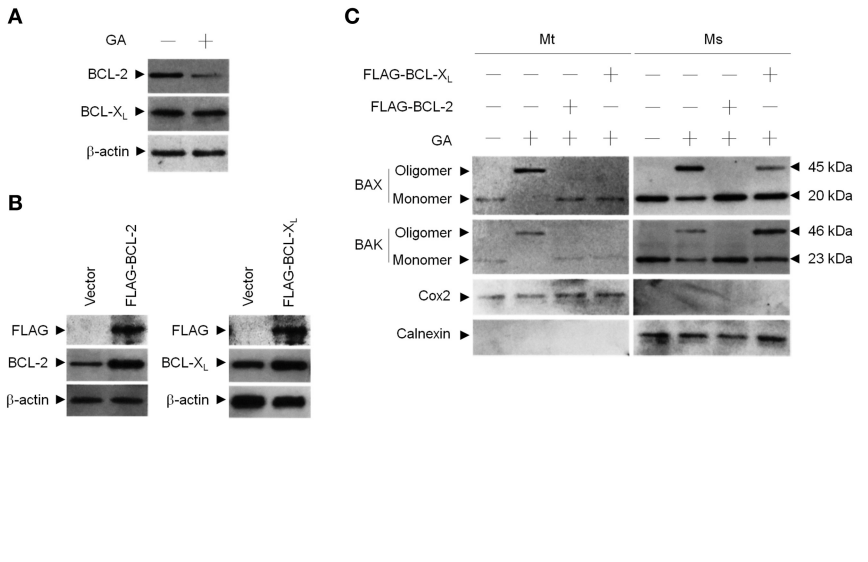

FIGURE 5 | Involvement of decreased B-cell lymphoma 2 (BCL-2) protein level in GA-induced BCL-2-associated x protein (BAX)/BCL-2 antagonist killer 1 (BAK) oligomerization in the endoplasmic reticulum (ER). At 12 h after transfection with vector alone, FLAG-BCL-2, or FLAG-BCL-X_L, cells were treated with vehicle or GA (300 μM) for 36 h. **(A,B)** Expression levels of BCL-2 and BCL-X_L in lysates prepared from cells treated with GA or transfected with vector alone, FLAG-BCL-2, or FLAG-BCL-X_L. FLAG-BCL-2, FLAG-BCL-X_L, BCL-2, and BCL-X_L were detected with the antibodies shown. **(C)** The levels of the indicated proteins in the lysates of the fractions of mitochondrial (Mt) and ER/microsomal (Ms) and total cell (T) extracts were determined by Western blot analysis using specific antibodies. Cox-2 and calnexin were used as internal controls for the mitochondria and ER, respectively. **(D)** The generation of cytosolic Ca^{++} level and ROS were monitored by measuring increased fluorescence of Indo-1 and 2,7-dichlorodihydrofluorescein by flow cytometry. DNA fragmentation was determined by using a Cell Death Detection ELISA kit. The values presented are the mean standard errors from three independent experiments. *Significantly different at $p < 0.05$.

GA-induced BIK (The 33/Ser 35) phosphorylation, BAX/BAK ER oligomerization, ER Ca^{++} and mitochondrial Cyt *c* release, ROS generation, and apoptosis by co-treatment with an ATP/GTP competitive inhibitor of CK II inhibitor (TBB), it is logical to suggest that CK II activity has physiological relevance related to modulating survival of OC cells *via* the regulation of BIK–BAX/BAK-dependent ER pathway. Characterization of CK II as an *in vivo* target molecule for GA does not rule possible involvement of protein kinase B (Akt) in the process, as evidence exists that BAX and BAK change their conformation conformations to form oligomers at the ER required AKT inactivation by reducing in its phosphorylation at Ser 473 (Lin et al., 2014). Although the Akt hyper-activation can be promoted by the induction of the phosphorylation of Akt Ser 129 with CK II to contribute anti-apoptotic function of Akt (Di Maira et al., 2005; Ruzzene et al., 2017). This observation, however, is used a phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*)-null human leukemia Jurkat T cells for functional assays (Ruzzene et al., 2017). The finding that Akt was found to be constitutively upregulated in PTEN-deficient human leukemia Jurkat T cells (Di Maira et al., 2005). Further studies are required to better understand the coordinated effect of CK II and Akt on the ER BAX/BAK-mediated apoptosis caused by GA in wild-type PTEN-carrying OC SCC-4 cells (Kubo et al., 1999).

Ser phosphorylation is implicated in stimulating transcriptional activation of p53-targeted genes (*p21*, *BAX*, and *BAK*) (Loughery et al., 2014) and adopting in a wild-type conformation of p53 (Ullrich et al., 1993). This phosphorylation also contributes to the pro-apoptotic function of p53 in the DNA-damage response (Meek, 2009). In the present study, we have used the SSC-4 cells, which possess a missense mutation in codon 151 of exone 5 (C→T transition) resulting in generation of mutant p53 (P151L) (Kim et al., 1993) and loss of p53 transcriptional activity (Xie et al., 2013). The function of mutant p53 (P151L) has been studied and found to exhibit oncogenic activity in orthotopic xenograft nude mouse (Sano et al., 2011). Consistency, our data indicate that p53 (P151L) lose its transcriptional activity for targeted genes, as evidence fails to induce an increase in the level of p21, BAX, and BAK proteins after treatment with GA; although the treatment induced ER oligomeric BAX/BAK-mediated apoptosis. Despite the fact that the result of p53 (P151L) gain-of-function in the promotion of tumor progression in SCC cell lines (Xie et al., 2013), loss of p53 (P151L) expression by shRNA sensitizes diverse SCC cells to anoikis induction. The oligomerization of BAX and BAK in the ER and apoptosis of SCC-4 cells induced by GA was attenuated by p53 shRNA. A structure-function analysis of p53 mutant proteins reveals that p53 transactivation domain mutants still had some suppression activity (Unger et al., 1993). It is known that BCL-2 can specifically inhibit p53-dependent apoptosis (Hemann and Lowe, 2006). The present study found a decrease in BCL-2 level in GA-treated SSC-4 cells. Using ectopically expressed FLAG-BCl-2 or FLAG-BCL-X_L, it was found that the GA-induced oligomerization of BAX/BAK in the ER was suppressed by BCL-2. Evidently, these results raised the possibility that GA-induced Ser 15- and 392-phosphorylated

forms of p53 (P151L) can act in a negative regulatory effect to control BCL-2 expression and modulates the recruitment of oligomeric BAX and BAK to the ER, although mutant form of p53 (P151L) have lost their transactivation function. In summary, our data provide exciting new insights into therapeutic activity and anti-OC mechanism of GA.

AUTHOR CONTRIBUTIONS

SC and ML developed the concept of the study, designed the experiments, and wrote the manuscript. ML performed the experiments, collected the data, and performed statistical analysis. SC interpreted the data, supervised this work, and critically revised the manuscript. All authors have read and approved the final manuscript.

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REFERENCES

Abdelwahed, A., Bouhlel, I., Skandrani, I., Valenti, K., Kadri, M., Guiraud, P., et al. (2007). Study of antimutagenic and antioxidant activities of gallic acid and 1,2,3,4,6-pentagalloylglucose from *Pistacia lentiscus*. Confirmation by microarray expression profiling. *Chem. Biol. Interact.* 165, 1–13. doi: 10.1016/j.cbi.2006.10.003

Agarwal, C., Tyagi, A., and Agarwal, R. (2006). Gallic acid causes inactivating phosphorylation of cdc25A/cdc25C-cdc2 via ATM-Chk2 activation, leading to cell cycle arrest, and induces apoptosis in human prostate carcinoma DU145 cells. *Mol. Cancer Ther.* 5, 3294–3302. doi: 10.1158/1535-7163.MCT-06-0483

Cheng, E. H., Wei, M. C., Weiler, S., Flavell, R. A., Mak, T. W., Lindsten, T., et al. (2001). BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Mol. Cell* 8, 705–711. doi: 10.1016/S1097-2765(01)00320-3

Chipuk, J. E., Kuwana, T., Bouchier-Hayes, L., Droin, N. M., Newmeyer, D. D., Schuler, M., et al. (2004). Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science* 303, 1010–1014. doi: 10.1126/science.1092734

Cory, S., Huang, D. C., and Adams, J. M. (2003). The Bcl-2 family: roles in cell survival and oncogenesis. *Oncogene* 22, 8590–8607. doi: 10.1038/sj.onc.1207102

Daneshfar, A., Ghaziaskar, H. S., and Homayoun, N. (2008). Solubility of gallic acid in methanol, ethanol, water, and ethyl acetate. *J. Chem. Eng. Data* 53, 776–778. doi: 10.1021/je700633w

Di Maira, G., Salvi, M., Arrigoni, G., Marin, O., Sarno, S., Brustolon, F., et al. (2005). Protein kinase CK2 phosphorylates and upregulates Akt/PKB. *Cell Death Differ.* 12, 668–677. doi: 10.1038/sj.cdd.4401604

Ding, J., Zhang, Z., Roberts, G. J., Falcone, M., Miao, Y., Shao, Y., et al. (2010). Bcl-2 and Bax interact via the BH1-3 groove-BH3 motif interface and a novel interface involving the BH4 motif. *J. Biol. Chem.* 285, 28749–28763. doi: 10.1074/jbc.M110.148361

Elangovan, B., and Chinnadurai, G. (1997). Functional dissection of the pro-apoptotic protein Bik. Heterodimerization with anti-apoptosis proteins is insufficient for induction of cell death. *J. Biol. Chem.* 272, 24494–24498. doi: 10.1074/jbc.272.39.24494

Giorgi, C., Bonora, M., Sorrentino, G., Missiroli, S., Poletti, F., Suski, J. M., et al. (2015). p53 at the endoplasmic reticulum regulates apoptosis in a Ca2+-dependent manner. *Proc. Natl. Acad. Sci. U.S.A.* 112, 1779–1784. doi: 10.1073/pnas.1410723112

Guo, Z., Deshpande, R., and Paull, T. T. (2010a). ATM activation in the presence of oxidative stress. *Cell Cycle* 9, 4805–4811. doi: 10.4161/cc.9.24.14323

Guo, Z., Kozlov, S., Lavin, M. F., Person, M. D., and Paull, T. T. (2010b). ATM activation by oxidative stress. *Science* 330, 517–521. doi: 10.1126/science.1192912

Haupt, S., Berger, M., Goldberg, Z., and Haupt, Y. (2003). Apoptosis - the p53 network. *J. Cell Sci.* 116, 4077–4085. doi: 10.1242/jcs.00739

Heinonen, I. M., Meyer, A. S., and Frankel, E. N. (1998). Antioxidant activity of berry phenolics on human low-density lipoprotein and liposome oxidation. *J. Agric. Food Chem.* 46, 4107–4112. doi: 10.1021/jf980181c

Hemann, M. T., and Lowe, S. W. (2006). The p53-Bcl-2 connection. *Cell Death Differ.* 13, 1256–1259. doi: 10.1038/sj.cdd.4401962

Inoue, M., Suzuki, R., Sakaguchi, N., Li, Z., Takeda, T., Ogihara, Y., et al. (1995). Selective induction of cell death in cancer cells by gallic acid. *Biol. Pharm. Bull.* 18, 1526–1530. doi: 10.1248/bpb.18.1526

Keller, D. M., and Lu, H. (2002). p53 serine 392 phosphorylation increases after UV through induction of the assembly of the CK2.hSPT16.SSRP1 complex. *J. Biol. Chem.* 277, 50206–50213. doi: 10.1074/jbc.M209820200

Kim, M. S., Li, S. L., Bertolami, C. N., Cherrick, H. M., and Park, N. H. (1993). State of p53, Rb and DCC tumor suppressor genes in human oral cancer cell lines. *Anticancer Res.* 13, 1405–1413.

Kim, Y. J. (2007). Antimelanogenic and antioxidant properties of gallic acid. *Biol. Pharm. Bull.* 30, 1052–1055. doi: 10.1248/bpb.30.1052

Kroemer, G., Galluzzi, L., and Brenner, C. (2007). Mitochondrial membrane permeabilization in cell death. *Physiol. Rev.* 87, 99–163. doi: 10.1152/physrev.00013.2006

Kubo, Y., Urano, Y., Hida, Y., and Arase, S. (1999). Lack of somatic mutation in the PTEN gene in squamous cell carcinomas of human skin. *J. Dermatol. Sci.* 19, 199–201. doi: 10.1016/S0923-1811(98)00058-9

Lin, M. L., Chen, S. S., Huang, R. Y., Lu, Y. C., Liao, Y. R., Reddy, M. V., et al. (2014). Suppression of PI3K/Akt signaling by synthetic bichalcone analog TSWU-CD4 induces ER stress- and Bax/Bak-mediated apoptosis of cancer cells. *Apoptosis* 19, 1637–1653. doi: 10.1007/s10495-014-1031-y

Lin, M. L., Lu, Y. C., Chung, J. G., Li, Y. C., Wang, S. G., N, G. S., et al. (2010). Aloe-emodin induces apoptosis of human nasopharyngeal carcinoma cells via caspase-8-mediated activation of the mitochondrial death pathway. *Cancer Lett.* 291, 46–58. doi: 10.1016/j.canlet.2009.09.016

Lin, M. L., Lu, Y. C., Su, H. L., Lin, H. T., Lee, C. C., Kang, S. E., et al. (2011). Destabilization of CARP mRNAs by aloe-emodin contributes to caspase-8-mediated p53-independent apoptosis of human carcinoma cells. *J. Cell. Biochem.* 112, 1176–1191. doi: 10.1002/jcb.23031

Loughery, J., Cox, M., Smith, L. M., and Meek, D. W. (2014). Critical role for p53-serine 15 phosphorylation in stimulating transactivation at p53-responsive promoters. *Nucleic Acids Res.* 42, 7666–7680. doi: 10.1093/nar/gku501

Lu, H. L., Chen, S. S., Hsu, W. T., Lu, Y. C., Lee, C. C., Wu, T. S., et al. (2016). Suppression of phospho-p85alpha-GTP-Rac1 lipid raft interaction by bichalcone analog attenuates cancer cell invasion. *Mol. Carcinog.* 55, 2106–2120. doi: 10.1002/mc.22455

Lu, Y. C., Lin, M. L., Su, H. L., and Chen, S. S. (2016). ER-dependent Ca^{++}-mediated cytosolic ROS as an Effector for induction of mitochondrial apoptotic and ATM-JNK signal pathways in gallic acid-treated human oral cancer cells. *Anticancer Res.* 36, 697–705. Available online at: http://ar.iiarjournals.org/

Mathai, J. P., Germain, M., and Shore, G. C. (2005). BH3-only BIK regulates BAX,BAK-dependent release of Ca2+ from endoplasmic reticulum stores and mitochondrial apoptosis during stress-induced cell death. *J. Biol. Chem.* 280, 23829–23836. doi: 10.1074/jbc.M500800200

Meek, D. W. (2009). Tumour suppression by p53: a role for the DNA damage response? *Nat. Rev. Cancer* 9, 714–723. doi: 10.1038/nrc2716

Meek, D. W., Simon, S., Kikkawa, U., and Eckhart, W. (1990). The p53 tumour suppressor protein is phosphorylated at serine 389 by casein kinase II. *EMBO J* 9, 3253–3260.

Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P., et al. (2003). p53 has a direct apoptogenic role at the mitochondria. *Mol. Cell* 11, 577–590. doi: 10.1016/S1097-2765(03)00050-9

Moll, U. M., Wolff, S., Speidel, D., and Deppert, W. (2005). Transcription-independent pro-apoptotic functions of p53. *Curr. Opin. Cell Biol.* 17, 631–636. doi: 10.1016/j.ceb.2005.09.007

Ozcelik, B., Kartal, M., and Orhan, I. (2011). Cytotoxicity, antiviral and antimicrobial activities of alkaloids, flavonoids, and phenolic acids. *Pharm. Biol.* 49, 396–402. doi: 10.3109/13880209.2010.519390

Pinton, P., and Rizzuto, R. (2006). Bcl-2 and Ca2+ homeostasis in the endoplasmic reticulum. *Cell Death Differ.* 13, 1409–1418. doi: 10.1038/sj.cdd.4401960

Rizzuto, R., and Pozzan, T. (2006). Microdomains of intracellular Ca2+: molecular determinants and functional consequences. *Physiol. Rev.* 86, 369–408. doi: 10.1152/physrev.00004.2005

Ruzzene, M., Bertacchini, J., Toker, A., and Marmiroli, S. (2017). Cross-talk between the CK2 and AKT signaling pathways in cancer. *Adv. Biol. Regul.* 64, 1–8. doi: 10.1016/j.jbior.2017.03.002

Sano, D., Xie, T. X., Ow, T. J., Zhao, M., Pickering, C. R., Zhou, G., et al. (2011). Disruptive TP53 mutation is associated with aggressive disease characteristics in an orthotopic murine model of oral tongue cancer. *Clin. Cancer Res.* 17, 6658–6670. doi: 10.1158/1078-0432.CCR-11-0046

Sato, H., and Seiki, M. (1993). Regulatory mechanism of 92 kDa type IV collagenase gene expression which is associated with invasiveness of tumor cells. *Oncogene* 8, 395–405.

Scorrano, L., Oakes, S. A., Opferman, J. T., Cheng, E. H., Sorcinelli, M. D., Pozzan, T., et al. (2003). BAX and BAK regulation of endoplasmic reticulum Ca2+: a control point for apoptosis. *Science* 300, 135–139. doi: 10.1126/science.1081208

Shi, J., Yu, J., Pohorly, J. E., and Kakuda, Y. (2003). Polyphenolics in grape seeds-biochemistry and functionality. *J. Med. Food* 6, 291–299. doi: 10.1089/109662003772519831

Ullrich, S. J., Sakaguchi, K., Leesmiller, S. P., Fiscella, M., Mercer, W. E., Anderson, C. W., et al. (1993). Phosphorylation at Ser-15 and Ser-392 in mutant P53-molecules from human tumors is altered compared to wild-type P53. *Proc. Natl. Acad. Sci. U.S.A.* 90, 5954–5958. doi: 10.1073/pnas.90.13.5954

Unger, T., Mietz, J. A., Scheffner, M., Yee, C. L., and Howley, P. M. (1993). Functional domains of wild-type and mutant p53 proteins involved in transcriptional regulation, transdominant inhibition, and transformation suppression. *Mol. Cell. Biol.* 13, 5186–5194. doi: 10.1128/MCB.13.9.5186

Verma, S., Zhao, L. J., and Chinnadurai, G. (2001). Phosphorylation of the pro-apoptotic protein BIK: mapping of phosphorylation sites and effect on apoptosis. *J. Biol. Chem.* 276, 4671–4676. doi: 10.1074/jbc.M008983200

Wang, N. S., Unkila, M. T., Reineks, E. Z., and Distelhorst, C. W. (2001). Transient expression of wild-type or mitochondrially targeted Bcl-2 induces apoptosis, whereas transient expression of endoplasmic reticulum-targeted Bcl-2 is protective against Bax-induced cell death. *J. Biol. Chem.* 276, 44117–44128. doi: 10.1074/jbc.M101958200

Xie, T. X., Zhou, G., Zhao, M., Sano, D., Jasser, S. A., Brennan, R. G., et al. (2013). Serine substitution of proline at codon 151 of TP53 confers gain of function activity leading to anoikis resistance and tumor progression of head and neck cancer cells. *Laryngoscope* 123, 1416–1423. doi: 10.1002/lary.23846

Yin, X. M., Oltvai, Z. N., and Korsmeyer, S. J. (1994). BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. *Nature* 369, 321–323. doi: 10.1038/369321a0

Yoshioka, K., Kataoka, T., Hayashi, T., Hasegawa, M., Ishi, Y., and Hibasami, H. (2000). Induction of apoptosis by gallic acid in human stomach cancer KATO III and colon adenocarcinoma COLO 205 cell lines. *Oncol. Rep.* 7, 1221–1223. doi: 10.3892/or.7.6.1221

Zong, W. X., Li, C., Hatzivassiliou, G., Lindsten, T., Yu, Q. C., Yuan, J., et al. (2003). Bax and Bak can localize to the endoplasmic reticulum to initiate apoptosis. *J. Cell Biol* 162, 59–69. doi: 10.1083/jcb.200302084

Zuo, Y., Chen, H., and Deng, Y. (2002). Simultaneous determination of catechins, caffeine and gallic acids in green, Oolong, black and pu-erh teas using HPLC with a photodiode array detector. *Talanta* 57, 307–316. doi: 10.1016/S0039-9140(02)00030-9

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REVIEW
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Oxidative Stress in Oral Diseases: Understanding Its Relation with Other Systemic Diseases

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Oxidative stress occurs in diabetes, various cancers, liver diseases, stroke, rheumatoid arthritis, chronic inflammation, and other degenerative diseases related to the nervous system. The free radicals have deleterious effect on various organs of the body. This is due to lipid peroxidation and irreversible protein modification that leads to cellular apoptosis or programmed cell death. During recent years, there is a rise in the oral diseases related to oxidative stress. Oxidative stress in oral disease is related to other systemic diseases in the body such as periodontitis, cardiovascular, pancreatic, gastric, and liver diseases. In the present review, we discuss the various pathways that mediate oxidative cellular damage. Numerous pathways mediate oxidative cellular damage and these include caspase pathway, PERK/NRF2 pathway, NADPH oxidase 4 pathways and JNK/mitogen-activated protein (MAP) kinase pathway. We also discuss the role of inflammatory markers, lipid peroxidation, and role of oxygen species linked to oxidative stress. Knowledge of different pathways, role of inflammatory markers, and importance of low-density lipoprotein, fibrinogen, creatinine, nitric oxide, nitrates, and highly sensitive C-reactive proteins may be helpful in understanding the pathogenesis and plan better treatment for oral diseases which involve oxidative stress.

Keywords: oral, disease, oxidative stress, pathways, free radicals, inflammation

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OXIDATIVE STRESS AND RELATED DISEASES IN THE BODY

Oxidative stress occurs as a state of disturbance between free radical produced and the capability of antioxidant system to counteract such (Pisoschi and Pop, 2015). Free radicals are also classified as reactive oxygen species (ROS) or reactive nitrogen species (RNS) and both possess unpaired valence electrons. ROS can be further classified into oxygen centered radicals (superoxide anion, hydroxyl radicals, alkoxyl radicals, and peroxyl radicals) and oxygen centered non-radicals (hydrogen peroxide and singlet oxygen), while RNS consists of nitric oxide, nitric dioxide and peroxynitrite (El-Bahr, 2013). ROS are naturally occurring oxidants involved in numerous cellular biochemical events that are essential to life but at the same time capable of causing harmful oxidative stress when overproduced (McCord, 2000). Free radicals cause damage to all essential biocompounds such as DNA, proteins, and membrane lipids, thereby causing cause cell death. These free radicals are countered by inherent antioxidant system that exists in two major groups: enzymatic

(glutathione peroxidase, myeloperoxidase, superoxide dismutase, and catalase) and non-enzymatic (minerals, vitamins, polyphenols, and thiols; Gilgun-Sherki et al., 2001; Pisoschi and Pop, 2015).

Oxidative stress forms the basis of cancer, diabetes, rheumatoid arthritis, non-alcoholic fatty liver disease, chronic inflammation, stroke, aging, and numerous neurodegenerative diseases (Fridovich, 1999; Fang et al., 2002; Gentric et al., 2015; Pisoschi and Pop, 2015). Different epidemiological and clinical studies showed evidence of important role of oxidative stress and impairment of antioxidant defense systems in the pathogenesis, neoangiogenesis, and dissemination of local or distant cancers, such as cancers of the ovary and prostate (Oh et al., 2016; Saed et al., 2017). In any cancer, oxidative stress induced by hypoxia, was reported to promote oncogenic protein (MUC4) degradation via autophagy, enhancing the survival of cancer cells in the pancreas (Joshi et al., 2016). In Parkinson's disease, oxidative stress and aggregation of protein are the key pathogenic processes, where aggregation of α-synuclein results in aberrant free radical production and neuronal death (Deas et al., 2016). Similarly in Alzheimer's disease, amyloid-β inserts into the membrane systems to begin the oxidative stress during the disease progression in the brain (Swomley and Butterfield, 2015). In addition, increased oxidative stress and reduced superoxide dismutase levels were observed in human peripheral blood mononuclear cells which were obtained from patients with mild cognitive impairment (Mota et al., 2015).

OXIDATIVE STRESS RELATED TO ORAL DISEASES

Oral diseases such as periodontitis, dental caries, cancer in the oral cavity, HIV/AIDS, diseases involving mucosal and salivary glands, orofacial pain, and clefts, affect the oral health and hygiene (Jin et al., 2016). Global Burden of Disease 2015 study showed individuals with untreated oral conditions to increase from 2.5 billion in the year 1990 to 3.5 billion in 2015, with a 64% increase in disability-adjusted life year (Kassebaum et al., 2017). In addition, the direct and indirect treatment expenses due to dental diseases worldwide, were approximately US$442 billion in 2010 (Listl et al., 2015).

Among all oral diseases, the periodontal disease (comprising gingivitis and periodontitis), accounted for 3.5 million years lived with disability, US$54 billion/year in lost productivity and a major portion of the US$442 billion/year cost for oral diseases (Tonetti et al., 2017). Oxidative stress was involved in the progression of periodontitis, a chronic inflammatory disease of the periodontal tissue, caused by disturbance in the regulation of the host inflammatory in response to bacterial infection (Kataoka et al., 2016; Kanzaki et al., 2017). In chronic periodontitis, there was lower serum total antioxidant level and salivary capacity when compared to the control individuals (Ahmadi-Motamayel et al., 2017). Biomarkers of lipid peroxidation (one of the oxidative stress-mediated pathways) such as 8-isoprostane and malondialdehyde (MDA) were high in patients affected by chronic periondontitis (Akalin et al., 2007; Matthews et al., 2007; Pradeep et al., 2013). In addition, assessment of blood and gingival tissues of chronic periodontitis patients also revealed mitochondrial DNA deletion (5 kbp; Canakci et al., 2006). Gingival blood analysis of periodontitis patients also marked high level of 7-8-dihydro-8-ossiguanina (8-oxoG), a pre-mutagen base that results from ROS-mediated DNA damage (Takane et al., 2002; Krol, 2004). Similarly, higher level of 8-isoprostane concentration (an alternative approach to estimate lipid peroxidation) in the crevicular fluid of gingiva was detected in chronic periodontitis patients compared to those with gingivitis and healthy individuals (Pradeep et al., 2013). Serum reactive oxygen metabolite levels in periodontitis patients positively correlated to antibody levels with regard to bacteria such as *Porphyromonas gingivalis*, *Prevotella intermedia,* and *Eikenella corrodens* (Tamaki et al., 2014). Following scaling and root planning after systemic antioxidant lycopene administration, there was decrease in the oxidative stress and improvement in clinical parameters, which was maintained up to 4 months after discontinuation of antioxidant treatment (Ambati et al., 2017).

The pathogenesis of chronic inflammatory disease like oral lichen planus (OLP) is not well-understood (Tvarijonaviciute et al., 2017). Various studies showed that oxidative stress is involved in the pathogenesis of OLP. Significantly higher salivary ROS, lipid peroxidation, nitric oxide, and nitrite levels were found in OLP patients compared to the control subjects (Batu et al., 2016; Mehdipour et al., 2017; Tvarijonaviciute et al., 2017). The total antioxidant activity was significantly decreased in OLP patients with increased level of salivary malondialdehyde (MDA) compared to the healthy control group suggesting the possible role of the oxidants to orchestrate the disease via lipid peroxidation-mediated pathway (Lopez-Jornet et al., 2014; Shiva and Arab, 2016).

Oxidative stress was also correlated with oral cancer, as increased lipid peroxidation and reduced antioxidants was reported in patients suffering from stage II, III, and IV oral cancer (Manoharan et al., 2005). In addition to these findings, nitric oxide-mediated DNA damage was reported in patients with oral leukoplakia. Samples of oral epithelium taken from these patients recorded high levels of 8-nitroguanine and 8-oxoG (Ma et al., 2006).

OXIDATIVE STRESS MEDIATE CELLULAR DAMAGE

The deleterious effects of ROS in the event of oxidative stress are through lipid peroxidation and irreversible protein modification that leads to cellular apoptosis or programmed cell death (Ferrari, 2000). Numerous pathways mediate oxidative cellular damage and these include as caspase pathway, PERK/NRF2 pathway, NADPH oxidase 4 pathway and JNK/mitogen-activated protein (MAP) kinase pathway.

PATHWAYS THAT MEDIATE OXIDATIVE CELLULAR DAMAGE

Caspase Pathway

Caspases are a family of cysteine protease enzymes that carry out programmed cell death and inflammation. During apoptosis, caspases are activated to ensure that programmed cell death occurs with less damage to nearby tissues and also to ensure the degradation of components of the cell in a well-controlled manner (Rathore et al., 2015). Functionally, the caspases are classified into two major groups in apoptosis: Initiator caspases such as caspase-8 and -9, activate downstream caspases known as the executioner. These include caspase-3, -6, and -7 which are responsible for the breakdown of the cellular proteins (Creagh and Martin, 2001). In chronic periodontitis, caspase-3 concentration was significantly increased in gingival crevicular fluid and serum, and significantly correlated to the probing depth, gingival index, and clinical attachment level, thereby indicating apoptosis plays an important role in the destruction of periodontium tissues in chronic periodontitis (Pradeep et al., 2016).

There are two major caspase-associated apoptotic pathways related to oxidative damage (1) mitochondrial mediated pathways, and (2) the death receptor mediated pathway. In cells, caspases exist as zymogens (pro-caspases) which are activated only in the presence of appropriate stimulus such as the insult of oxidative stress (Slee et al., 1999). During activation, the caspases undergo proteolytic cleaving to dimerize into an active enzyme (Alnemri et al., 1996). In the mitochondria-mediated apoptotic pathway, hydrogen peroxide (H_2O_2) releases cytochrome C which binds to the apoptotic protease activating-factor 1 (Apaf-1) to initiate caspase-9 activation (Madesh and Hajnoczky, 2001; Andoh et al., 2002). ROS-mediated oxidative modification of caspase-9 at C403 residue promotes the interaction of caspase-9 with Apaf-1 via disulfide bonding that results in the apoptosome formation which leads to activation of caspase-9 (Zuo et al., 2009). The executioner, caspase-3 is speculated to be the converging point in both mitochondria-dependent and independent pathways in oxidative stress-driven apoptosis (Ueda et al., 2002; Kanthasamy et al., 2003). H_2O_2 activation of caspase-3 lead to activation of PKC delta and this contributes to the nuclear DNA breakdown and apoptotic cell death (Carvour et al., 2008).

In death receptor-mediated apoptotic pathway, caspase-8 channels apoptosis following oxidative stress (Baumgartner et al., 2007). Death receptors (also known as death-domain receptors) can promote the cleavage of pro-caspase-8 with appropriate stimulation (Boldin et al., 1996). Then, caspase-8 activates downstream executioner caspases (caspase-3; Jiang and Wang, 2004) or cleaves a pro-apoptotic protein known as Bid, which once activated translocates to the mitochondria and causes the release of cytochrome C, followed by fragmentation of DNA and apoptosis (Li et al., 1998). Interestingly, numerous studies reported crosstalk between caspase-8 and caspase-9 (**Figure 1**; Basu et al., 2006; Mareninova et al., 2006). Using pancreatic acinar cells, Baumgartner et al. (2007) showed reported partial inhibition of caspase-8 activation by caspase-9 and vice versa during H_2O_2-mediated apoptosis. The same study also reported the involvement of lysosomal proteins such as cathedpsin D and E (aspartyl proteases from lysosomes) in activation of caspase-8.

NADPH Oxidase 4 (NOX4) Pathway

NADPH oxidases (NOX) are enzymes that are known to catalyze the electron transfer from NADPH to molecular oxygen, to generate ROS as a microcode in immune response (DeLeo and Quinn, 1996). NOX protein family consists of NOX1, -2, -3, and -4 (Sahoo et al., 2016). The NOX4 isoform is expressed everywhere in the body, including heart, neuron, kidney, liver, and endothelial cells (Byrne et al., 2003; Vallet et al., 2005; Ray et al., 2011; Babelova et al., 2012; Crosas-Molist et al., 2014). NOX4 predominantly generates H_2O_2 in mitochondria where it is usually localized (Ago et al., 2008; Nisimoto et al., 2014; Sanders et al., 2015).

Recent research highlighted the mechanistic effects of NOX4 in oxidative stress (Vendrov et al., 2015; Theccanat et al., 2016). Unlike other isoforms of NOX, the NOX4 do not require cytosolic regulatory subunits in order to be activated. Instead, the enzyme is regulated by transcription factors which include E2F (Zhang et al., 2008), AP-1/Smad3 complex (Bai et al., 2014), retinoblastoma protein kinase (by regulating the activity of E2F), G-protein coupled receptor kinase 2 (Theccanat et al., 2016), and also via epigenetic regulation through increased association of histone H4K16 (Sanders et al., 2015). Enhanced NOX4 expression/activity and mitochondrial localization positively correlates to ROS production in mitochondria (Vendrov et al., 2015). Increased mitochondrial ROS leads to mitochondrial DNA damage, oxidation of mitochondrial proteins, and eventually apoptotic cell death (Madamanchi and Runge, 2013). This overproduced ROS is also likely to enter cytoplasm and activate numerous pro-apoptotic proteins such as caspase-9 and -3 (Tariq et al., 2013), caspase-1 (Moon et al., 2016). Excess ROS also causes a pro-inflammatory shift in the gene expression through nuclear factor-kappa-β activation (NF-κβ; Ungvari et al., 2007). Significant increased NOX4 levels was observed following inflammatory or hypoxic stimulation in periodontal ligament cells, which was accompanied by up-regulation of ROS and catalase levels (**Figure 2**; Golz et al., 2014). However, prolonged exposure to both stimuli leads to a decreased in catalase level suggesting the collapse of the antioxidative mechanism favoring oxidative stress and inflammatory response as observed in periodontitis (Golz et al., 2014).

NRF2—Antioxidant Response Element Signaling Pathway

The nuclear factor erythroid 2 (NFE2)-related factor (NRF2), a basic leucine zipper (bZIP) protein from the cap "n" collar (CNC) subfamily, protects against oxidative stress. NFE2 regulate the expression of antioxidant and detoxification proteins (Gold et al., 2012). NRF2 plays an important role in numerous diseases such as rheumatoid arthritis, atherosclerosis, oral cancer, and chronic periodontitis (Kim et al., 2010; Huang et al., 2013; Sima et al., 2016). NRF2 plays a role as a positive regulator of human Antioxidant Response Elements (AREs; Venugopal and Jaiswal, 1996). NRF2 was reported to regulate the expression

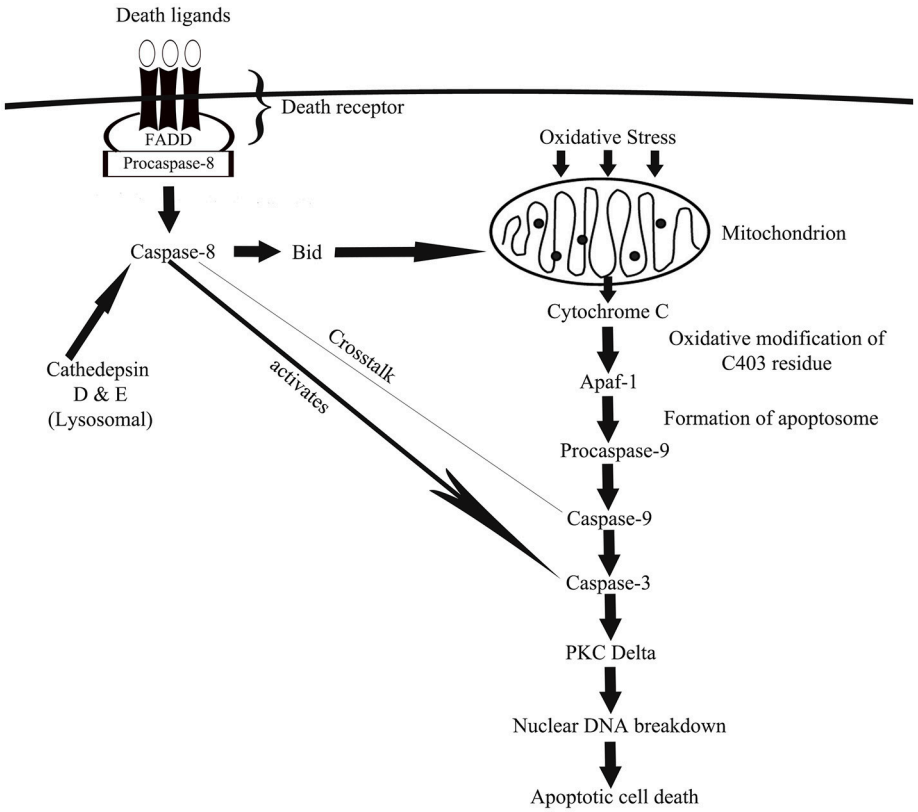

FIGURE 1 | A schematic depiction of how caspase-associated pathways leads to oxidative damage via mitochondrial- and death receptor-mediated cellular apoptosis. In mitochondrial-mediated apoptosis, generation of ROS causes the release of cytochrome C from mitochondria, which via a cascade of cellular actions activates caspase-9 and then caspase-3 that eventually channels cell death. In death receptor-mediated apoptosis, oxidative stress-driven death ligands activates caspase-8, which then activates a pro-apoptotic protein known as Bid, which once activated travels to mitochondria to facilitate mitochondria-mediated apoptotic pathway. Death receptor-activated caspase-8 also activates caspase-3 to induce cell death.

of numerous antioxidant enzymes, which include glutathione peroxidase, glutathione S-transferase, catalase, and superoxide dismutase (Niture et al., 2014).

The NRF2 activation was reported to involve a protein known as Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1 (Keap 1) by Itoh et al. (1999). The same study reported that under basal condition, Keap 1 anchored to cytoplasm act as a suppressor of NRF2 by physically binding to NRF2 and prevent the translocation of NRF2 to the nucleus to activate ARE-containing gene promotor regions (Itoh et al., 1999). Under this condition, NRF2 is rapidly degraded by proteasomes (Kobayashi et al., 2004) through polyubiquitination via Keap1/Cul3 ubiquitin ligase (Wakabayashi et al., 2003). Keap1/NRF2 complex "senses" the oxidative stress directly via reactive cysteine residues in Keap1 (Dinkova-Kostova et al., 2004) and NRF2 (Huang et al., 2002). NRF2 is activated by protein kinase C, MAP kinase and phosphatidylinositol-3 kinase (Yu et al., 1999; Kang et al., 2002; Numazawa et al., 2003). Following the "oxidant sensing," NRF2 is phosphorylated at serine40 to be released from Keap1 (Huang et al., 2002) and translocates to the nucleus where there is formation of a heterodimer with Maf and binds to AREs of numerous antioxidant gene promoter regions to begin their transcription (**Figure 3**; Itoh et al., 1997).

The critical role of NRF2 in protection against oxidative stress was shown through myriad studies (Chan et al., 2001; Talalay et al., 2003; Motohashi and Yamamoto, 2004). A recent study reported significant down-regulation of NRF2 pathway in patients with severe chronic periodontitis (Sima et al., 2016). NRF2 over-expression significantly improved anti-oxidative levels, increased cell proliferation, and inhibited periodontal ligament stem cell apoptosis (Liu et al., 2017).

Role of Different Inflammatory Markers

Inflammation and oxidative stress were found to be associated with numerous chronic diseases such as diabetes, cardiovascular disease (CVD), hypertension, alcoholic liver diseases, neurodegenerative diseases, cancer, and aging (Biswas and de Faria, 2007; Cachofeiro et al., 2008; Ambade and Mandrekar, 2012; Tucker et al., 2015). The accepted hypothesis is that inflammation can trigger oxidative stress and the oxidation also can induce inflammation.

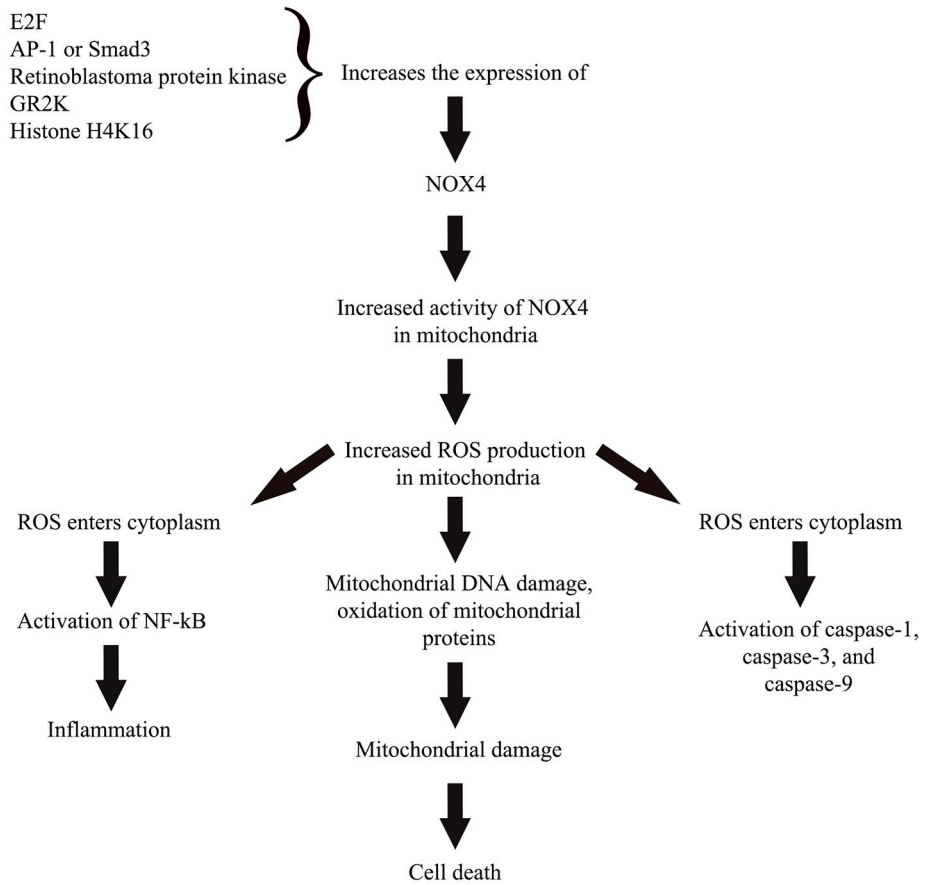

FIGURE 2 | A simplified representation on the role of NOX4 in cellular pathway toward ROS-induced oxidative stress. Activity of NOX4 is regulated through regulation of the enzyme's expression by various transcription factors, protein kinase, cellular receptor, and epigenetic regulator. Enhanced NOX4 expression or activity in mitochondria leads to increased ROS production that subsequently causes mitochondrial damage and cell death. Excess ROS also likely to travel toward cytoplasm and activate numerous pro-apoptotic proteins and also activate nuclear factor kappa beta to trigger a pro-inflammatory state of the cell.

Inflammation is the protective response of our biological system toward harmful exogenous and endogenous stimuli (Ferrero-Miliani et al., 2007). Inflammation is also an inherent immune response employed to safeguard our health. However, unregulated or exaggerated prolonged inflammation can cause tissue damage and chronic diseases. During the onset of inflammation, transcription factors such as activating protein-1 (AP-1) and NF-κβ induce pro-inflammatory gene expressions, which increases production of ROS while inducing oxidative stress (Tabas and Glass, 2013). Certain ROS such as H_2O_2 can enhance the pro-inflammatory gene expression (Flohe et al., 1997) through activation of numerous cellular pathways. Oxidative stress activates NOD-like receptor protein 3 (NRLP3) inflammasome (Shimada et al., 2012) which are responsible for maturation of pro-inflammatory proteins such as interleukin (IL)-18 and IL-1β (Schroder and Tschopp, 2010). The expression of pro-IL-1β was upregulated in human oral squamous cell carcinoma tumors and it increased the proliferation of dysplasia oral cells, stimulated oncogenic cytokines, and promoted the severity of oral squamous cell carcinoma (Lee et al., 2015).

Role of Oxygen Species Signal Assembly of the NLRP3 Inflammasome

The inflammasome is a part of the innate immune system and it responds to microbes or cellular stress through regulation of caspase-1 activation and induction of inflammation (Lamkanfi and Dixit, 2009). Among the numerous NLR inflammasome complexes such as NLRP1, -P2, -P3, -P6, -P12, and -C4 (Correa et al., 2012; Allen et al., 2013; Chen, 2014), the NLRP3 inflammasome influences the chronic inflammation and maturation of pro-inflammatory IL-1β and IL-18 (Davis et al., 2011). The expression of NLRP3 was significantly higher in patients with chronic periodontitis and generalized aggressive periodontitis, which were mainly distributed in inflammatory cells (Xue et al., 2015; Ran et al., 2017). *P. gingivalis* infection increased loss of alveolar bone, production of IL-1β, IL-6, IL-18, gingival gene expression of pro-IL-18 and pro-IL-1β, and activity of caspase-1 in peritoneal macrophages of wild-type mice, unlike in NLRP3-deficient mice. This suggests that *P. gingivalis* activate innate immune cells through the NLRP3 inflammasome in periodontal disease (Yamaguchi et al.,

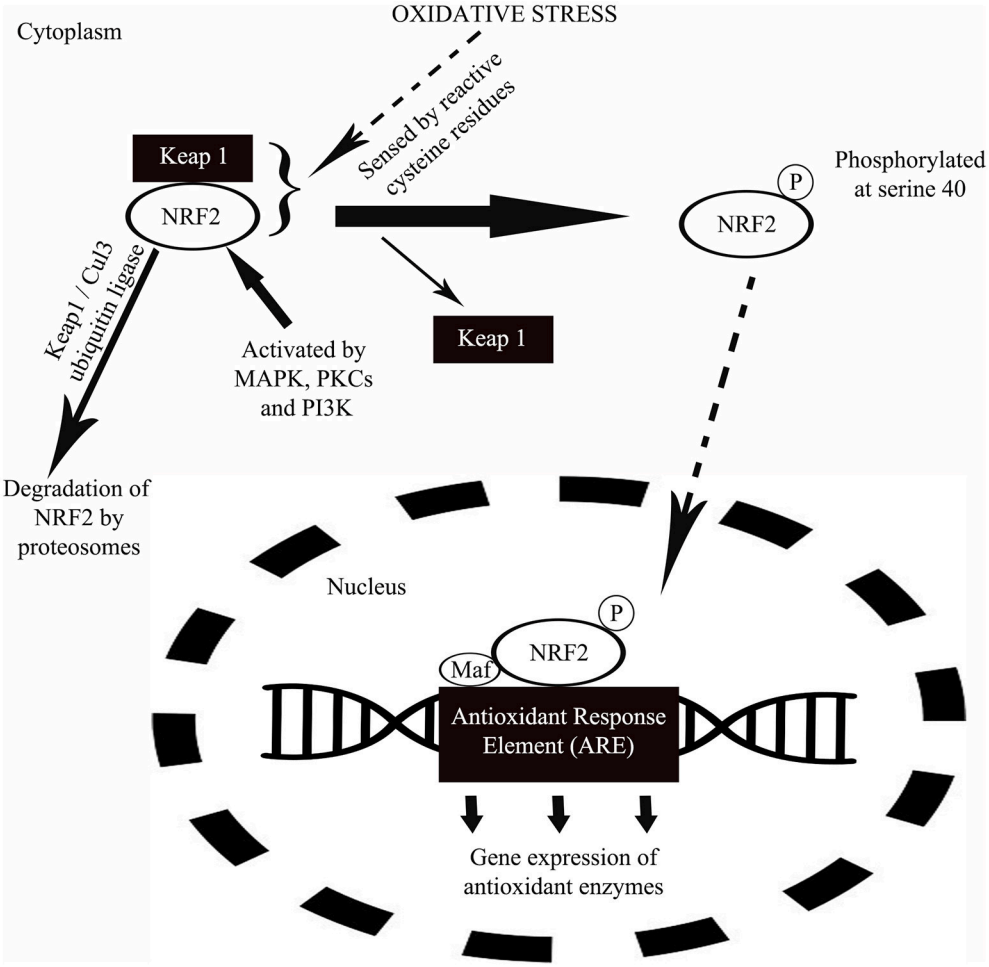

FIGURE 3 | A schematic diagram showing the mechanism of NRF2 in protection against oxidative stress. Under normal condition, a protein called Keap1 suppresses the activity of NRF2 by physically binding to NRF2 and at the same time anchored to cytoplasm. Following a period of inactivity, NRF2 is degraded by proteasomes. During oxidative stress, oxidants will be sensed by the Keap1/NRF2 complex via reactive cysteine residues in Keap1. Upon oxidant sensing, NRF2 undergoes phosphorylation at serine40 releasing Keap1 from the complex. This is followed by the translocation of NRF2 to nucleus where the protein forms a heterodimer with small transcription factor Maf and binds to antioxidant response enzyme (ARE) of numerous antioxidant gene promoter regions to initiate their transcription in response to oxidative stress.

2017). Similarly, *Fusobacterium nucleatum* infection involving the gingival epithelial cells, leads to NLRP3 inflammasome-dependent secretion of IL-1β (Bui et al., 2016). Activation of NLRP3 causes the activation of caspase-1 which is integral for the maturation of IL-18 and IL-1β into active cytokines and also the initiation of pryoptosis (Lamkanfi, 2011; Zhao et al., 2016).

Improper regulation of inflammasome could lead to the imbalance in between pro- and anti-inflammatory cytokines and result in inflammation and pryoptosis. NLRP3 inflammasome was reported to be activated by a host of molecules such as excess ROS, glucose, ATP, ceramides, sphingosine, crystals of cholesterol, uric acid, and oxidized LDL (Duewell et al., 2010; Jiang et al., 2012; Luheshi et al., 2012; Bandyopadhyay et al., 2013; Fukumoto et al., 2013). The exact underlying molecular mechanisms that regulate the assembly and activation of NLRP3 were not fully elucidated. However, recent studies reported the ROS signaling to activate NLRP3 inflammasome (Fukumoto et al., 2013; Heid et al., 2013).

The cellular source of ROS in influencing the activation of NLRP3 inflammasome arises from the byproduct of mitochondrial oxidative phosphorylation, NOX, xanthine oxidase, cyclooxygenase, and lipooxygenase (Habu et al., 1990; Lacy et al., 1998; Andrew and Mayer, 1999; Paravicini and Touyz, 2008; Sorbara and Girardin, 2011; Heid et al., 2013). For mitochondrial-ROS dependent NRLP3 inflammasome activation, numerous molecules such as saturated fatty acid palmitate (Wen et al., 2011), liposome (Zhong et al., 2013), and mitochondrial cardiolipin (Iyer et al., 2013) were reported to be involved. NOX acts as a mediator in various molecules associated activation of NRLP3 complex. For an instance, excess of extracellular ATP binding to P2X7 receptors in mammals leads to rapid accumulation of ROS that eventually activate

NRLP3 inflammasome (Riteau et al., 2012). The origin of ROS caused by excess ATP is reported to be NOX-derived (Cruz et al., 2007). Akin to ATP, alum, particulated metals and uric acid crystals were also shown to activate NLRP3 inflammasome via NOX-driven ROS generation (Martinon, 2010).

Lipid Peroxidation as a Result of Infection

Lipid peroxidation (LPO) is the oxidative deterioration of lipids caused by ROS. LPO is a chain reaction that mostly affects polyunsaturated fatty acids due to the presence of methylene bridges (-CH_2-) that possess reactive hydrogen atoms (Halliwell and Gutteridge, 1984). The chain reaction consists of three major steps including initiation, propagation and termination. For an in-depth information on the mechanism of LPO, we would suggest the readers to refer to review written by Repetto et al. (2012). The end-products of LPO are aldehyde, ethane, pentane, 2,3-transconjugated diens, isoprostains, and chlesteroloxides (Ustinova and Riabinin, 2003).

LPO has been implicated in numerous non-communicable diseases and aging-related disorders such as cataract, rheumatoid arthritis, atherosclerosis, and neurodegenerative diseases (Niki et al., 2005). In addition to these ailments, LPO was linked to infections such as influenza virus (Mileva et al., 2000; Kumar et al., 2003), acute and chronic fascioliasis (Kaya et al., 2007) and *Helicobacter pylori* infection (Davi et al., 2005). In addition, LPO as shown by salivary MDA level, was significantly increased in patients suffering from chronic periodontitis, OLP, oral leukoplakia, and oral squamous cell carcinoma (Baltacioglu et al., 2014; Malik et al., 2014; Metgud and Bajaj, 2014; Shirzad et al., 2014; Trivedi et al., 2015).

Role of Polyphenols

Polyphenols are naturally occurring compounds that are found in vegetables, fruits, beverages, herbs and spices. Examples of polyphenols include isoflavones, flavanols, flavones, phenolic acids, resveratrol, tannins, curcumin, anthovyanidins, and lignans (Tanigawa et al., 2007). In plants, polyphenols provide front line of protection from pathogens and ultraviolet light (Pandey and Rizvi, 2009).

Recent advances in research focusing on the anti-inflammatory and antioxidant effects of the polyphenols have shed light on the mechanisms of the phenolic compounds in scavenging free radicals, regulation of cytokine activities, and the maintenance of antioxidant enzyme system. Phenolic compounds scavenge free radicals through donation of an electron or hydrogen atom to various reactive oxygen, chlorine and nitrogen species (Tsao and Li, 2012). Phenolic compounds also directly inhibit Fe^{3+} reduction and thus generate reactive OH· (Perron and Brumaghim, 2009). These free radical scavenging and metal chelating effects of phenolic compounds interrupts the propagation stage of the LPO. Dietary phenolic compounds are able to restore inherent antioxidant enzymatic activities such as the superoxide dismutase, glutathione peroxidase, catalase, and glutathione reductase. Phenolic compounds control the expression of these enzymes through regulation of transcription factor NRF2 activities which in turn influences the ARE-mediated expression of the mentioned enzymes (Kohle and Bock, 2006). Flavanols, isoflavones, and flavones were reported to regulate the transcriptional activities of NRF2 (Zhang et al., 2003; Kohle and Bock, 2006).

In addition to antioxidant effects, dietary phenolic compounds were also shown to possess protective effects on inflammation through modulation of NLRP3 inflammasome. Recently, Hori et al. (2013) showed that green propolis rich in cinnamic acids inhibited inflammasome mediated secretion of IL-1β and activation of caspase-1. In separate studies, flavonoids such as procyanidin B2 and apigenin inhibited inflammasome-mediated secretion of IL-1β in LPS-induced human macrophages (Zhang et al., 2014; Martinez-Micaelo et al., 2015). Dietary phenolic compounds also reduced inflammation by attenuating pro-inflammatory cytokine-induced activation of NF-κβ by acting as AhR agonist regulator. By doing such, phenolic compounds modulate AhR-mediated signaling pathways that are involved in the activation of NF-κβ (Kohle and Bock, 2006; Vogel et al., 2014).

HOW ORAL INFECTIONS ARE LINKED TO OTHER DISEASES

Oral health is an important aspect of overall well-being of an organism. Numerous systemic conditions and diseases have oral origins (Beck et al., 1996; Li et al., 2000). At oral cavity, saliva act as the first line of defense against free radicals (Amerongen and Veerman, 2002; Battino et al., 2002) through antioxidants such as catalase, superoxide dismutase, and glutathione peroxidase (Battino et al., 2002). In the event of an infection, increased generation of free radicals outnumber antioxidants to initiate oxidative stress.

Periodontitis and Circulating Oxidants

Geerts et al. (2002) assessed the level of endotoxins in blood (pro-inflammatory factors) following mastication in patients with periodontitis. Endotoxin level was significantly higher following mastication in patients with severe periodontitis, thereby suggesting the possible detrimental effect of the oral disease on systemic health (Geerts et al., 2002). Myriad of clinical and pre-clinical findings were reported periodontal inflammation-generated ROS to diffuse into bloodstream, and gradually affecting other organs (Sobaniec and Sobaniec-Lotowska, 2000; Tomofuji et al., 2007; Baltacioglu et al., 2014). In addition to oxidants, the level of circulatory antioxidants were reported to be lower in periodontitis patients (Baltacioglu et al., 2006; Konopka et al., 2007).

Smoking is regarded as one of the most significant risk factors for the development of periodontitis. Smoking can also increase oxidative stress. Smoking may affect the alveola and tooth loss may be a feature. Smoking is perhaps the only modifiable cause which can check periodontitis.

Cardiovascular Disease

Numerous cross-sectional studies and systematic reviews highlighted oral diseases, particularly periodontitis which could be a risk factor for development of atherosclerotic CVD (Ahn et al., 2016; Bengtsson et al., 2016; Berlin-Broner et al.,

2016; Gomes et al., 2016; Hansen et al., 2016; Khatri et al., 2016; Zeng et al., 2016; Beukers et al., 2017; Natarajan and Midhun, 2017). A cross-sectional analytical study showed an association between periodontitis and dental parameters (gingival recession, pocket depth, clinical attachment level, and bleeding on probing) with the severity of coronary artery obstruction being measured by angiography (Ketabi et al., 2016). Similarly, periodontitis was associated with increased thickness of carotid intima-media and arterial stiffness, which are indicators of subclinical atherosclerosis and predicts CVD risk (Houcken et al., 2016; Wu et al., 2016). However, studies also showed lack of significant association between periodontal variables and obstruction of coronary vessels (Zanella et al., 2016). The risk of a myocardial infarction for the first time and peripheral arterial disease was significantly increased in patients with periodontitis (Ryden et al., 2016; Calapkorur et al., 2017). The link between periodontal disease and CVD with respect of detailed clinical findings in the patient was summarized in **Table 1**. The role of different microorganisms, involvement of ROS, different mechanisms involved, inflammatory markers, and the development of CVD was also represented in **Figure 4**.

The main mechanisms linking oral diseases to CVD involve actions of oral bacteria on the blood vessels, and systemic inflammation (Kholy et al., 2015). Bacterial (*T. denticola*,

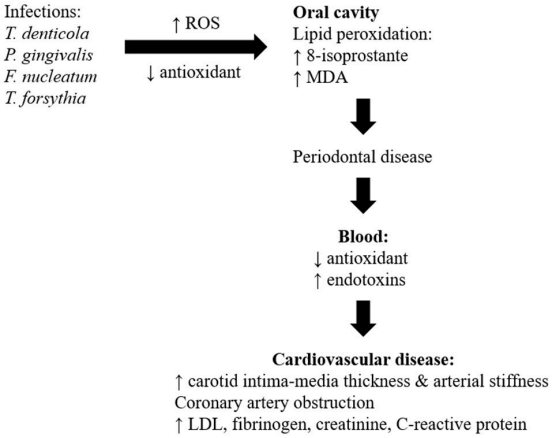

FIGURE 4 | Schematic diagram to show how oral disease may be linked to cardiovascular disease.

TABLE 1 | Association between periodontal disease and cardiovascular diseases.

Participants	Sample size	Findings	References
Adults with aged >40 years in Korea.	1,343	Periodontitis was associated with subclinical atherosclerosis and peripheral arterial disease.	Ahn et al., 2016
Adults with aged 60–96 years in Sweden.	499	Significant association between periodontitis and carotid calcification.	Bengtsson et al., 2016
Participants in the baltimore longitudinal study of aging.	278	Periodontal disease, endodontic burden, number of teeth and oral inflammatory burden were associated with incident cardiovascular events.	Gomes et al., 2016
Periodontitis patients in Denmark	100,694	Periodontitis patients were at higher risk of myocardial infarction, ischemic stroke, cardiovascular death, major adverse cardiovascular events, and all-cause mortality.	Hansen et al., 2016
Dental patients in University of Amsterdam.	109	Periodontitis is associated with increased arterial stiffness.	Houcken et al., 2016
Coronary artery obstruction patients in Isfahan, Iran.	82	Positive correlation between variables gingival recession, pocket depth, clinical attachment level, decayed, missing, decayed-missing-filled, bleeding on probing, and degree of coronary artery obstruction.	Ketabi et al., 2016
Adults with aged 35–65 years in Bhopal, India.	40	Periodontitis patients was associated with higher carotid intima-media thickness and diastolic blood pressure.	Khatri et al., 2016
Adults with mean age 46 years, mean BMI 21.1 kg/m2 in Bangladesh.	917	Mean attachment loss was associated with increased carotid intima-media thickness.	Wu et al., 2016
Patients that underwent coronary angiography.	195	No significant associations were found between periodontal variables and vessel obstruction. Tooth loss was found to be a risk indicator for coronary heart disease.	Zanella et al., 2016
Meta-analysis of 15 observational studies	17,330	Presence of periodontal disease was associated with carotid atherosclerosis.	Zeng et al., 2016
Dental patients aged >35 years in Netherlands.	60,174	Periodontitis showed significant association with atherosclerosis.	Beukers et al., 2017
Patients referred from the Department of Cardiovascular Surgery to Department of Periodontology.	60	Periodontitis raised the odds ratio for having peripheral arterial disease.	Calapkorur et al., 2017
Adults with aged 20–40 years in India.	60	Severe generalized periodontitis was associated with subclinical atherosclerosis	Natarajan and Midhun, 2017

P. gingivalis, *F. nucleatum,* and *T. forsythia*) genomic DNA were detected in visceras such as aorta, heart, kidney, lung, and liver in mice induced with periodontitis suggesting that oral bacteria were able to gain entry into the blood stream from inflamed gingival thereby producing low-level transient bacteremia (Chukkapalli et al., 2017). Various periodontal bacteria were identified in atherosclerotic plaques and were deemed possible contributors to the disease. Different bacterial co-occurrences were detected in the plaques of subgingiva and plaque of atherosclerosis patients, including *T. forsythia*, *T. denticol, P. gingivalis* and *P. nigrescens* (Mahalakshmi et al., 2017). The bacteria in the atherosclerotic plaques created biofilms that stimulated the innate immune system reactant Toll-like receptor 2. This contributed to chronic inflammation and continued the immune system activation (Allen et al., 2016). Study also showed positive correlation between the periodontal bacteria levels and CVD risk associated mediators (low-density lipoprotein, fibrinogen, creatinine, and highly sensitive C-reactive proteins) levels in subjects with periodontitis and atherosclerosis (Bozoglan et al., 2017).

The local production and accumulation of inflammatory mediators in severe generalized periodontitis can cause systemic inflammation and endothelial dysfunction (Tonetti, 2009). *P. gingivalis* oral infection in mice, induced alterations in systemic cytokine production, i.e., up-regulation of matrix metalloproteinase 3, intercellular adhesion molecule 1, insulin-like growth factor binding protein 2, chemokine (C-X-C motif) ligand 7 and the down-regulation of interleukin-17, L-selectin, and tumor necrosis factor-α (Miyauchi et al., 2012). Similarly in patients with coronary heart disease, periodontitis was associated with increased systemic inflammation (elevated IFN-γ, IL-10, and TNF-α levels; Kampits et al., 2016).

Recent studies indicated that periodontal treatment attenuated pro-artherosclerotic factors. Following periodontal treatment, white blood cells, low-density lipoprotein, platelet, fibrinogen, creatinine, and highly sensitive C-reactive proteins levels were significantly reduced and high-density lipoprotein levels significantly increased in patients with periodontitis and atherosclerosis, as well as in patients diagnosed with periodontitis alone (Bozoglan et al., 2017). Similarly, systemic markers of atherosclerosis: adrenomedullin and chemokine (C-C motif) ligand 28 levels changed significantly in the periodontitis and atherosclerotic patients, compared to the non-artherosclerotic periodontitis patients following non-surgical periodontal treatment. Decrease in serum neopterin induced by periodontal treatment contributed to the increased arterial elasticity in periodontitis patients (Ren et al., 2015). The results of these studies suggests that removal and reduction of these periodontal bacteria in subgingival plaque may be an important prophylactic measure to periodontitis and atherosclerosis (Mahalakshmi et al., 2017).

Liver Diseases

Using rat model of periodontitis (lipopolysaccharide/protease-induced), Tomofuji et al. (2008) reported oxidative DNA damage in the liver of experimental rats. Supporting the notion, another rat model of periodontitis, that is the ligature-induced model showed a decrease in the liver antioxidant, glutathione and increase in circulating level of hexanoyl-lysine suggesting a possible link between periodontitis-generated oxidants and liver damage (Tomofuji et al., 2008). In humans, limited literature has related periodontitis and liver diseases (Furuta et al., 2010; Han et al., 2016).

Pancreatic Disease

A recently published meta-analysis associated periodontitis with pancreatic cancer. The study estimated the relative risk for periodontitis and pancreatic cancer to be 1.74 (95% Cl, 1.41–2.15) based on findings from three continents (Maisonneuve et al., 2017). Despite a large number of studies associating periodontitis and pancreatic, however, the underlying mechanism of the disease is poorly understood.

Gastric Disorders

H. pylori (bacteria implicated in gastritis and peptic ulcers) has been shown to be harbored by periodontal pockets in periodontitis patients (Soory, 2010). In parallel to this finding, periodontitis patients showed presence of *H. pylori* in subgingival biofilm (Riggio and Lennon, 1999; Gebara et al., 2004). Gastric carcinoma has been correlated with *H. pylori* infection-mediated ROS production, DNA damage along other endogenous and exogenous factors (Farinati et al., 2008). In a study investigating the effects of outer membrane vesicles of *H. pylori* in human gastric epithelial cells, oxidative stress-associated genomic damage with glutathione was noticed (Chitcholtan et al., 2008). In a separate study, *H. pylori* elicited mitochondrial damage in gastric epithelial cells, causing oxidative burst and mitochondrial-ROS mediated apoptosis (Calvino-Fernandez et al., 2008).

Alzheimer's Disease

Studies have suggested significant association between periodontitis and Alzheimer's disease, which may be mediated through effects on systemic inflammation (Ide et al., 2016; Leira et al., 2017). The risk of developing dementia were higher for periodontitis patients aged 65 and older, compared to healthy individuals (Shin et al., 2016; Lee et al., 2017). Alzheimer's patients showed high serum IL-6 levels while periodontitis patients had high serum TNF-α levels (Cestari et al., 2016). The association between these cytokine levels in periodontitis and Alzheimer's patients suggests their implication in the overlapping mechanisms between periodontitis and Alzheimer's disease (Cestari et al., 2016). In addition, patients with severe periodontitis had higher blood $A\beta_{1-42}$ levels and higher $A\beta_{42/40}$ ratio (Gil-Montoya et al., 2017).

SUMMARY AND PERSPECTIVE

Oxidative stress causes damage to various organs in the human body. Proper understanding of oxidative stress and its pathways, free radicals and inflammatory markers related to oral diseases are important for effective treatment. Future drug targets may be planned according to the different pathways involved in inflammation and oxidative process.

AUTHOR CONTRIBUTIONS

Conceptual framework and design: SD, PM. Searched references: JK and SLT. Drafted manuscript: SD, JK, and SLT. Critically revised the manuscript: SD, JK, SLT, PM.

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REFERENCES

Ago, T., Liu, T., Zhai, P., Chen, W., Li, H., Molkentin, J. D., et al. (2008). A redox-dependent pathway for regulating class II HDACs and cardiac hypertrophy. *Cell* 133, 978–993. doi: 10.1016/j.cell.2008.04.041

Ahmadi-Motamayel, F., Goodarzi, M. T., Jamshidi, Z., and Kebriaei, R. (2017). Evaluation of salivary and serum antioxidant and oxidative stress statuses in patients with chronic periodontitis: a case-control study. *Front. Physiol.* 8:189. doi: 10.3389/fphys.2017.00189

Ahn, Y. B., Shin, M. S., Han, D. H., Sukhbaatar, M., Kim, M. S., Shin, H. S., et al. (2016). Periodontitis is associated with the risk of subclinical atherosclerosis and peripheral arterial disease in Korean adults. *Atherosclerosis* 251, 311–318. doi: 10.1016/j.atherosclerosis.2016.07.898

Akalin, F. A., Baltacioglu, E., Alver, A., and 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. doi: 10.1111/j.1600-051X.2007.01091.x

Allen, H. B., Boles, J., Morales, D., Ballal, S., and Joshi, S. G. (2016). Arteriosclerosis: the novel finding of biofilms and innate immune system activity within the plaques. *J. Med. Surg. Pathol.* 1:135. doi: 10.4172/jmsp.1000121

Allen, I. C., McElvania-TeKippe, E., Wilson, J. E., Lich, J. D., Arthur, J. C., Sullivan, J. T., et al. (2013). Characterization of NLRP12 during the *in vivo* host immune response to *Klebsiella pneumoniae* and *Mycobacterium tuberculosis*. *PLoS ONE* 8:e60842. doi: 10.1371/annotation/2190ebe1-452c-4966-9f74-9813378524d4

Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salvesen, G., Thornberry, N. A., Wong, W. W., et al. (1996). Human ICE/CED-3 protease nomenclature. *Cell* 87:171. doi: 10.1016/S0092-8674(00)81334-3

Ambade, A., and Mandrekar, P. (2012). Oxidative stress and inflammation: essential partners in alcoholic liver disease. *Int. J. Hepatol.* 2012:853175. doi: 10.1155/2012/853175

Ambati, M., Rani, K. R., Reddy, P. V., Suryaprasanna, J., Dasari, R., and Gireddy, H. (2017). Evaluation of oxidative stress in chronic periodontitis patients following systemic antioxidant supplementation: a clinical and biochemical study. *J. Nat. Sci. Biol. Med.* 8, 99–103. doi: 10.4103/0976-9668.198366

Amerongen, A. V., and Veerman, E. C. (2002). Saliva-the defender of the oral cavity. *Oral Dis.* 8, 12–22. doi: 10.1034/j.1601-0825.2002.1o816.x

Andoh, T., Chock, P. B., and Chiueh, C. C. (2002). The roles of thioredoxin in protection against oxidative stress-induced apoptosis in SH-SY5Y cells. *J. Biol. Chem.* 277, 9655–9660. doi: 10.1074/jbc.M110701200

Andrew, P. J., and Mayer, B. (1999). Enzymatic function of nitric oxide synthases. *Cardiovasc. Res.* 43, 521–531. doi: 10.1016/S0008-6363(99)00115-7

Babelova, A., Avaniadi, D., Jung, O., Fork, C., Beckmann, J., Kosowski, J., et al. (2012). Role of Nox4 in murine models of kidney disease. *Free Radic. Biol. Med.* 53, 842–853. doi: 10.1016/j.freeradbiomed.2012.06.027

Bai, G., Hock, T. D., Logsdon, N., Zhou, Y., and Thannickal, V. J. (2014). A far-upstream AP-1/Smad binding box regulates human NOX4 promoter activation by transforming growth factor-β. *Gene* 540, 62–67. doi: 10.1016/j.gene.2014.02.026

Baltacioglu, E., Akalin, F. A., Alver, A., Balaban, F., Unsal, M., and 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, 385–392. doi: 10.1111/j.1600-051X.2006.00923.x

Baltacioglu, E., Yuva, P., Aydin, G., Alver, A., Kahraman, C., Karabulut, E., et al. (2014). Lipid peroxidation levels and total oxidant/antioxidant status in serum and saliva from patients with chronic and aggressive periodontitis. Oxidative stress index: a new biomarker for periodontal disease? *J. Periodontol.* 85, 1432–1441. doi: 10.1902/jop.2014.130654

Bandyopadhyay, S., Lane, T., Venugopal, R., Parthasarathy, P. T., Cho, Y., Galam, L., et al. (2013). MicroRNA-133a-1 regulates inflammasome activation through uncoupling protein-2. *Biochem. Biophys. Res. Commun.* 439, 407–412. doi: 10.1016/j.bbrc.2013.08.056

Basu, A., Castle, V. P., Bouziane, M., Bhalla, K., and Haldar, S. (2006). Crosstalk between extrinsic and intrinsic cell death pathways in pancreatic cancer: synergistic action of estrogen metabolite and ligands of death receptor family. *Cancer Res.* 66, 4309–4318. doi: 10.1158/0008-5472.CAN-05-2657

Battino, M., Ferreiro, M. S., Gallardo, I., Newman, H. N., and Bullon, P. (2002). The antioxidant capacity of saliva. *J. Clin. Periodontol.* 29, 189–194. doi: 10.1034/j.1600-051X.2002.290301x.x

Batu, S., Ofluoglu, D., Ergun, S., Warnakulasuriya, S., Uslu, E., Guven, Y., et al. (2016). Evaluation of prolidase activity and oxidative stress in patients with oral lichen planus and oral lichenoid contact reactions. *J. Oral Pathol. Med.* 45, 281–288. doi: 10.1111/jop.12391

Baumgartner, H. K., Gerasimenko, J. V., Thorne, C., Ashurst, L. H., Barrow, S. L., Chvanov, M. A., et al. (2007). Caspase-8-mediated apoptosis induced by oxidative stress is independent of the intrinsic pathway and dependent on cathepsins. *Am. J. Physiol. Gastrointest. Liver Physiol.* 293, G296–G307. doi: 10.1152/ajpgi.00103.2007

Beck, J., Garcia, R., Heiss, G., Vokonas, P. S., and Offenbacher, S. (1996). Periodontal disease and cardiovascular disease. *J. Periodontol.* 67, 1123–1137. doi: 10.1902/jop.1996.67.10s.1123

Bengtsson, V. W., Persson, G. R., Berglund, J., and Renvert, S. (2016). A cross-sectional study of the associations between periodontitis and carotid arterial calcifications in an elderly population. *Acta Odontol. Scand.* 74, 115–120. doi: 10.3109/00016357.2015.1050603

Berlin-Broner, Y., Febbraio, M., and Levin, L. (2016). Association between apical periodontitis and cardiovascular diseases: a systematic review of the literature. *Int. Endod. J.* 50, 847–859. doi: 10.1111/iej.12710

Beukers, N. G., van der Heijden, G. J., van Wijk, A. J., and Loos, B. G. (2017). Periodontitis is an independent risk indicator for atherosclerotic cardiovascular diseases among 60 174 participants in a large dental school in the Netherlands. *J. Epidemiol. Commun. Health* 71, 37–42. doi: 10.1136/jech-2015-206745

Biswas, S. K., and de Faria, J. B. (2007). Which comes first: renal inflammation or oxidative stress in spontaneously hypertensive rats? *Free Radic. Res.* 41, 216–224. doi: 10.1080/10715760601059672

Boldin, M. P., Goncharov, T. M., Goltsev, Y. V., and Wallach, D. (1996). Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. *Cell* 85, 803–815. doi: 10.1016/S0092-8674(00)81265-9

Bozoglan, A., Ertugrul, A. S., Taspinar, M., and Yuzbasioglu, B. (2017). Determining the relationship between atherosclerosis and periodontopathogenic microorganisms in chronic periodontitis patients. *Acta Odontol. Scand.* 75, 233–242. doi: 10.1080/00016357.2017.1280739

Bui, F. Q., Johnson, L., Roberts, J., Hung, S. C., Lee, J., Atanasova, K. R., et al. (2016). *Fusobacterium nucleatum* infection of gingival epithelial cells leads to NLRP3 inflammasome-dependent secretion of IL-1β and the danger signals ASC and HMGB1. *Cell. Microbiol.* 18, 970–981. doi: 10.1111/cmi.12560

Byrne, J. A., Grieve, D. J., Bendall, J. K., Li, J. M., Gove, C., Lambeth, J. D., et al. (2003). Contrasting roles of NADPH oxidase isoforms in pressure-overload versus angiotensin II-induced cardiac hypertrophy. *Circ. Res.* 93, 802–805. doi: 10.1161/01.RES.0000099504.30207.F5

Cachofeiro, V., Goicochea, M., de Vinuesa, S. G., Oubina, P., Lahera, V., and Luno, J. (2008). Oxidative stress and inflammation, a link between chronic kidney disease and cardiovascular disease. *Kidney Int. Suppl.* 111, S4–S9. doi: 10.1038/ki.2008.516

Calapkorur, M. U., Alkan, B. A., Tasdemir, Z., Akcali, Y., and Saatci, E. (2017). Association of peripheral arterial disease with periodontal disease: analysis of inflammatory cytokines and an acute phase protein in gingival crevicular fluid and serum. *J. Periodont. Res.* 52, 532–539. doi: 10.1111/jre.12419

Calvino-Fernandez, M., Benito-Martinez, S., and Parra-Cid, T. (2008). Oxidative stress by *Helicobacter pylori* causes apoptosis through mitochondrial pathway in gastric epithelial cells. *Apoptosis* 13, 1267–1280. doi: 10.1007/s10495-008-0255-0

Canakci, C. F., Tatar, A., Canakci, V., Cicek, Y., Oztas, S., and Orbak, R. (2006). New evidence of premature oxidative DNA damage: mitochondrial DNA deletion in gingival tissue of patients with periodontitis. *J. Periodontol.* 77, 1894–1900. doi: 10.1902/jop.2006.060108

Carvour, M., Song, C., Kaul, S., Anantharam, V., Kanthasamy, A., and Kanthasamy, A. (2008). Chronic low-dose oxidative stress induces caspase-3-dependent PKCd proteolytic activation and apoptosis in a cell culture model of dopaminergic neurodegeneration. *Ann. N. Y. Acad. Sci.* 1139, 197–205. doi: 10.1196/annals.1432.020

Cestari, J. A., Fabri, G. M., Kalil, J., Nitrini, R., Jacob-Filho, W., de Siqueira, J. T., et al. (2016). Oral infections and cytokine levels in patients with Alzheimer's disease and mild cognitive impairment compared with controls. *J. Alzheimers Dis.* 52, 1479–1485. doi: 10.3233/JAD-160212

Chan, K., Han, X. D., and Kan, Y. W. (2001). An important function of Nrf2 in combating oxidative stress: detoxification of acetaminophen. *Proc. Natl. Acad. Sci. U.S.A.* 98, 4611–4616. doi: 10.1073/pnas.081082098

Chen, G. Y. (2014). Role of Nlrp6 and Nlrp12 in the maintenance of intestinal homeostasis. *Eur. J. Immunol.* 44, 321–327. doi: 10.1002/eji.201344135

Chitcholtan, K., Hampton, M. B., and Keenan, J. I. (2008). Outer membrane vesicles enhance the carcinogenic potential of *Helicobacter pylori*. *Carcinogenesis* 29, 2400–2405. doi: 10.1093/carcin/bgn218

Chukkapalli, S. S., Easwaran, M., Rivera-Kweh, M. F., Velsko, I. M., Ambadapadi, S., Dai, J., et al. (2017). Sequential colonization of periodontal pathogens in induction of periodontal disease and atherosclerosis in LDLR^{null} mice. *Pathog. Dis.* 75:ftx003. doi: 10.1093/femspd/ftx003

Correa, R. G., Milutinovic, S., and Reed, J. C. (2012). Roles of NOD1 (NLRC1) and NOD2 (NLRC2) in innate immunity and inflammatory diseases. *Biosci. Rep.* 32, 597–608. doi: 10.1042/BSR20120055

Creagh, E. M., and Martin, S. J. (2001). Caspases: cellular demolition experts. *Biochem. Soc. Trans.* 29, 696–702. doi: 10.1042/bst0290696

Crosas-Molist, E., Bertran, E., Sancho, P., Lopez-Luque, J., Fernando, J., Sanchez, A., et al. (2014). The NADPH oxidase NOX4 inhibits hepatocyte proliferation and liver cancer progression. *Free Radic. Biol. Med.* 69, 338–347. doi: 10.1016/j.freeradbiomed.2014.01.040

Cruz, C. M., Rinna, A., Forman, H. J., Ventura, A. L., Persechini, P. M., and Ojcius, D. M. (2007). ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages. *J. Biol. Chem.* 282, 2871–2879. doi: 10.1074/jbc.M608083200

Davi, G., Neri, M., Falco, A., Festi, D., Taraborelli, T., Ciabattoni, G., et al. (2005). *Helicobacter pylori* infection causes persistent platelet activation *in vivo* through enhanced lipid peroxidation. *Arterioscler. Thromb. Vasc. Biol.* 25, 246–251. doi: 10.1161/01.ATV.0000147128.10278.99

Davis, B. K., Wen, H., and Ting, J. P. (2011). The inflammasome NLRs in immunity, inflammation, and associated diseases. *Annu. Rev. Immunol.* 29, 707–735. doi: 10.1146/annurev-immunol-031210-101405

Deas, E., Cremades, N., Angelova, P. R., Ludtmann, M. H., Yao, Z., Chen, S., et al. (2016). Alpha-synuclein oligomers interact with metal ions to induce oxidative stress and neuronal death in Parkinson's disease. *Antioxid. Redox Signal.* 24, 376–391. doi: 10.1089/ars.2015.6343

DeLeo, F. R., and Quinn, M. T. (1996). Assembly of the phagocyte NADPH oxidase: molecular interaction of oxidase proteins. *J. Leukoc. Biol.* 60, 677–691.

Dinkova-Kostova, A. T., Fahey, J. W., and Talalay, P. (2004). Chemical structures of inducers of nicotinamide quinone oxidoreductase 1 (NQO1). *Methods Enzymol.* 382, 423–448. doi: 10.1016/S0076-6879(04)82023-8

Duewell, P., Kono, H., Rayner, K. J., Sirois, C. M., Vladimer, G., Bauernfeind, F. G., et al. (2010). NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature* 464, 1357–1361. doi: 10.1038/nature08938

El-Bahr, S. M. (2013). Biochemistry of free radicals and oxidative stress. *Sci. Int.* 1, 111–117. doi: 10.5567/sciintl.2013.111.117

Fang, Y. Z., Yang, S., and Wu, G. (2002). Free radicals, antioxidants, and nutrition. *Nutrition* 18, 872–879. doi: 10.1016/S0899-9007(02)00916-4

Farinati, F., Cardin, R., Cassaro, M., Bortolami, M., Nitti, D., Tieppo, C., et al. (2008). *Helicobacter pylori*, inflammation, oxidative damage and gastric cancer: a morphological, biological and molecular pathway. *Eur. J. Cancer Prev.* 17, 195–200. doi: 10.1097/CEJ.0b013e3282f0bff5

Ferrari, C. K. B. (2000). Free radicals, lipid peroxidation and antioxidants in apoptosis: implications in cancer, cardiovascular and neurological diseases. *Biologia* 55, 581–590.

Ferrero-Miliani, L., Nielsen, O. H., Andersen, P. S., and Girardin, S. E. (2007). Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1β generation. *Clin. Exp. Immunol.* 147, 227–235. doi: 10.1111/j.1365-2249.2006.03261.x

Flohe, L., Brigelius-Flohe, R., Saliou, C., Traber, M. G., and Packer, L. (1997). Redox regulation of NF-kappa B activation. *Free Radic. Biol. Med.* 22, 1115–1126. doi: 10.1016/S0891-5849(96)00501-1

Fridovich, I. (1999). Fundamental aspects of reactive oxygen species, or what's the matter with oxygen? *Ann. N. Y. Acad. Sci.* 893, 13–18.

Fukumoto, J., Fukumoto, I., Parthasarathy, P. T., Cox, R., Huynh, B., Ramanathan, G. K., et al. (2013). NLRP3 deletion protects from hyperoxia-induced acute lung injury. *Am. J. Physiol. Cell Physiol.* 305, C182–189. doi: 10.1152/ajpcell.00086.2013

Furuta, M., Ekuni, D., Yamamoto, T., Irie, K., Koyama, R., Sanbe, T., et al. (2010). Relationship between periodontitis and hepatic abnormalities in young adults. *Acta Odontol. Scand.* 68, 27–33. doi: 10.3109/00016350903291913

Gebara, E. C., Pannuti, C., Faria, C. M., Chehter, L., Mayer, M. P., and Lima, L. A. (2004). Prevalence of *Helicobacter pylori* detected by polymerase chain reaction in the oral cavity of periodontitis patients. *Oral Microbiol. Immunol.* 19, 277–280. doi: 10.1111/j.1399-302X.2004.00153.x

Geerts, S. O., Nys, M., De, M. P., Charpentier, J., Albert, A., Legrand, V., et al. (2002). Systemic release of endotoxins induced by gentle mastication: association with periodontitis severity. *J. Periodontol.* 73, 73–78. doi: 10.1902/jop.2002.73.1.73

Gentric, G., Maillet, V., Paradis, V., Couton, D., L'Hermitte, A., Panasyuk, G., et al. (2015). Oxidative stress promotes pathologic polyploidization in nonalcoholic fatty liver disease. *J. Clin. Invest.* 125, 981–992. doi: 10.1172/JCI73957

Gilgun-Sherki, Y., Melamed, E., and Offen, D. (2001). Oxidative stress induced-neurodegenerative diseases: the need for antioxidants that penetrate the blood brain barrier. *Neuropharmacology* 40, 959–975. doi: 10.1016/S0028-3908(01)00019-3

Gil-Montoya, J. A., Barrios, R., Santana, S., Sanchez-Lara, I., Pardo, C. C., Fornieles-Rubio, F., et al. (2017). Association between periodontitis and amyloid-β peptide in elderly people with and without cognitive impairment. *J. Periodontol.* doi: 10.1902/jop.2017.170071. [Epub ahead of print].

Gold, R., Kappos, L., Arnold, D. L., Bar-Or, A., Giovannoni, G., Selmaj, K., et al. (2012). Placebo-controlled phase 3 study of oral BG-12 for relapsing multiple sclerosis. *N. Engl. J. Med.* 367, 1098–1107. doi: 10.1056/NEJMoa1114287

Golz, L., Memmert, S., Rath-Deschner, B., Jager, A., Appel, T., Baumgarten, G., et al. (2014). LPS from *P. gingivalis* and hypoxia increases oxidative stress in periodontal ligament fibroblasts and contributes to periodontitis. *Mediators Inflamm.* 2014:986264. doi: 10.1155/2014/986264

Gomes, M. S., Hugo, F. N., Hilgert, J. B., Sant'Ana Filho, M., Padilha, D. M., Simonsick, E. M., et al. (2016). Apical periodontitis and incident cardiovascular events in the baltimore longitudinal study of ageing. *Int. Endod. J.* 49, 334–342. doi: 10.1111/iej.12468

Habu, H., Saito, N., Sato, Y., Takeshita, K., Sunagawa, M., and Endo, M. (1990). Results of surgery in patients with gastric cancer extending to the adjacent organs. *Hepatogastroenterology* 37, 417–420.

Halliwell, B., and Gutteridge, J. M. (1984). Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J.* 219, 1–14. doi: 10.1042/bj2190001

Han, P., Sun, D., and Yang, J. (2016). Interaction between periodontitis and liver diseases. *Biomed. Rep.* 5, 267–276. doi: 10.3892/br.2016.718

Hansen, G. M., Egeberg, A., Holmstrup, P., and Hansen, P. R. (2016). Relation of periodontitis to risk of cardiovascular and all-cause mortality (from a Danish nationwide cohort study). *Am. J. Cardiol.* 118, 489–493. doi: 10.1016/j.amjcard.2016.05.036

Heid, M. E., Keyel, P. A., Kamga, C., Shiva, S., Watkins, S. C., and Salter, R. D. (2013). Mitochondrial reactive oxygen species induces NLRP3-dependent lysosomal damage and inflammasome activation. *J. Immunol.* 191, 5230–5238. doi: 10.4049/jimmunol.1301490

Hori, J. I., Zamboni, D. S., Carrao, D. B., Goldman, G. H., and Berretta, A. A. (2013). The Inhibition of Inflammasome by Brazilian Propolis (EPP-AF). *Evid. Based Complement. Alternat. Med.* 2013:418508. doi: 10.1155/2013/418508

Houcken, W., Teeuw, W. J., Bizzarro, S., Alvarez Rodriguez, E., Mulders, T. A., van den Born, B. J., et al. (2016). Arterial stiffness in periodontitis patients and controls. A case-control and pilot intervention study. *J. Hum. Hypertens* 30, 24–29. doi: 10.1038/jhh.2015.41

Huang, C. F., Zhang, L., Ma, S. R., Zhao, Z. L., Wang, W. M., He, K. F., et al. (2013). Clinical significance of Keap1 and Nrf2 in oral squamous cell carcinoma. *PLoS ONE* 8:e83479. doi: 10.1371/journal.pone.0083479

Huang, H. C., Nguyen, T., and Pickett, C. B. (2002). Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. *J. Biol. Chem.* 277, 42769–42774. doi: 10.1074/jbc.M206911200

Ide, M., Harris, M., Stevens, A., Sussams, R., Hopkins, V., Culliford, D., et al. (2016). Periodontitis and cognitive decline in Alzheimer's disease. *PLoS ONE* 11:e0151081. doi: 10.1371/journal.pone.0151081

Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., et al. (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. doi: 10.1006/bbrc.1997.6943

Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J. D., et al. (1999). Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev.* 13, 76–86. doi: 10.1101/gad.13.1.76

Iyer, S. S., He, Q., Janczy, J. R., Elliott, E. I., Zhong, Z., Olivier, A. K., et al. (2013). Mitochondrial cardiolipin is required for Nlrp3 inflammasome activation. *Immunity* 39, 311–323. doi: 10.1016/j.immuni.2013.08.001

Jiang, X., and Wang, X. (2004). Cytochrome C-mediated apoptosis. *Annu. Rev. Biochem.* 73, 87–106. doi: 10.1146/annurev.biochem.73.011303.073706

Jiang, Y., Wang, M., Huang, K., Zhang, Z., Shao, N., Zhang, Y., et al. (2012). Oxidized low-density lipoprotein induces secretion of interleukin-1β by macrophages via reactive oxygen species-dependent NLRP3 inflammasome activation. *Biochem. Biophys. Res. Commun.* 425, 121–126. doi: 10.1016/j.bbrc.2012.07.011

Jin, L. J., Lamster, I. B., Greenspan, J. S., Pitts, N. B., Scully, C., and Warnakulasuriya, S. (2016). Global burden of oral diseases: emerging concepts, management and interplay with systemic health. *Oral Dis.* 22, 609–619. doi: 10.1111/odi.12428

Joshi, S., Kumar, S., Ponnusamy, M. P., and Batra, S. K. (2016). Hypoxia-induced oxidative stress promotes MUC4 degradation via autophagy to enhance pancreatic cancer cells survival. *Oncogene* 35, 5882–5892. doi: 10.1038/onc.2016.119

Kampits, C., Montenegro, M. M., Ribeiro, I. W., Furtado, M. V., Polanczyk, C. A., Rosing, C. K., et al. (2016). Periodontal disease and inflammatory blood cytokines in patients with stable coronary artery disease. *J. Appl. Oral Sci.* 24, 352–358. doi: 10.1590/1678-775720160082

Kang, K. W., Lee, S. J., Park, J. W., and Kim, S. G. (2002). Phosphatidylinositol 3-kinase regulates nuclear translocation of NF-E2-related factor 2 through actin rearrangement in response to oxidative stress. *Mol. Pharmacol.* 62, 1001–1010. doi: 10.1124/mol.62.5.1001

Kanthasamy, A. G., Kitazawa, M., Kanthasamy, A., and Anantharam, V. (2003). Role of proteolytic activation of protein kinase Cδ in oxidative stress-induced apoptosis. *Antioxid. Redox Signal.* 5, 609–620. doi: 10.1089/152308603770310275

Kanzaki, H., Wada, S., Narimiya, T., Yamaguchi, Y., Katsumata, Y., Itohiya, K., et al. (2017). Pathways that regulate ROS scavenging enzymes, and their role in defense against tissue destruction in periodontitis. *Front. Physiol.* 8:351. doi: 10.3389/fphys.2017.00351

Kassebaum, N. J., Smith, A. G. C., Bernabe, E., Fleming, T. D., Reynolds, A. E., Vos, T., et al. (2017). Global, regional, and national prevalence, incidence, and disability-adjusted life years for oral conditions for 195 countries, 1990–2015: a systematic analysis for the global burden of diseases, injuries, and risk factors. *J. Dent. Res.* 96, 380–387. doi: 10.1177/0022034517693566

Kataoka, K., Ekuni, D., Tomofuji, T., Irie, K., Kunitomo, M., Uchida, Y., et al. (2016). Visualization of oxidative stress induced by experimental periodontitis in Keap1-dependent oxidative stress detector-luciferase mice. *Int. J. Mol. Sci.* 17:1907. doi: 10.3390/ijms17111907

Kaya, S., Sutcu, R., Cetin, E. S., Aridogan, B. C., Delibas, N., and Demirci, M. (2007). Lipid peroxidation level and antioxidant enzyme activities in the blood of patients with acute and chronic fascioliasis. *Int. J. Infect. Dis.* 11, 251–255. doi: 10.1016/j.ijid.2006.05.003

Ketabi, M., Meybodi, F. R., and Asgari, M. R. (2016). The association between periodontal disease parameters and severity of atherosclerosis. *Dent. Res. J.* 13, 250–255. doi: 10.4103/1735-3327.182185

Khatri, R., Deshmukh, J., Shrivastava, R., Gupta, S., Kawadkar, A., and Vinaya Kumar, K. (2016). Is periodontitis an independent risk factor for subclinical atherosclerosis? *Singapore Dent. J.* 37, 9–13. doi: 10.1016/j.sdj.2016.10.004

Kholy, K. E., Genco, R. J., and Van Dyke, T. E. (2015). Oral infections and cardiovascular disease. *Trends Endocrinol. Metab.* 26, 315–321. doi: 10.1016/j.tem.2015.03.001

Kim, J., Cha, Y. N., and Surh, Y. J. (2010). A protective role of nuclear factor-erythroid 2-related factor-2 (Nrf2) in inflammatory disorders. *Mutat. Res.* 690, 12–23. doi: 10.1016/j.mrfmmm.2009.09.007

Kobayashi, A., Kang, M. I., Okawa, H., Ohtsuji, M., Zenke, Y., Chiba, T., et al. (2004). Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. *Mol. Cell. Biol.* 24, 7130–7139. doi: 10.1128/MCB.24.16.7130-7139.2004

Kohle, C., and Bock, K. W. (2006). Activation of coupled Ah receptor and Nrf2 gene batteries by dietary phytochemicals in relation to chemoprevention. *Biochem. Pharmacol.* 72, 795–805. doi: 10.1016/j.bcp.2006.04.017

Konopka, T., Krol, K., Kopec, W., and Gerber, H. (2007). Total antioxidant status and 8-hydroxy-2'-deoxyguanosine levels in gingival and peripheral blood of periodontitis patients. *Arch. Immunol. Ther. Exp.* 55, 417–422. doi: 10.1007/s00005-007-0047-1

Krol, K. (2004). Reactive oxygen species and antioxidant mechanisms in the pathogenesis of periodontitis. *Ann. Acad. Med. Stetin.* 50, 135–148.

Kumar, P., Sharma, S., Khanna, M., and Raj, H. G. (2003). Effect of Quercetin on lipid peroxidation and changes in lung morphology in experimental influenza virus infection. *Int. J. Exp. Pathol.* 84, 127–133. doi: 10.1046/j.1365-2613.2003.00344.x

Lacy, F., Gough, D. A., and Schmid-Schonbein, G. W. (1998). Role of xanthine oxidase in hydrogen peroxide production. *Free Radic. Biol. Med.* 25, 720–727. doi: 10.1016/S0891-5849(98)00154-3

Lamkanfi, M. (2011). Emerging inflammasome effector mechanisms. *Nat. Rev. Immunol.* 11, 213–220. doi: 10.1038/nri2936

Lamkanfi, M., and Dixit, V. M. (2009). Inflammasomes: guardians of cytosolic sanctity. *Immunol. Rev.* 227, 95–105. doi: 10.1111/j.1600-065X.2008.00730.x

Lee, C. H., Chang, J. S., Syu, S. H., Wong, T. S., Chan, J. Y., Tang, Y. C., et al. (2015). IL-1β promotes malignant transformation and tumor aggressiveness in oral cancer. *J. Cell. Physiol.* 230, 875–884. doi: 10.1002/jcp.24816

Lee, Y. T., Lee, H. C., Hu, C. J., Huang, L. K., Chao, S. P., Lin, C. P., et al. (2017). Periodontitis as a modifiable risk factor for dementia: a nationwide population-based cohort study. *J. Am. Geriatr. Soc.* 65, 301–305. doi: 10.1111/jgs.14449

Leira, Y., Dominguez, C., Seoane, J., Seoane-Romero, J., Pias-Peleteiro, J. M., Takkouche, B., et al. (2017). Is periodontal disease associated with Alzheimer's disease? A systematic review with meta-analysis. *Neuroepidemiology* 48, 21–31. doi: 10.1159/000458411

Li, H., Zhu, H., Xu, C. J., and Yuan, J. (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 94, 491–501. doi: 10.1016/S0092-8674(00)81590-1

Li, X., Kolltveit, K. M., Tronstad, L., and Olsen, I. (2000). Systemic diseases caused by oral infection. *Clin. Microbiol. Rev.* 13, 547–558. doi: 10.1128/CMR.13.4.547-558.2000

Listl, S., Galloway, J., Mossey, P. A., and Marcenes, W. (2015). Global economic impact of dental diseases. *J. Dent. Res.* 94, 1355–1361. doi: 10.1177/0022034515602879

Liu, Y., Yang, H., Wen, Y., Li, B., Zhao, Y., Xing, J., et al. (2017). Nrf2 inhibits periodontal ligament stem cell apoptosis under excessive oxidative stress. *Int. J. Mol. Sci.* 18:1076. doi: 10.3390/ijms18051076

Lopez-Jornet, P., Martinez-Canovas, A., and Pons-Fuster, A. (2014). Salivary biomarkers of oxidative stress and quality of life in patients with oral lichen planus. *Geriatr. Gerontol. Int.* 14, 654–659. doi: 10.1111/ggi.12153

Luheshi, N. M., Giles, J. A., Lopez-Castejon, G., and Brough, D. (2012). Sphingosine regulates the NLRP3-inflammasome and IL-1β release from macrophages. *Eur. J. Immunol.* 42, 716–725. doi: 10.1002/eji.201142079

Ma, N., Tagawa, T., Hiraku, Y., Murata, M., Ding, X., and Kawanishi, S. (2006). 8-Nitroguanine formation in oral leukoplakia, a premalignant lesion. *Nitric Oxide* 14, 137–143. doi: 10.1016/j.niox.2005.09.012

Madamanchi, N. R., and Runge, M. S. (2013). Redox signaling in cardiovascular health and disease. *Free Radic. Biol. Med.* 61, 473–501. doi: 10.1016/j.freeradbiomed.2013.04.001

Madesh, M., and Hajnoczky, G. (2001). VDAC-dependent permeabilization of the outer mitochondrial membrane by superoxide induces rapid and massive cytochrome c release. *J. Cell Biol.* 155, 1003–1015. doi: 10.1083/jcb.200105057

Mahalakshmi, K., Krishnan, P., and Arumugam, S. B. (2017). "Association of periodontopathic anaerobic bacterial co-occurrence to atherosclerosis" - a cross-sectional study. *Anaerobe* 44, 66–72. doi: 10.1016/j.anaerobe.2017.02.003

Maisonneuve, P., Amar, S., and Lowenfels, A. B. (2017). Periodontal disease, edentulism, and pancreatic cancer: a meta-analysis. *Ann. Oncol.* 28, 985–995. doi: 10.1093/annonc/mdx019

Malik, U. U., Siddiqui, I. A., Hashim, Z., and Zarina, S. (2014). Measurement of serum paraoxonase activity and MDA concentrations in patients suffering with oral squamous cell carcinoma. *Clin. Chim. Acta* 430, 38–42. doi: 10.1016/j.cca.2013.12.033

Manoharan, S., Kolanjiappan, K., Suresh, K., and Panjamurthy, K. (2005). Lipid peroxidation & antioxidants status in patients with oral squamous cell carcinoma. *Indian J. Med. Res.* 122, 529–534.

Mareninova, O. A., Sung, K. F., Hong, P., Lugea, A., Pandol, S. J., Gukovsky, I., et al. (2006). Cell death in pancreatitis: caspases protect from necrotizing pancreatitis. *J. Biol. Chem.* 281, 3370–3381. doi: 10.1074/jbc.M511276200

Martinez-Micaelo, N., Gonzalez-Abuin, N., Pinent, M., Ardevol, A., and Blay, M. (2015). Procyanidin B2 inhibits inflammasome-mediated IL-1β production in lipopolysaccharide-stimulated macrophages. *Mol. Nutr. Food Res.* 59, 262–269. doi: 10.1002/mnfr.201400370

Martinon, F. (2010). Signaling by ROS drives inflammasome activation. *Eur. J. Immunol.* 40, 616–619. doi: 10.1002/eji.200940168

Matthews, J. B., Wright, H. J., Roberts, A., Ling-Mountford, N., Cooper, P. R., and Chapple, I. L. (2007). Neutrophil hyper-responsiveness in periodontitis. *J. Dent. Res.* 86, 718–722. doi: 10.1177/154405910708600806

McCord, J. M. (2000). The evolution of free radicals and oxidative stress. *Am. J. Med.* 108, 652–659. doi: 10.1016/S0002-9343(00)00412-5

Mehdipour, M., Taghavi Zenouz, A., Dastanpour, S., Bahramian, A., Gholizadeh, N., and Azari-Marhabi, S. (2017). Evaluation of serum malondialdehyde level in patients with oral lichen planus. *Caspian J. Dent. Res.* 6, 45–48.

Metgud, R., and Bajaj, S. (2014). Evaluation of salivary and serum lipid peroxidation, and glutathione in oral leukoplakia and oral squamous cell carcinoma. *J. Oral Sci.* 56, 135–142. doi: 10.2334/josnusd.56.135

Mileva, M., Tancheva, L., Bakalova, R., Galabov, A., Savov, V., and Ribarov, S. (2000). Effect of vitamin E on lipid peroxidation and liver monooxigenase activity in experimental influenza virus infection. *Toxicol. Lett.* 114, 39–45. doi: 10.1016/S0378-4274(99)00265-9

Miyauchi, S., Maekawa, T., Aoki, Y., Miyazawa, H., Tabeta, K., Nakajima, T., et al. (2012). Oral infection with *Porphyromonas gingivalis* and systemic cytokine profile in C57BL/6.KOR-ApoE^{shl} mice. *J. Periodont. Res.* 47, 402–408. doi: 10.1111/j.1600-0765.2011.01441.x

Moon, J. S., Nakahira, K., Chung, K. P., DeNicola, G. M., Koo, M. J., Pabon, M. A., et al. (2016). NOX4-dependent fatty acid oxidation promotes NLRP3 inflammasome activation in macrophages. *Nat. Med.* 22, 1002–1012. doi: 10.1038/nm.4153

Mota, S. I., Costa, R. O., Ferreira, I. L., Santana, I., Caldeira, G. L., Padovano, C., et al. (2015). Oxidative stress involving changes in Nrf2 and ER stress in early stages of Alzheimer's disease. *Biochim. Biophys. Acta* 1852, 1428–1441. doi: 10.1016/j.bbadis.2015.03.015

Motohashi, H., and Yamamoto, M. (2004). Nrf2-Keap1 defines a physiologically important stress response mechanism. *Trends Mol. Med.* 10, 549–557. doi: 10.1016/j.molmed.2004.09.003

Natarajan, U. M., and Midhun, M. (2017). A study of association between severe periodontitis with subclinical atherosclerosis in young subjects. *Int. J. Comtemporary Med.* 5, 36–41. doi: 10.5958/2321-1032.2017.00008.0

Niki, E., Yoshida, Y., Saito, Y., and Noguchi, N. (2005). Lipid peroxidation: mechanisms, inhibition, and biological effects. *Biochem. Biophys. Res. Commun.* 338, 668–676. doi: 10.1016/j.bbrc.2005.08.072

Nisimoto, Y., Diebold, B. A., Cosentino-Gomes, D., and Lambeth, J. D. (2014). Nox4: a hydrogen peroxide-generating oxygen sensor. *Biochemistry* 53, 5111–5120. doi: 10.1021/bi500331y

Niture, S. K., Khatri, R., and Jaiswal, A. K. (2014). Regulation of Nrf2-an update. *Free Radic. Biol. Med.* 66, 36–44. doi: 10.1016/j.freeradbiomed.2013.02.008

Numazawa, S., Ishikawa, M., Yoshida, A., Tanaka, S., and Yoshida, T. (2003). Atypical protein kinase C mediates activation of NF-E2-related factor 2 in response to oxidative stress. *Am. J. Physiol. Cell Physiol.* 285, C334–C342. doi: 10.1152/ajpcell.00043.2003

Oh, B., Figtree, G., Costa, D., Eade, T., Hruby, G., Lim, S., et al. (2016). Oxidative stress in prostate cancer patients: a systematic review of case control studies. *Prostate Int.* 4, 71–87. doi: 10.1016/j.prnil.2016.05.002

Pandey, K. B., and Rizvi, S. I. (2009). Plant polyphenols as dietary antioxidants in human health and disease. *Oxid. Med. Cell. Longev.* 2, 270–278. doi: 10.4161/oxim.2.5.9498

Paravicini, T. M., and Touyz, R. M. (2008). NADPH oxidases, reactive oxygen species, and hypertension: clinical implications and therapeutic possibilities. *Diabetes Care* 31(Suppl. 2), S170–S180. doi: 10.2337/dc08-s247

Perron, N. R., and Brumaghim, J. L. (2009). A review of the antioxidant mechanisms of polyphenol compounds related to iron binding. *Cell Biochem. Biophys.* 53, 75–100. doi: 10.1007/s12013-009-9043-x

Pisoschi, A. M., and Pop, A. (2015). The role of antioxidants in the chemistry of oxidative stress: a review. *Eur. J. Med. Chem.* 97, 55–74. doi: 10.1016/j.ejmech.2015.04.040

Pradeep, A. R., Rao, N. S., Bajaj, P., and Agarwal, E. (2013). 8-Isoprostane: a lipid peroxidation product in gingival crevicular fluid in healthy, gingivitis and chronic periodontitis subjects. *Arch. Oral Biol.* 58, 500–504. doi: 10.1016/j.archoralbio.2013.01.011

Pradeep, A. R., Suke, D. K., Prasad, M. V., Singh, S. P., Martande, S. S., Nagpal, K., et al. (2016). Expression of key executioner of apoptosis caspase-3 in periodontal health and disease. *J. Investig. Clin. Dent.* 7, 174–179. doi: 10.1111/jicd.12134

Ran, S., Liu, B., Gu, S., Sun, Z., and Liang, J. (2017). Analysis of the expression of NLRP3 and AIM2 in periapical lesions with apical periodontitis and microbial analysis outside the apical segment of teeth. *Arch. Oral Biol.* 78, 39–47. doi: 10.1016/j.archoralbio.2017.02.006

Rathore, S., Datta, G., Kaur, I., Malhotra, P., and Mohmmed, A. (2015). Disruption of cellular homeostasis induces organelle stress and triggers apoptosis like cell-death pathways in malaria parasite. *Cell Death Dis.* 6:e1803. doi: 10.1038/cddis.2015.142

Ray, R., Murdoch, C. E., Wang, M., Santos, C. X., Zhang, M., Alom-Ruiz, S., et al. (2011). Endothelial Nox4 NADPH oxidase enhances vasodilatation and reduces blood pressure *in vivo*. *Arterioscler. Thromb. Vasc. Biol.* 31, 1368–1376. doi: 10.1161/ATVBAHA.110.219238

Ren, J., Chen, Y. B., Zhang, Y. Y., Zhou, Q. B., Chen, S., Yang, J. Y., et al. (2015). Decreased circulating neopterin is associated with increased arterial elasticity: a beneficial role of periodontal treatment. *Aust. Dent. J.* 61, 76–83. doi: 10.1111/adj.12303

Repetto, M., Semprine, J., and Boveris, A. (2012). "Lipid peroxidation: chemical mechanism, biological implications and analytical determination," in *Biochemistry, Genetics and Molecular Biology,* ed A. Catala (INTECH). doi: 10.5772/45943. Available online at: https://www.intechopen.com/books/lipid-peroxidation/lipid-peroxidation-chemical-mechanism-biological-implications-and-analytical-determination

Riggio, M. P., and Lennon, A. (1999). Identification by PCR of *Helicobacter pylori* in subgingival plaque of adult periodontitis patients. *J. Med. Microbiol.* 48, 317–322. doi: 10.1099/00222615-48-3-317

Riteau, N., Baron, L., Villeret, B., Guillou, N., Savigny, F., Ryffel, B., et al. (2012). ATP release and purinergic signaling: a common pathway for particle-mediated inflammasome activation. *Cell Death Dis.* 3:e403. doi: 10.1038/cddis.2012.144

Ryden, L., Buhlin, K., Ekstrand, E., de Faire, U., Gustafsson, A., Holmer, J., et al. (2016). Periodontitis increases the risk of a first myocardial

infarction: A report from the PAROKRANK study. *Circulation* 133, 576–583. doi: 10.1161/CIRCULATIONAHA.115.020324

Saed, G. M., Diamond, M. P., and Fletcher, N. M. (2017). Updates of the role of oxidative stress in the pathogenesis of ovarian cancer. *Gynecol. Oncol.* 145, 595–602. doi: 10.1016/j.ygyno.2017.02.033

Sahoo, S., Meijles, D. N., and Pagano, P. J. (2016). NADPH oxidases: key modulators in aging and age-related cardiovascular diseases? *Clin. Sci.* 130, 317–335. doi: 10.1042/CS20150087

Sanders, Y. Y., Liu, H., Liu, G., and Thannickal, V. J. (2015). Epigenetic mechanisms regulate NADPH oxidase-4 expression in cellular senescence. *Free Radic. Biol. Med.* 79, 197–205. doi: 10.1016/j.freeradbiomed.2014.12.008

Schroder, K., and Tschopp, J. (2010). The inflammasomes. *Cell* 140, 821–832. doi: 10.1016/j.cell.2010.01.040

Shimada, K., Crother, T. R., Karlin, J., Dagvadorj, J., Chiba, N., Chen, S., et al. (2012). Oxidized mitochondrial DNA activates the NLRP3 inflammasome during apoptosis. *Immunity* 36, 401–414. doi: 10.1016/j.immuni.2012.01.009

Shin, H. S., Shin, M. S., Ahn, Y. B., Choi, B. Y., Nam, J. H., and Kim, H. D. (2016). Periodontitis is associated with cognitive impairment in elderly Koreans: results from the Yangpyeong cohort study. *J. Am. Geriatr. Soc.* 64, 162–167. doi: 10.1111/jgs.13781

Shirzad, A., Pouramir, M., Seyedmajidi, M., Jenabian, N., Bijani, A., and Motallebnejad, M. (2014). Salivary total antioxidant capacity and lipid peroxidation in patients with erosive oral lichen planus. *J. Dent. Res. Dent. Clin. Dent. Prospects* 8, 35–39. doi: 10.5681/joddd.2014.006

Shiva, A., and Arab, S. (2016). Evaluation of uric acid, total antioxidant and lipid peroxidation parameters in serum and saliva of patients with oral lichen planus. *Glob. J. Health Sci.* 8, 225–231. doi: 10.5539/gjhs.v8n12p225

Sima, C., Aboodi, G. M., Lakschevitz, F. S., Sun, C., Goldberg, M. B., and Glogauer, M. (2016). Nuclear factor erythroid 2-related factor 2 down-regulation in oral neutrophils is associated with periodontal oxidative damage and severe chronic periodontitis. *Am. J. Pathol.* 186, 1417–1426. doi: 10.1016/j.ajpath.2016.01.013

Slee, E. A., Harte, M. T., Kluck, R. M., Wolf, B. B., Casiano, C. A., Newmeyer, D. D., et al. (1999). Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. *J. Cell Biol.* 144, 281–292. doi: 10.1083/jcb.144.2.281

Sobaniec, H., and Sobaniec-Lotowska, M. E. (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.

Soory, M. (2010). Oxidative stress induced mechanisms in the progression of periodontal diseases and cancer: a common approach to redox homeostasis? *Cancers (Basel).* 2, 670–692. doi: 10.3390/cancers2020670

Sorbara, M. T., and Girardin, S. E. (2011). Mitochondrial ROS fuel the inflammasome. *Cell Res.* 21, 558–560. doi: 10.1038/cr.2011.20

Swomley, A. M., and Butterfield, D. A. (2015). Oxidative stress in Alzheimer disease and mild cognitive impairment: evidence from human data provided by redox proteomics. *Arch. Toxicol.* 89, 1669–1680. doi: 10.1007/s00204-015-1556-z

Tabas, I., and Glass, C. K. (2013). Anti-inflammatory therapy in chronic disease: challenges and opportunities. *Science* 339, 166–172. doi: 10.1126/science.1230720

Takane, M., Sugano, N., Iwasaki, H., Iwano, Y., Shimizu, N., and 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. doi: 10.1902/jop.2002.73.5.551

Talalay, P., Dinkova-Kostova, A. T., and Holtzclaw, W. D. (2003). Importance of phase 2 gene regulation in protection against electrophile and reactive oxygen toxicity and carcinogenesis. *Adv. Enzyme Regul.* 43, 121–134. doi: 10.1016/S0065-2571(02)00038-9

Tamaki, N., Hayashida, H., Fukui, M., Kitamura, M., Kawasaki, K., Nakazato, M., et al. (2014). Oxidative stress and antibody levels to periodontal bacteria in adults: the Nagasaki Islands study. *Oral Dis.* 20, e49–e56. doi: 10.1111/odi.12127

Tanigawa, S., Fujii, M., and Hou, D. X. (2007). Action of Nrf2 and Keap1 in ARE-mediated NQO1 expression by quercetin. *Free Radic. Biol. Med.* 42, 1690–1703. doi: 10.1016/j.freeradbiomed.2007.02.017

Tariq, M., Manzoor, S., Ahmed, Q. L., Khalid, M., and Ashraf, W. (2013). NOX4 induces oxidative stress and apoptosis through upregulation of caspases 3 and 9 and downregulation of TIGAR in HCV-infected Huh-7 cells. *Future Virol.* 8, 707–716. doi: 10.2217/fvl.13.50

Theccanat, T., Philip, J. L., Razzaque, A. M., Ludmer, N., Li, J., Xu, X., et al. (2016). Regulation of cellular oxidative stress and apoptosis by G protein-coupled receptor kinase-2; The role of NADPH oxidase 4. *Cell. Signal.* 28, 190–203. doi: 10.1016/j.cellsig.2015.11.013

Tomofuji, T., Ekuni, D., Yamanaka, R., Kusano, H., Azuma, T., Sanbe, T., et al. (2007). Chronic administration of lipopolysaccharide and proteases induces periodontal inflammation and hepatic steatosis in rats. *J. Periodontol.* 78, 1999–2006. doi: 10.1902/jop.2007.070056

Tomofuji, T., Sanbe, T., Ekuni, D., Azuma, T., Irie, K., Maruyama, T., et al. (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

Tonetti, M. S. (2009). Periodontitis and risk for atherosclerosis: an update on intervention trials. *J. Clin. Periodontol.* 36(Suppl. 10), 15–19. doi: 10.1111/j.1600-051X.2009.01417.x

Tonetti, M. S., Jepsen, S., Jin, L., and Otomo-Corgel, J. (2017). Impact of the global burden of periodontal diseases on health, nutrition and wellbeing of mankind: a call for global action. *J. Clin. Periodontol.* 44, 456–462. doi: 10.1111/jcpe.12732

Trivedi, S., Lal, N., Mahdi, A. A., Singh, B., and Pandey, S. (2015). Association of salivary lipid peroxidation levels, antioxidant enzymes, and chronic periodontitis. *Int. J. Periodontics Restorative Dent.* 35, e14–e19. doi: 10.11607/prd.2079

Tsao, R., and Li, H. (2012). "Antioxidant properties *in vitro* and *in vivo*: realistic assessments of efficacy of plant extracts," in *Plant Sciences Reviews 2012,* ed D. Hemming (Boston, MA: CABI International), 11–20.

Tucker, P. S., Scanlan, A. T., and Dalbo, V. J. (2015). Chronic kidney disease influences multiple systems: describing the relationship between oxidative stress, inflammation, kidney damage, and concomitant disease. *Oxid. Med. Cell. Longev.* 2015:806358. doi: 10.1155/2015/806358

Tvarijonaviciute, A., Aznar-Cayuela, C., Rubio, C. P., Ceron, J. J., and Lopez-Jornet, P. (2017). Evaluation of salivary oxidate stress biomarkers, nitric oxide and C-reactive protein in patients with oral lichen planus and burning mouth syndrome. *J. Oral Pathol. Med.* 46, 387–392. doi: 10.1111/jop.12522

Ueda, S., Masutani, H., Nakamura, H., Tanaka, T., Ueno, M., and Yodoi, J. (2002). Redox control of cell death. *Antioxid. Redox Signal.* 4, 405–414. doi: 10.1089/15230860260196209

Ungvari, Z., Orosz, Z., Labinskyy, N., Rivera, A., Xiangmin, Z., Smith, K., et al. (2007). Increased mitochondrial H_2O_2 production promotes endothelial NF-kappaB activation in aged rat arteries. *Am. J. Physiol. Heart Circ. Physiol.* 293, H37–H47. doi: 10.1152/ajpheart.01346.2006

Ustinova, A. A., and Riabinin, V. E. (2003). Effect of chronic γ-irradiation on lipid peroxidation in CBA mouse blood serum. *Radiats. Biol. Radioecol.* 43, 459–463.

Vallet, P., Charnay, Y., Steger, K., Ogier-Denis, E., Kovari, E., Herrmann, F., et al. (2005). Neuronal expression of the NADPH oxidase NOX4, and its regulation in mouse experimental brain ischemia. *Neuroscience* 132, 233–238. doi: 10.1016/j.neuroscience.2004.12.038

Vendrov, A. E., Vendrov, K. C., Smith, A., Yuan, J., Sumida, A., Robidoux, J., et al. (2015). NOX4 NADPH oxidase-dependent mitochondrial oxidative stress in aging-associated cardiovascular disease. *Antioxid. Redox Signal.* 23, 1389–1409. doi: 10.1089/ars.2014.6221

Venugopal, R., and Jaiswal, A. K. (1996). Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. *Proc. Natl. Acad. Sci. U.S.A.* 93, 14960–14965. doi: 10.1073/pnas.93.25.14960

Vogel, C. F., Khan, E. M., Leung, P. S., Gershwin, M. E., Chang, W. L., Wu, D., et al. (2014). Cross-talk between aryl hydrocarbon receptor and the inflammatory response: a role for nuclear factor-κB. *J. Biol. Chem.* 289, 1866–1875. doi: 10.1074/jbc.M113.505578

Wakabayashi, N., Itoh, K., Wakabayashi, J., Motohashi, H., Noda, S., Takahashi, S., et al. (2003). Keap1-null mutation leads to postnatal lethality due to constitutive Nrf2 activation. *Nat. Genet.* 35, 238–245. doi: 10.1038/ng1248

Wen, H., Gris, D., Lei, Y., Jha, S., Zhang, L., Huang, M. T., et al. (2011). Fatty acid-induced NLRP3-ASC inflammasome activation interferes with insulin signaling. *Nat. Immunol.* 12, 408–415. doi: 10.1038/ni.2022

Wu, F., Chen, Y., Demmer, R. T., Parvez, F., Paul, R. R., Shaheen, I., et al. (2016). Periodontal diseases and carotid intima-media thickness in Bangladesh. *J. Clin. Periodontol.* 43, 909–917. doi: 10.1111/jcpe.12597

Xue, F., Shu, R., and Xie, Y. (2015). The expression of NLRP3, NLRP1 and AIM2 in the gingival tissue of periodontitis patients: RT-PCR study and immunohistochemistry. *Arch. Oral Biol.* 60, 948–958. doi: 10.1016/j.archoralbio.2015.03.005

Yamaguchi, Y., Kurita-Ochiai, T., Kobayashi, R., Suzuki, T., and Ando, T. (2017). Regulation of the NLRP3 inflammasome in *Porphyromonas gingivalis*-accelerated periodontal disease. *Inflamm. Res.* 66, 59–65. doi: 10.1007/s00011-016-0992-4

Yu, R., Lei, W., Mandlekar, S., Weber, M. J., Der, C. J., Wu, J., et al. (1999). Role of a mitogen-activated protein kinase pathway in the induction of phase II detoxifying enzymes by chemicals. *J. Biol. Chem.* 274, 27545–27552. doi: 10.1074/jbc.274.39.27545

Zanella, S. M., Pereira, S. S., Barbisan, J. N., Vieira, L., Saba-Chujfi, E., Haas, A. N., et al. (2016). Periodontal disease, tooth loss and coronary heart disease assessed by coronary angiography: a cross-sectional observational study. *J. Periodont. Res.* 51, 221–227. doi: 10.1111/jre.12301

Zeng, X. T., Leng, W. D., Lam, Y. Y., Yan, B. P., Wei, X. M., Weng, H., et al. (2016). Periodontal disease and carotid atherosclerosis: a meta-analysis of 17,330 participants. *Int. J. Cardiol.* 203, 1044–1051. doi: 10.1016/j.ijcard.2015.11.092

Zhang, L., Sheppard, O. R., Shah, A. M., and Brewer, A. C. (2008). Positive regulation of the NADPH oxidase NOX4 promoter in vascular smooth muscle cells by E2F. *Free Radic. Biol. Med.* 45, 679–685. doi: 10.1016/j.freeradbiomed.2008.05.019

Zhang, S., Qin, C., and Safe, S. H. (2003). Flavonoids as aryl hydrocarbon receptor agonists/antagonists: effects of structure and cell context. *Environ. Health Perspect.* 111, 1877–1882. doi: 10.1289/ehp.6322

Zhang, X., Wang, G., Gurley, E. C., and Zhou, H. (2014). Flavonoid apigenin inhibits lipopolysaccharide-induced inflammatory response through multiple mechanisms in macrophages. *PLoS ONE* 9:e107072. doi: 10.1371/journal.pone.0107072

Zhao, D., Wu, Y., Zhuang, J., Xu, C., and Zhang, F. (2016). Activation of NLRP1 and NLRP3 inflammasomes contributed to cyclic stretch-induced pyroptosis and release of IL-1β in human periodontal ligament cells. *Oncotarget* 7, 68292–68302. doi: 10.18632/oncotarget.11944

Zhong, Z., Zhai, Y., Liang, S., Mori, Y., Han, R., Sutterwala, F. S., et al. (2013). TRPM2 links oxidative stress to NLRP3 inflammasome activation. *Nat. Commun.* 4:1611. doi: 10.1038/ncomms2608

Zuo, Y., Xiang, B., Yang, J., Sun, X., Wang, Y., Cang, H., et al. (2009). Oxidative modification of caspase-9 facilitates its activation via disulfide-mediated interaction with Apaf-1. *Cell Res.* 19, 449–457. doi: 10.1038/cr.2009.19

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Withaferin A Induces Oxidative Stress-Mediated Apoptosis and DNA Damage in Oral Cancer Cells

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Withaferin A (WFA) is one of the most active steroidal lactones with reactive oxygen species (ROS) modulating effects against several types of cancer. ROS regulation involves selective killing. However, the anticancer and selective killing effects of WFA against oral cancer cells remain unclear. We evaluated whether the killing ability of WFA is selective, and we explored its mechanism against oral cancer cells. An MTS tetrazolium cell proliferation assay confirmed that WFA selectively killed two oral cancer cells (Ca9-22 and CAL 27) rather than normal oral cells (HGF-1). WFA also induced apoptosis of Ca9-22 cells, which was measured by flow cytometry for subG1 percentage, annexin V expression, and pan-caspase activity, as well as western blotting for caspases 1, 8, and 9 activations. Flow cytometry analysis shows that WFA-treated Ca9-22 oral cancer cells induced G2/M cell cycle arrest, ROS production, mitochondrial membrane depolarization, and phosphorylated histone H2A.X (γH2AX)-based DNA damage. Moreover, pretreating Ca9-22 cells with *N*-acetylcysteine (NAC) rescued WFA-induced selective killing, apoptosis, G2/M arrest, oxidative stress, and DNA damage. We conclude that WFA induced oxidative stress-mediated selective killing of oral cancer cells.

Keywords: oral cancer, selective killing, apoptosis, oxidative stress, withaferin A, *N*-acetylcysteine

INTRODUCTION

Most anticancer drugs effectively kill cancer cells; however, they also non-selectively kill normal cells, which limits their therapeutic value. It is currently believed that deregulated cell proliferation and deregulated apoptosis contributes to carcinogenesis (Reed, 1999). When the balance between proliferation and apoptosis is interrupted during tumor development, cell proliferation is deregulated (Scully et al., 2000; Evan and Vousden, 2001). Therapeutics that target oral squamous cell carcinoma (OSCC) cell proliferation and apoptosis regulators can enable these cancer cells to evade the regulatory system (Evan and Vousden, 2001).

Reactive oxygen species (ROS) are primarily generated in the mitochondria, and they cause a loss of mitochondrial membrane potential (MMP) (Li et al., 2006; Oh and Lim, 2006). ROS is an important inducer for the early stages of apoptosis (Samhan-Arias et al., 2004) and DNA damage (Barzilai and Yamamoto, 2004; Chen et al., 2016). Several drugs that modulate ROS have been reported (Nicco et al., 2005; Trachootham et al., 2006; Wu and Hua, 2007; Widodo et al., 2010) to regulate apoptosis for selective killing. Thus, ROS might mediate the selective activation of apoptosis for selective killing in cancer chemotherapy (Pollack et al., 2001; Daniel et al., 2003; Real et al., 2004).

Recently, several natural products have also been reported to induce apoptosis involving ROS (Ding et al., 2009; Chiu et al., 2013; Lee et al., 2013; Vyas and Singh, 2014). For example, the antiproliferation effect of cancer cells for the natural product from *Withania somnifera* (*W. somnifera*) is commonly reported to steroidal lactones (withanolides) (Vyas and Singh, 2014). Of the various withanolides derived from the root or leaf of *W. somnifera*, withaferin A (WFA) appears to be the most active against cancer (Vyas and Singh, 2014). The molecular anticancer activities of WFA have been reported through its antioxidant, anti-inflammatory and metabolic activities (Vanden Berghe et al., 2012; Vyas and Singh, 2014). WFA also induces ROS production and mitochondria-mediated caspase activation in apoptosis of HL-60 myeloid leukemia cells (Malik et al., 2007). Thus, WFA has potential for modulating apoptosis and oxidative stress. Moreover, WFA has been widely used in several types of cancer (Malik et al., 2007; Uma Devi et al., 2008; Woo et al., 2014; Lee et al., 2015), but its use in oral cancer cells has not been sufficiently investigated.

We therefore hypothesized that WFA killed oral cancer cells by regulating oxidative stress-mediated apoptosis. We tested this hypothesis by evaluating cell viability, cell cycle changes, annexin V, caspases, ROS, MMP, and DNA damage. Moreover, the role of oxidative stress in WFA-induced cell killing of oral cancer cells was examined by the addition of an antioxidant *N*-acetylcysteine (NAC). Therefore, this work sheds light on exploring the roles of oxidative stress-mediated WFA-induced cell killing mechanism of oral cancer cells.

MATERIALS AND METHODS

Cell Cultures and Chemicals

Ca9-22 and CAL 27 oral cancer cell lines and HGF-1 normal human gingival fibroblast cell lines were kept in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY, USA) plus 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2, as previously described (Chang Y. T. et al., 2016). WFA was purchased from Selleckchem.com (Houston, TX, USA) and dissolved in dimethyl sulfoxide (DMSO) for experiments. An antioxidant or free radical scavenger NAC (Sigma-Aldrich; St. Louis, MO, USA) was pretreated before WFA treatment to diminish cellular ROS and confirm the role of oxidative stress in WFA treatment.

Cell Viability

Cell viability was measured using an MTS assay (CellTiter 96 Aqueous One Solution; Promega, Madison, WI, USA), as previously described (Chiu et al., 2013).

Cell Cycle Distribution

DNA content was detected using 7-aminoactinomycin D (7AAD), a DNA dye, (Vignon et al., 2013). After fixation in 70% ethanol, cells were treated with 1 μg/ml 7AAD for 30 min at 37°C. Finally, the cells were resuspended in phosphate-buffered saline (PBS), and signals were examined using the FL3 channel of the Accuri C6 flow cytometer, and software (BD Biosciences, Franklin Lakes, New Jersey, USA). The G2/M percentage was calculated as the G2/M population among the whole cell cycle phases (G1, S, and G2/M).

Annexin V/DNA Content Assay to Measure Apoptosis

Apoptosis was measured using annexin V/propidum iodide (PI) (Strong Biotech Corp., Taipei, Taiwan; and Sigma-Aldrich) or annexin V/7AAD, as previously described (Chiu et al., 2011). After cells were treated with the WFA- or NAC/WFA (NAC pretreatment and WFA posttreatment), they were treated for 30 min at 37°C with annexin V-fluorescein isothiocyanate (annexin V-FITC) (10 μg/ml) and PI (5 μg/ml) or annexin V-FITC/7AAD (1 μg/ml), and then resuspended in PBS for analysis using the FL1/FL2 or FL1/FL3 channels of the Accuri C6 flow cytometer and software, respectively.

Pan-caspase Assay to Measure Apoptosis

Apoptosis was measured using a generic caspase activity assay kit (Fluorometric-Green: ab112130; Abcam, Cambridge, UK) (Yeh et al., 2012) for detecting the activity of caspases-1, 3-9. After the cells had been treated with WFA or NAC/WFA, they were treated with 2 μl of 500X TF2-VAD-FMK per 2 ml of medium for 2 h in a cell culture incubator. After the cells had been washed with PBS, they were resuspended in 0.5 ml of assay buffer, and signals were immediately detected using the FL1 channel of the Accuri C6.

Western Blotting of Caspase Signaling to Measure Apoptosis

The detailed procedures of western blotting are described previously (Chen et al., 2016). Briefly, 30 μg of protein lysates was loaded for 10% SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis), and transferred to PVDF (polyvinylidene fluoride) membranes (Pall Corp., Port Washington, NY, USA). Protein blocking was done using 5% non-fat milk in Tris-buffered saline with Tween-20. Subsequently, the lysates were treated with primary antibodies: cleaved caspase-8 (Asp391) (18C8) rabbit monoclonal antibody (mAb); cleaved PARP [poly(ADP-ribose) polymerase] (Asp214) (D64E10) XP® rabbit mAb; cleaved caspase-3 (Asp175) (5A1E) rabbit mAb; and cleaved caspase-9 (Asp330) (D2D4) rabbit mAb (Cell Signaling Technology, Inc., Danvers, MA, USA) (diluted 1:1000). mAb-β-actin (clone AC-15) (#A5441; Sigma-Aldrich) (diluted 1:5,000) was used as a control. Their matched secondary antibodies were also used. Signal was detected using a substrate

(WesternBright™ ECL HRP: #K-12045-D50; Advansta, Menlo Park, CA, USA). The densitometry quantification of blot was determined by ImageJ freeware.

ROS Production Assay

2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCF-DA) was used for ROS detection, as previously described (Shih et al., 2014). After the cells were treated with WFA or NAC/WFA, they were treated with 100 nM of H_2DCF-DA in PBS for 30 min in a cell culture incubator. After the cells were harvested and washed, they were resuspended in PBS and their signals were immediately analyzed using the FL1 channel of the Accuri C6.

Mitochondrial Membrane Potential (MMP) Assay

MMP was detected using an assay kit (MitoProbe™ 3,3′-diethyloxacarbocyanine iodide ($DiOC_2(3)$) (Invitrogen, San Diego, CA, USA)) as previously described (Yen et al., 2012). After the cells were treated with WFA or NAC/WFA, they were washed with PBS and treated with 10 of 10 μM $DiOC_2(3)$ per 1 ml of medium/well in a 6-well plate in a cell culture incubator for 20–30 min. After the cells were harvested and washed, they were resuspended in PBS and their signals were immediately analyzed using the FL1 channel of the Accuri C6.

Phosphorylated Histone H2A.X (γH2AX) Assay

γH2AX, DNA double-strand break biomarker, was detected using flow cytometry as previously described (Chen et al., 2016). After the cells were treated with WFA or NAC/WFA, they were fixed with 70% ethanol. After they had been washed, they were treated with 2 μg/ml of phospho-Histone 2A.X (Ser139) Antibody (H2AX) (sc-101696; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C for 1 h. After the cells were washed, they were treated with a secondary antibody (Jackson Laboratory, Bar Harbor, ME, USA) at room temperature for 30 min. Finally, the cells were treated in 20 μg/ml of PI, and their signals were detected using the FL1/FL2 channels of the Accuri C.

Statistical Analysis

All data are presented as means ± SD. Each analysis was performed in three separate experiments at different times ($n = 3$). All data were analyzed using Student paired t-test of Sigmaplot 10.0 (Scientific Data Analysis and Graphing Software, Systat Software Inc., Chicago, IL, USA).

RESULTS

The Viability of Oral Cancer Cells and Normal Oral Cells Treated with WFA Was Significantly Affected in WFA-Treated Cells with NAC Pretreatment

MTS assays showed that the relative cell viability (%) of the Ca9-22 and CAL 27 oral cancer cells were significantly lower than control after 24 h WFA treatments of 0.5, 1, 2, and 3 μM in a dose-dependent manner (**Figure 1A**). In contrast, HGF-1 normal oral cells treated with WFA showed no reduction in viability.

The involvement of oxidative stress in drug treatment is usually validated by pretreating cells with an antioxidant like NAC (Chan et al., 2006; Shieh et al., 2014; Hung et al., 2015; Lien et al., 2017). Cells treated with NAC-only [NAC pretreatment (2 mM)/WFA posttreatment (0 μM)] differed non-significantly from untreated controls (no NAC pretreatment and no WFA posttreatment in all three types of cells (**Figure 1B**). Moreover, WFA-induced antiproliferation was significantly inhibited in two types of WFA-treated oral cancer cells with NAC pretreatment (NAC/WFA) ($p < 0.05$–0.001).

To further validate the low cytotoxicity of WFA-treated HGF-1 normal oral cells, the levels of WFA-induced apoptosis in HGF-1 cells were evaluated using the pan-caspase assay. The flow cytometric pan-caspase patterns of WFA-treated HGF-1 cells are shown in **Figure 1C**. Generic caspase activities in WFA-treated HGF-1 cells slightly increased at 1–3 μM WFA about 60% compared to the control (50%) ($p < 0.001$) (**Figure 1D**), suggesting that WFA only induced minor signs of apoptosis (only 10% induction) with low cytotoxicity to HGF-1 normal oral cells compared to the control.

Cell Cycle-Perturbed Distribution of CA9-22 Oral Cancer Cells Treated with WFA Was Inhibited in WFA-Treated Cells with NAC Pretreatment

The flow cytometric cell cycle patterns of Ca9-22 oral cancer cells treated with WFA are shown in **Figure 2A** (top panel). Sub-G1 populations were higher in Ca9-22 cells treated with WFA than the control (**Figure 2B**, top panel). The flow cytometric cell cycle patterns of WFA and NAC/WFA-treated Ca9-22 cells are shown in **Figure 2A** (bottom panel). WFA-induced sub-G1 accumulation (**Figure 2B**, top panel) was significantly inhibited in WFA-treated Ca9-22 cells with NAC pretreatment (NAC/WFA) ($p < 0.001$). Moreover, G2/M populations were higher in Ca9-22 cells treated with WFA ranging from 1 to 2 μM (**Figure 2B**, bottom panel). WFA-induced G2/M accumulation (**Figure 2B**, bottom panel) was significantly inhibited in WFA (2 μM)-treated Ca9-22 cells with NAC pretreatment (NAC/WFA) ($p < 0.05$).

Annexin V/PI-Induced Apoptosis of CA9-22 Oral Cancer Cells Treated with WFA Was Inhibited in WFA-Treated Cells with NAC Pretreatment

The flow cytometric annexin V/PI patterns of Ca9-22 oral cancer cells treated with WFA are shown in **Figure 3A**. The annexin V positive (+) expression (%) for WFA-treated Ca9-22 cells was higher than the control in a dose-dependent manner (**Figure 3B**).

The flow cytometric annexin V/PI patterns of WFA- and NAC/WFA-treated Ca9-22 cells are shown in **Figure 3C**. Annexin V (+) expression in cells treated with NAC differed non-significantly from those in untreated controls of WFA-treated Ca9-22 cells (**Figure 3D**, left). Moreover, WFA-induced annexin

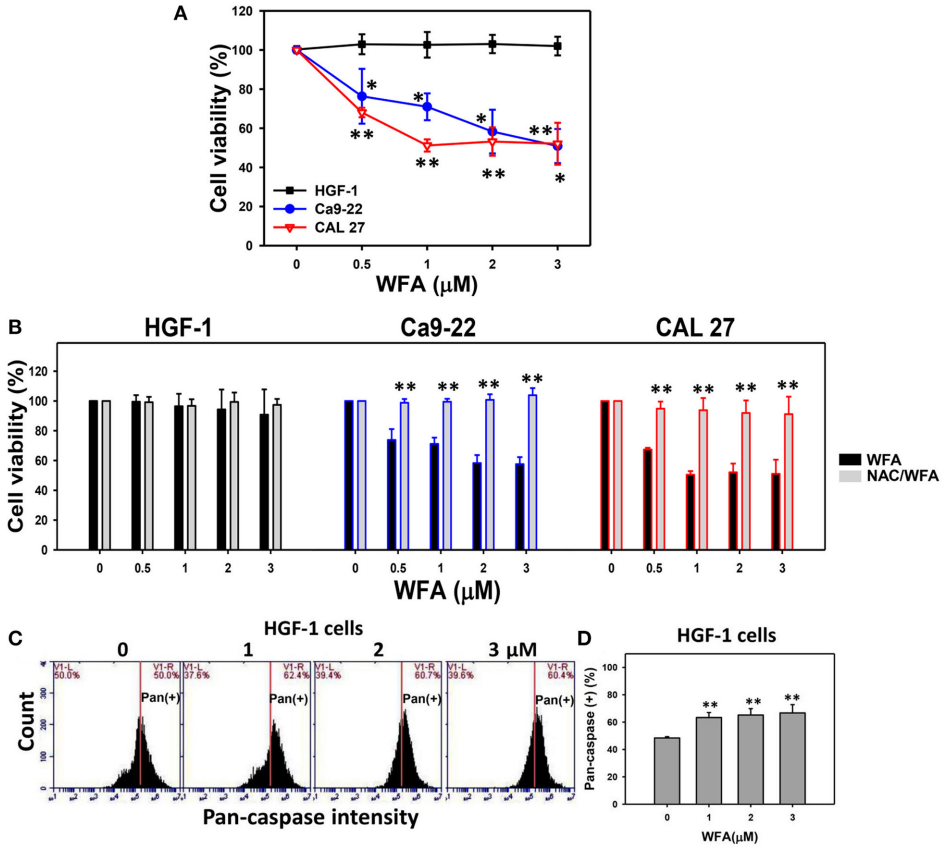

FIGURE 1 | MTS-based cell viability of WFA-treated oral cancer cells and normal cells and its changes after NAC pretreatment. **(A)** Oral cancer cells (Ca9-22 and CAL 27) and oral normal cells (HGF-1) were treated with WFA (0–3 μM) for 24 h. **(B)** NAC pretreatment effect on MTS-based cell viability of WFA-treated oral cancer and oral normal cells. Cells were pretreated with 2 mM NAC for 1 h and post-treated with WFA (0–3 μM) for 24 h. **(C)** Typical patterns of pan-caspase activity for WFA-treated oral normal cells (HGF-1). Cells were treated with different concentrations (0–3 μM) of WFA for 24 h for pan-caspase analysis. V1-L and V1-R listed in the corner of each plot, respectively, indicate the percentages of cell population (black color) on the left and right sides of the vertical line. In the control, the percentages of cell populations for V1-L and V1-R are 50% each (Chang H. W. et al., 2016). The position of this line is at the same position for all treatments as the control setting. Positive (+) %, the cell population on the right side of the line, is indicated in each **(D)** Pan-caspase-based apoptosis (+) (%) for **(C)**. Data are means ± SDs ($n = 3$). **(A,D)** $^{*}p < 0.05$ and $^{**}p < 0.001$ against control (0 μM). **(B)** $^{**}p < 0.001$ for comparison between WFA and NAC/WFA (NAC pretreatment and WFA posttreatment).

V-based apoptosis was significantly inhibited in WFA-treated Ca9-22 cells with NAC pretreatment (NAC/WFA) (**Figure 3D**, right) ($p < 0.001$).

Pan-caspase-Based Apoptosis of CA9-22 Oral Cancer Cells Treated with WFA Was Inhibited in WFA-Treated Cells with NAC Pretreatment

The involvement of caspases in the apoptosis of WFA-treated Ca9-22 cells was examined using a TF2-VAD-FMK flow cytometric assay (**Figure 4**). The flow cytometric pan-caspase patterns of WFA-treated Ca9-22 cells are shown in **Figure 4A**. Generic caspase activities in Ca9-22 cells treated with WFA ranging from 2 to 3 μM showed a significant increase above 80% compared to the control (50%) ($p < 0.05$–0.001), i.e., 30% induction (**Figure 4B**). The flow cytometric pan-caspase patterns of WFA- and NAC/WFA-treated Ca9-22 cells are provided in **Figure 4C**. In **Figure 4D**, the WFA-induced pan-caspase-based apoptosis in Ca9-22 cells was significantly inhibited in WFA-treated Ca9-22 cells with NAC pretreatment (NAC/WFA) ($p < 0.05$).

The involvement of caspases in the change of pan-caspase activity was validated using western blotting. In **Figures 4E,F**, the expression of apoptosis signaling proteins, such as cleaved PARP and cleaved-caspases 3, 8, and 9, was higher in WFA-treated Ca9-22 cells; although some of them declined at a concentration of 3 μM. In contrast, these caspase signaling proteins of the WFA-treated Ca9-22 cells were inhibited in WFA-treated Ca9-22 cells with NAC pretreatment (NAC/WFA).

ROS Generated Oxidative Stress of CA9-22 Oral Cancer Cells Treated with WFA Was Inhibited in WFA-Treated Cells with NAC Pretreatment

The ROS positive (+) patterns of WFA-treated Ca9-22 cells for 3, 6, and 12 h are displayed in **Figure 5A**. The ROS (+) (%) expression of WFA-treated Ca9-22 cells was significantly and time-dependently higher ($p < 0.001$) (**Figure 5B**).

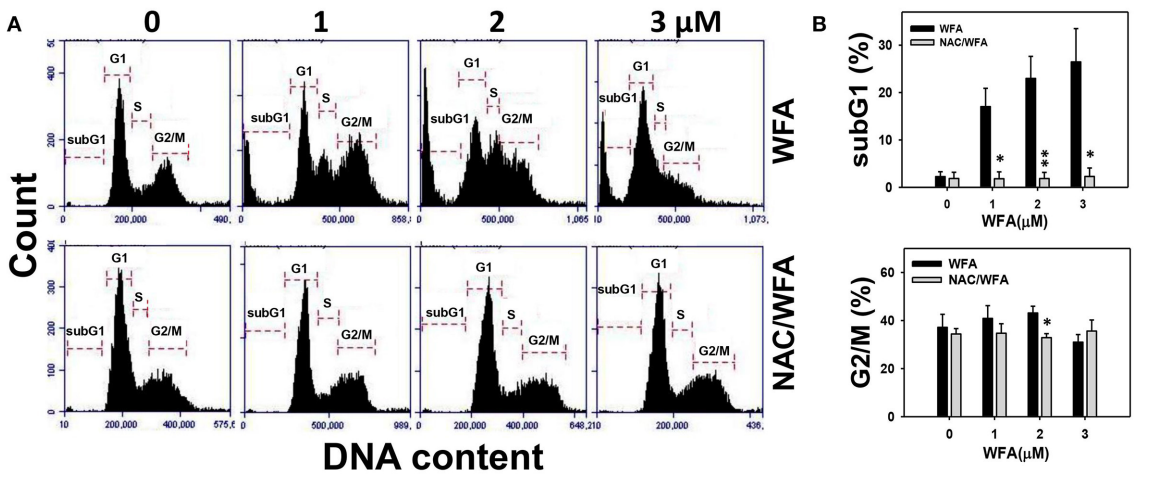

FIGURE 2 | The cell cycle distribution of WFA-treated Ca9-22 oral cancer cells and its changes after NAC pretreatment. **(A)** Typical cell cycle patterns of WFA-treated Ca9-22 oral cancer cells with and without NAC pretreatment. With and without NAC pretreatment (2 mM NAC for 1 h), cells were post-treated with WFA (0–3 μM) for 24 h. **(B)** SubG1 and G2/M phases (%) for **(A)**. Data are means ± SDs ($n = 3$). *$p < 0.05$ and **$p < 0.001$ for comparison between WFA and NAC/WFA for each concentration of WFA. NAC/WFA, NAC pretreatment and WFA posttreatment.

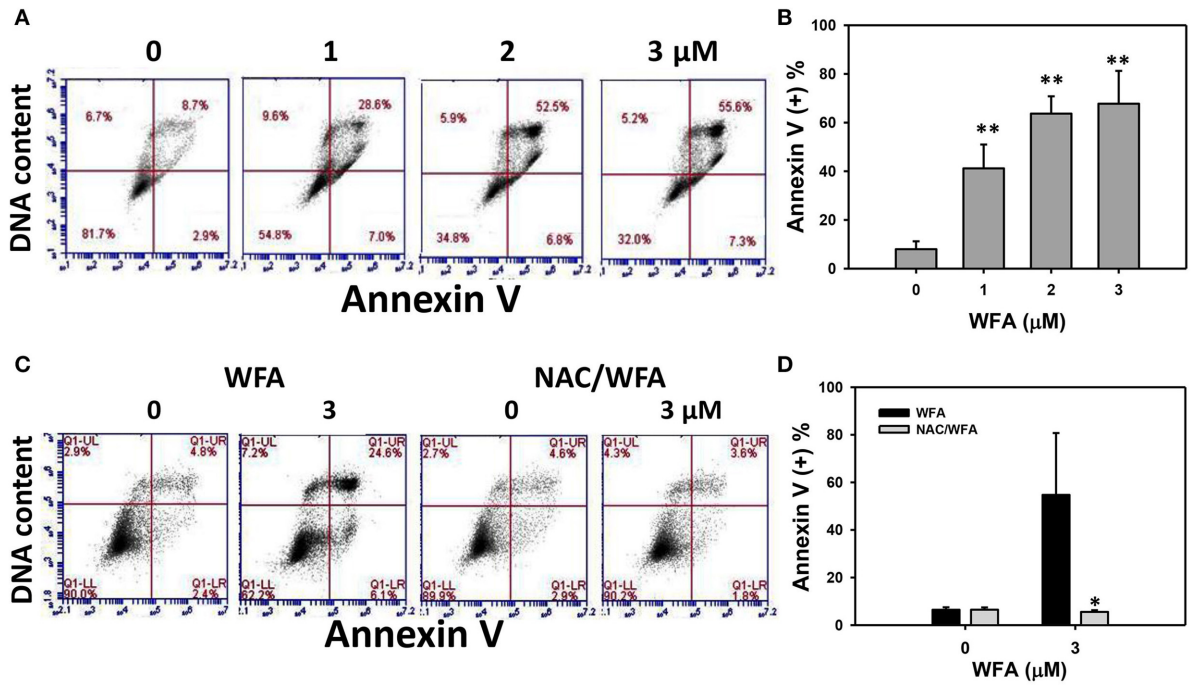

FIGURE 3 | Apoptosis of WFA-treated Ca9-22 oral cancer cells and its changes after NAC pretreatment. **(A)** Typical patterns of annexin V/DNA content method for WFA-treated Ca9-22 oral cancer cells. Cells were treated with WFA (0–3 μM) of 24 h for flow cytometry analyses. **(B)** Annexin V positive (+) (%) for **(A)**. **(C)** Typical annexin/DNA content-based apoptosis patterns of NAC effect on WFA-treated Ca9-22 cells. With or without NAC pretreatment (2 mM NAC for 1 h), cells were post-treated with WFA (0 and 3 μM) for 24 h. **(D)** Annexin/DNA content-based apoptosis (+) (%) for **(C)**. Data are means ± SDs ($n = 3$). **(B)** **$p < 0.001$ against control (0 μM). **(D)** *$p < 0.05$ for comparison between WFA and NAC/WFA (NAC pretreatment and WFA posttreatment).

Figure 5C shows the ROS (+) patterns of WFA- and NAC/WFA-treated Ca9-22 cells at 24 h of WFA treatment. **Figure 5D** shows higher ROS (+) expression in Ca9-22 cells after WFA treatment when compared to the control in a dose-dependent manner. ROS production induced by WFA treatment was significantly inhibited in WFA-treated Ca9-22 cells with NAC pretreatment (NAC/WFA) (**Figure 5D**) ($p < 0.001$).

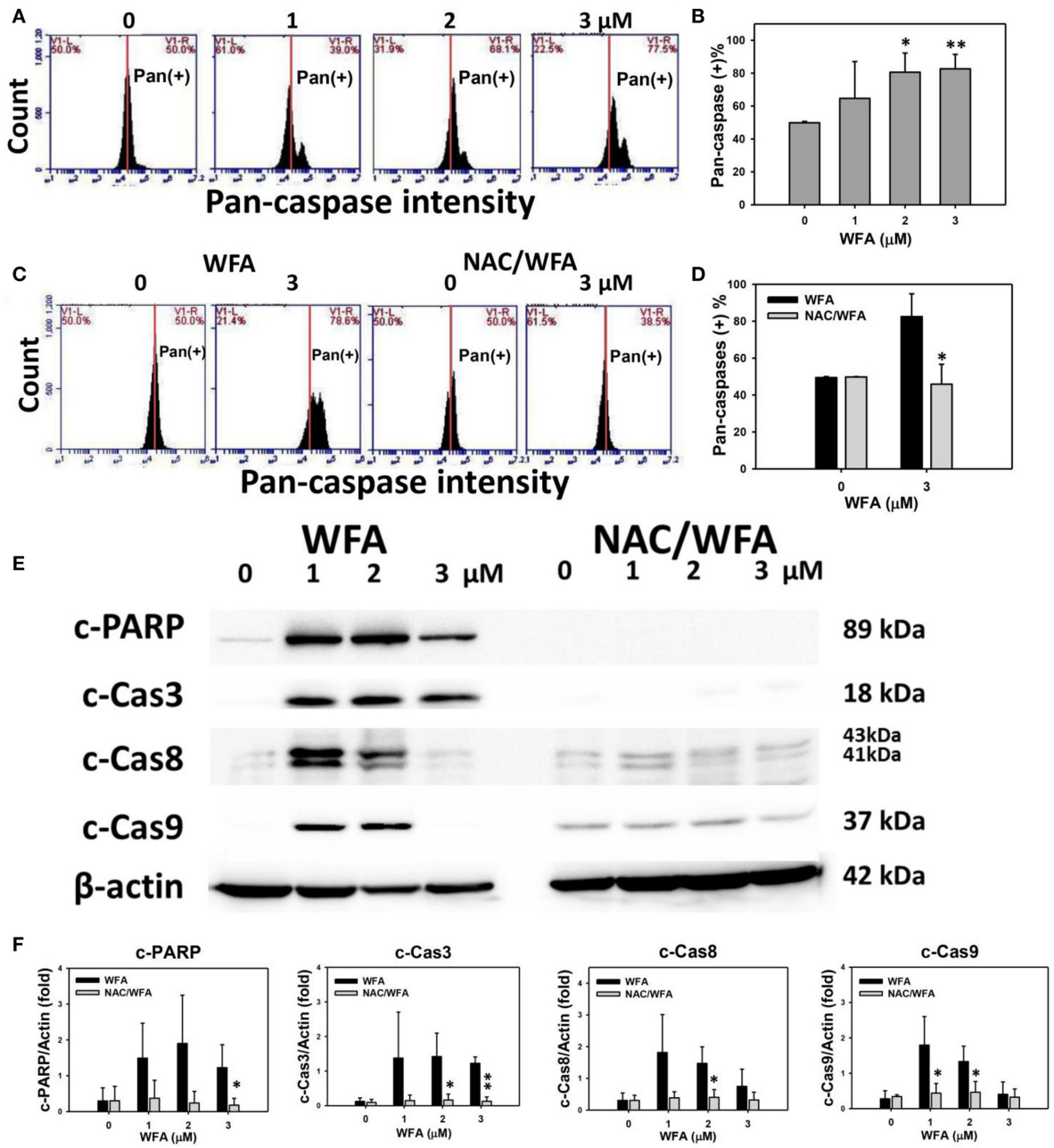

FIGURE 4 | Caspase activity in WFA-treated Ca9-22 oral cancer cells and its changes after NAC pretreatment. **(A)** Typical patterns of pan-caspase activity for WFA-treated oral cancer Ca9-22 cells. Cells were treated with different concentrations (0–3 μM) of WFA for 24 h for pan-caspase analysis. V1-L and V1-R labeled in the corner of each plot respectively indicate the percentages of cell population (black color) in the left and right sides of the vertical line. In control, the percentages of cell population for V1-L and V1-R are 50% each (Chang H. W. et al., 2016). The position of this line is at the same position for all treatments as the control setting. Positive (+) %, the cell population in the right side of the line, is indicated in each panel. **(B)** Pan-caspase-based apoptosis (+) (%) for **(A)**. **(C)** Typical pan-caspase-based apoptosis patterns of NAC effect on WFA-treated Ca9-22 cells. Cells were pretreated with 2 mM NAC for 1 h and post-treated with WFA (0–3 μM) for 24 h. **(D)** Pan-caspase-based apoptosis (+) (%) for **(C)**. **(E)** Expressions of apoptosis signaling proteins (cleaved caspases 3, 8, 9, and PARP) in Ca9-22 cells with WFA treatment with or without NAC pretreatment. Cells were pretreated with 2 mM NAC for 1 h and post-treated with WFA (0, 1, and 3 μM) for 24 h. β-actin was an internal control. Western blotting for WFA and NAC/WAF was performed in the same gel with the same exposure time. NAC/WFA, NAC pretreatment, and WFA posttreatment. **(F)** Statistic result for three separate western blottings in **(E)**. The densitometry quantification of blot was determined by ImageJ freeware. Data are means ± SDs ($n = 3$). **(B)** $^*p < 0.05$ and $^{**}p < 0.001$ against control (0 μM). **(D,F)** $^*p < 0.05$ and $^{**}p < 0.001$ for comparison between WFA and NAC/WFA.

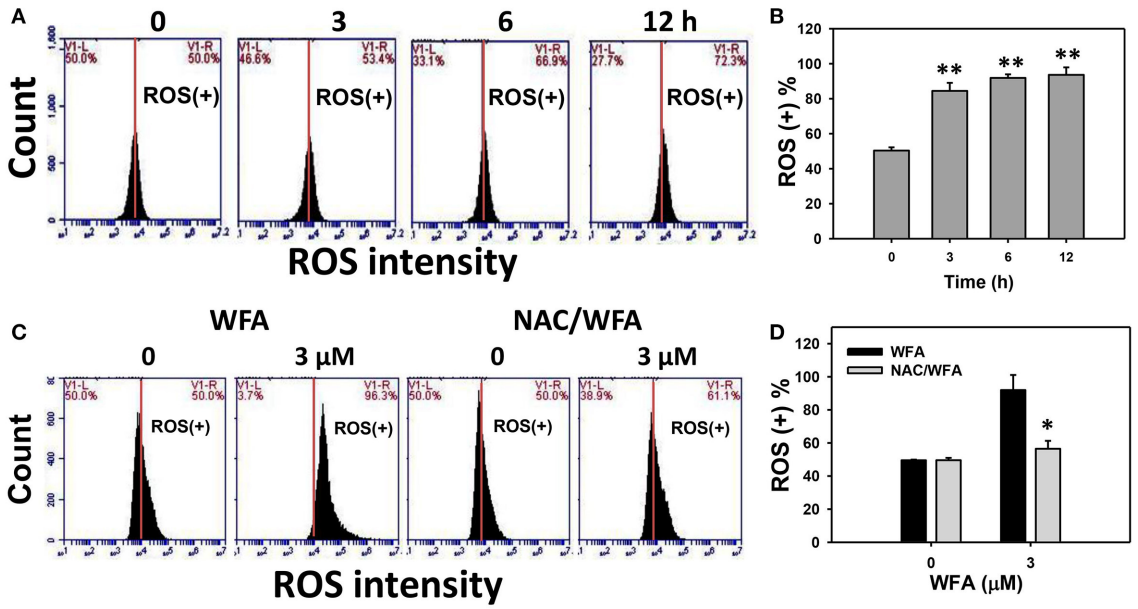

FIGURE 5 | ROS levels of WFA-treated Ca9-22 oral cancer cells and its changes after NAC pretreatment. **(A)** Typical ROS patterns of WFA-treated Ca9-22 oral cancer cells. Cells were treated with WFA (0–3 μM) for 3, 6, and 12 h for flow cytometry analysis. V1-L and V1-R labeled in the corner of each plot respectively indicate the percentages of cell population (black color) in the left and right sides of the vertical line. In the control, the percentages of cell populations for V1-L, and V1-R are 50% each (Chang H. W. et al., 2016). The position of this line is at the same position for all treatments as the control setting. Positive (+) %, the cell population in the right side of the line, is indicated in each panel. **(B)** ROS (+) intensity (%) for **(A)**. **(C)** Typical ROS patterns of NAC effect on WFA-treated Ca9-22 cells. Cells were pretreated with 2 mM NAC for 1 h and post-treated with WFA (0–3 μM) for **(24 h)**. **(D)** ROS (+) intensity (%) for **(C)**. NAC/WFA, NAC pretreatment and WFA posttreatment. Data are means ± SDs ($n = 3$). **(B)** $^{**}p < 0.001$ against control (0 μM). **(D)** $^{*}p < 0.05$ for comparison between WFA and NAC/WFA for each concentration of WFA.

Mitochondrial Membrane Potential (MMP) of CA9-22 Oral Cancer Cells Treated with WFA Was Inhibited in WFA-Treated Cells with NAC Pretreatment

The MMP negative (−) expression (%) of WFA-treated Ca9-22 is displayed in **Figure 6A**. After Ca9-22 cells were treated with WFA for 24 h, the MMP (−) expression (%) of WFA-treated Ca9-22 cells were increased (**Figure 6B**), suggesting that WFA induces mitochondrial membrane depolarization in Ca9-22 cells.

The flow cytometric MMP patterns of WFA- and NAC/WFA-treated Ca9-22 cells are displayed in **Figure 6C**. WFA-induced increase of MMP (−) expression was significantly inhibited in WFA-treated Ca9-22 cells with NAC pretreatment (NAC/WFA) ($p < 0.001$) (**Figure 6D**).

γH2AX-Based DNA Damage of CA9-22 Oral Cancer Cells Treated with WFA Was Inhibited in WFA-Treated Cells with NAC Pretreatment

γH2AX was flow cytometrically measured the DNA damage in WFA-treated Ca9-22 oral cancer cells. The percentages and profiles of γH2AX positive (+) stained cells were shown for the 24 h treatments with 0, 1, 2, and 3 μM of WFA (**Figure 7A**). After 24 h of WFA treatment, the % of γH2AX (+) stained cells was significantly higher than the control ($p < 0.05$–0.001) (**Figure 7B**).

The flow-cytometry γH2AX patterns of WFA- and NAC/WFA-treated Ca9-22 cells were provided in **Figure 7C**. WFA-induced γH2AX expression was significantly inhibited in WFA-treated Ca9-22 cells with NAC pretreatment (NAC/WFA) ($p < 0.05$) (**Figure 7D**).

DISCUSSION

WFA has anticancer (Uma Devi et al., 2008; Woo et al., 2014; Lee et al., 2015) and ROS production (Lee et al., 2009; Li et al., 2017; Liu et al., 2017) effects in several types of cancer. However, the selective killing effect of WFA was rarely investigated as yet, especially in oral cancer cells. The aim of this study is to examine the selective killing effects of WFA against oral cancer cells and to explore the role of oxidative stress in WFA-treated oral cancer cells. The cell killing, apoptosis, cell cycle, DNA damage, and oxidative stress effects of WFA in oral cancer cells are discussed as follows.

Comparison of IC_{50} Using WFA in Different Types of Cancer Cells

Different cancer cell types are sensitive to WFA. For example, the IC_{50} values of WFA were 2 μM for myeloid leukemia (HL-60) cells (24 h; MTT assay) (Malik et al., 2007), about 2 μM for pancreatic cancer cells (Panc-1 and MIAPaCa-2) (48 h; MTS assay) (Li et al., 2015), and 0.2–1.2 μM for cervical cancer cells (C33a, CaSki, HeLa, and SiHa) (24 h; MTT assay) (Munagala

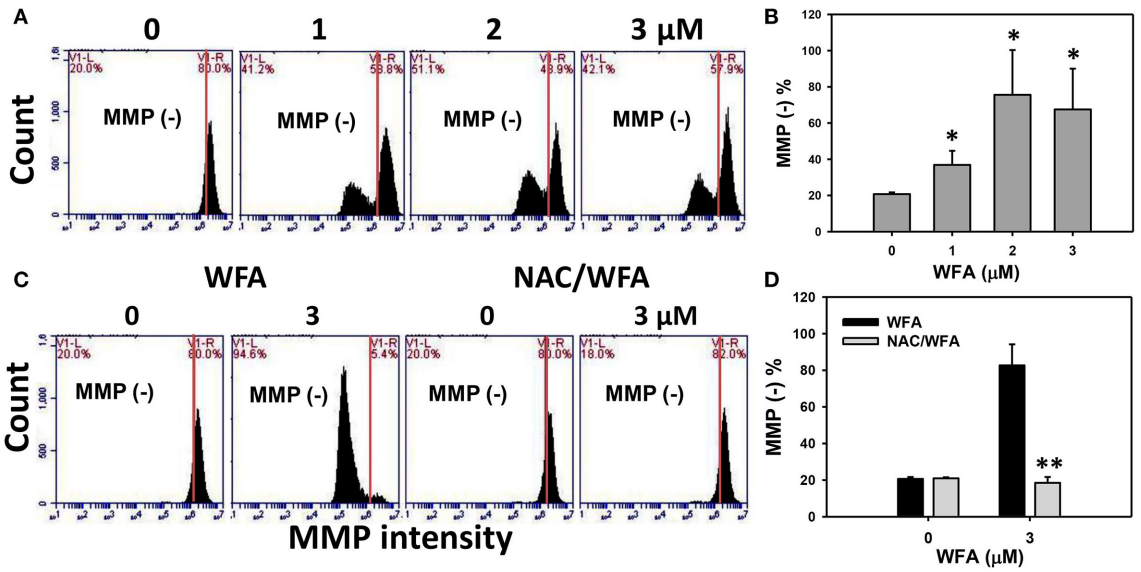

FIGURE 6 | MMP levels of WFA-treated Ca9-22 oral cancer cells and its changes after NAC pretreatment. **(A)** Typical MMP patterns of WFA-treated Ca9-22 oral cancer cells. Cells were treated with WFA (0–3 μM) for 24 h for flow cytometry analysis. V1-L and V1-R labeled in the corner of each plot respectively indicate the percentages of cell populations (black color) in the left and right sides of the vertical line. In control, the percentages of cell populations for V1-L and V1-R are 20 and 80%, respectively (Chang H. W. et al., 2016). The position of this line is at the same position for all treatments as the control setting. MMP-negative (–) (%), the cell population in the left side of the line, is indicated in each panel. **(B)** MMP (–) intensity (%) for **(A)**. **(C)** Typical MMP patterns of the effect of NAC on WFA-treated Ca9-22 cells. Cells were pretreated with 2 mM NAC for 1 h and post-treated with WFA (0–3 μM) for 24 h. **(D)** MMP (–) (%) for **(C)**. Data are means ± SDs ($n = 3$). **(B)** $^*p < 0.05$ against control (0 μM). **(D)** $^{**}p < 0.001$ for comparison between WFA and NAC/WFA (NAC pretreatment and WFA posttreatment).

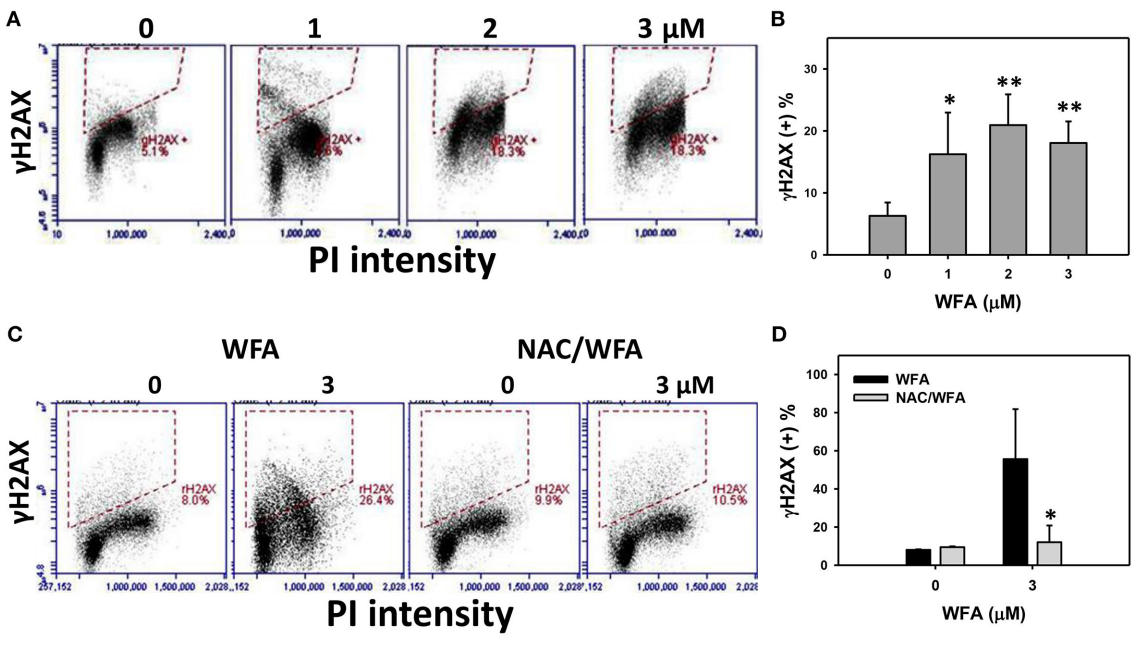

FIGURE 7 | DNA double strand breaks of WFA-treated Ca9-22 oral cancer cells and its changes after NAC pretreatment. **(A)** Ca9-22 oral cancer cells treated with different concentrations (0-3 μM) of WFA for 24 h were stained with γH2AX. **(B)** γH2AX positive (+) intensity (%) for **(A)**. **(C)** Typical DNA damage patterns of NAC effect on WFA-treated Ca9-22 cells. Cells were pretreated with 2 mM NAC for 1 h and post-treated with WFA (0–3 μM) for 12 h. **(D)** γH2AX (+) intensity (%) for **(C)**. Data are means ± SDs ($n = 3$). **(B)** $^*p < 0.05$ and $^{**}p < 0.001$ against control (0 μM). **(D)** $^*p < 0.05$ for comparison between WFA and NAC/WFA (NAC pretreatment and WFA posttreatment).

et al., 2011). However, the selective killing effect of WFA was not thoroughly investigated in these studies. We also found that the IC_{50}-values of WFA in Ca9-22 and CAL 27 oral cancer cells were 3 and 2 μM (24 h; MTS assay), respectively. In contrast, WFA did not harm HGF-1 normal oral cells. Accordingly, WFA induces selective killing against oral cancer cells without adverse effects for the viability of normal oral cells. Therefore, we provide here the first report that documents WFA's selective killing effects against oral cancer cells.

However, HGF-1 normal human gingival cells are a type of fibroblast cell lines and oral cancer cells arise from the epithelium. It was reported that some epithelial and fibroblast cells had different responses to various signaling molecules. For example, epithelial cells undergo growth arrest in response to TGF-β, whereas fibroblasts undergo morphological changes, and proliferate (Wilkes et al., 2003). Therefore, the possibility of selective killing of WFA against oral cancer cells warrants further detailed investigation. In a follow-up study we will compare oral cancer cells with normal epithelial cells using a similar experimental design.

WFA Induces Apoptosis in Cancer Cells

In the current study, WFA induced apoptosis in Ca9-22 oral cancer cells associated with subG1 accumulation and caspase activation. This is consistent with the findings that WFA induced apoptosis in CaSki cervical cancer cells (Munagala et al., 2011) and WFA induced apoptosis in MDA-MB-231 and MCF-7 breast cancer cells (Hahm and Singh, 2013). In the current study (**Figure 4E**), the cleaved caspases (3, 8, and 9) and cleaved PARP showed high cleavage (high apoptosis) in 1 and 2 μM of WFA, but showed less cleavage (less apoptosis) in 3 μM of WFA. Similarly, other drugs have related findings. For example, etoposide induced more cleavage of PARP at 2 and 4 days, but declined at day 6 in human non-small cell lung cancer cells (H1437) (Chiu et al., 2005). Ganciclovir induced more cleavage of PARP, caspases 3/9, and cytochrome *c* at 1 or 2 days, but declined at 3 days in human non-small cell lung cancer cells (CL-1) (Chiu et al., 2002). It is possible that higher dose treatment or longer exposure may lead to more cell death and fail to proportionally cleave apoptotic proteins. Furthermore, caspase-1 may mediate apoptosis (Bergsbaken et al., 2009; Miao et al., 2011; Sollberger et al., 2014) and it warrants further investigation for the role of caspase-1 in WFA induced apoptosis.

In addition to the induction of subG1 accumulation, WFA induces G2/M arrest and a mitotic catastrophe in prostate cancer cells in terms of cell cycle analysis and western blotting for several G2/M arresting proteins (Roy et al., 2013). WFA also induces G2/M arrest in gastric cancer cells (Kim et al., 2017), osteosarcoma cells (Lv and Wang, 2015), and breast cancer cells (Zhang et al., 2011). Similar to the current study, WFA induced G2/M arrest in Ca9-22 cells at lower concentrations (1 and 2 μM) but did not present at higher concentration (3 μM) (**Figure 2B**) where subG1 population (apoptosis) was gradually increased.

WFA Induces DNA Damage in Cancer Cells

Drug-induced ROS generation is associated with DNA damage (Yang et al., 2012; Chiu et al., 2013; Chen et al., 2016). Since WFA is known to induce ROS production (Lee et al., 2009; Li et al., 2017; Liu et al., 2017) (**Figure 5**), the DNA damage effect warrants for investigation. In the current study, we found that WFA treatment induced γH2AX expression in Ca9-22 oral cancer cells, which is consistent with the findings that WFA-induced γH2AX expression in MCF7 breast cancer cells, but not in normal human TIG-3 fibroblasts (Widodo et al., 2010). This DNA damaging ability was shown to be associated with apoptosis in several other cancer cell studies (Norbury and Zhivotovsky, 2004; Roos and Kaina, 2006).

WFA-Induced Cell Killing, Apoptosis, Cell Cycle Change, and DNA Damage Are Medidated by Oxidative Stress in Cancer Cells

NAC is a common free radical scavenger. NAC pretreatment is reported to effectively diminish cellular ROS and to confirm the role of oxidative stress in drug treatment. Recently, several WFA-based cancer therapies were reported to be rescued by NAC pretreatment. For example, WFA induced apoptosis in human melanoma cells was accomplished by generating ROS (Mayola et al., 2011), and NAC pretreatment rescued ROS-induced damage. NAC also inhibited WFA-induced ROS production and caspase activation of human HL-60 myeloid leukemia cells (Malik et al., 2007).

Similarly, we found that NAC pretreatment rescued WFA-induced apoptosis in Ca9-22 oral cancer cells by subG1 accumulation, annexin V/PI, pan-caspases, and caspase signaling in western blotting. In our study, low concentration of WFA-induced G2/M arrest in Ca9-22 cells was rescued by NAC pretreatment. NAC pretreatment rescued WFA-induced oxidative stress in Ca9-22 cells by ROS and MMP analysis. NAC pretreatment also rescued γH2AX-based DNA damage in Ca9-22 cells as shown by the flow cytometry assay. This is the first evidence that NAC pretreatment protects WFA-treated oral cancer cells against oxidative stress.

CONCLUSION

WFA selectively killed oral cancer cells with less toxic effects to normal oral cells. WFA also induced apoptosis, oxidative stress, and DNA damage, which ultimately inhibited the proliferation of oral cancer cells.

AUTHOR CONTRIBUTIONS

JL, HW, and YC carried out the experiments. JT and HH analyzed the data. HC, RL, and CY conceived and designed the study. HC and CY wrote and revised the manuscript.

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REFERENCES

Barzilai, A., and Yamamoto, K. (2004). DNA damage responses to oxidative stress. *DNA Repair* 3, 1109–1115. doi: 10.1016/j.dnarep.2004.03.002

Bergsbaken, T., Fink, S. L., and Cookson, B. T. (2009). Pyroptosis: host cell death and inflammation. *Nat. Rev. Microbiol.* 7, 99–109. doi: 10.1038/nrmicro2070

Chan, W. H., Shiao, N. H., and Lu, P. Z. (2006). CdSe quantum dots induce apoptosis in human neuroblastoma cells via mitochondrial-dependent pathways and inhibition of survival signals. *Toxicol. Lett.* 167, 191–200. doi: 10.1016/j.toxlet.2006.09.007

Chang, H. W., Tang, J. Y., Yen, C. Y., Chang, H. S., Huang, H. W., Chung, Y. A., et al. (2016). Synergistic anti-oral cancer effects of UVC and methanolic extracts of *Cryptocarya concinna* roots via apoptosis, oxidative stress and DNA damage. *Int. J. Radiat. Biol.* 92, 263–272. doi: 10.3109/09553002.2016.1145753

Chang, Y. T., Huang, C. Y., Li, K. T., Li, R. N., Liaw, C. C., Wu, S. H., et al. (2016). Sinuleptolide inhibits proliferation of oral cancer Ca9-22 cells involving apoptosis, oxidative stress, and DNA damage. *Arch. Oral Biol.* 66, 147–154. doi: 10.1016/j.archoralbio.2016.02.019

Chen, C. Y., Yen, C. Y., Wang, H. R., Yang, H. P., Tang, J. Y., Huang, H. W., et al. (2016). Tenuifolide B from *Cinnamomum tenuifolium* stem selectively inhibits proliferation of oral cancer cells via apoptosis, ROS generation, mitochondrial depolarization, and DNA damage. *Toxins* 8:319. doi: 10.3390/toxins8110319

Chiu, C. C., Haung, J. W., Chang, F. R., Huang, K. J., Huang, H. M., Huang, H. W., et al. (2013). Golden berry-derived 4β-hydroxywithanolide E for selectively killing oral cancer cells by generating, ROS DNA damage, and apoptotic pathways. *PLoS ONE* 8:e64739. doi: 10.1371/journal.pone.0064739

Chiu, C. C., Kang, Y. L., Yang, T. H., Huang, C. H., and Fang, K. (2002). Ectopic expression of herpes simplex virus-thymidine kinase gene in human non-small cell lung cancer cells conferred caspase-activated apoptosis sensitized by ganciclovir. *Int. J. Cancer* 102, 328–333. doi: 10.1002/ijc.10701

Chiu, C. C., Li, C. H., Ung, M. W., Fuh, T. S., Chen, W. L., and Fang, K. (2005). Etoposide (VP-16) elicits apoptosis following prolonged G2-M cell arrest in p53-mutated human non-small cell lung cancer cells. *Cancer Lett.* 223, 249–258. doi: 10.1016/j.canlet.2004.10.049

Chiu, C. C., Liu, P. L., Huang, K. J., Wang, H. M., Chang, K. F., Chou, C. K., et al. (2011). Goniothalamin inhibits growth of human lung cancer cells through DNA damage, apoptosis, and reduced migration ability. *J. Agric. Food Chem.* 59, 4288–4293. doi: 10.1021/jf200566a

Daniel, D., Susal, C., Kopp, B., Opelz, G., and Terness, P. (2003). Apoptosis-mediated selective killing of malignant cells by cardiac steroids: maintenance of cytotoxicity and loss of cardiac activity of chemically modified derivatives. *Int. Immunopharmacol.* 3, 1791–1801. doi: 10.1016/j.intimp.2003.08.004

Ding, H., Han, C., Guo, D., Chin, Y. W., Ding, Y., Kinghorn, A. D., et al. (2009). Selective induction of apoptosis of human oral cancer cell lines by avocado extracts via a ROS mediated mechanism. *Nutr. Cancer* 61, 348–356. doi: 10.1080/01635580802567158

Evan, G. I., and Vousden, K. H. (2001). Proliferation, cell cycle and apoptosis in cancer. *Nature* 411, 342–348. doi: 10.1038/35077213

Hahm, E. R., and Singh, S. V. (2013). Withaferin A induced apoptosis in human breast cancer cells is associated with suppression of inhibitor of apoptosis family protein expression. *Cancer Lett.* 334, 101–108. doi: 10.1016/j.canlet.2012.08.026

Hung, J. H., Chen, C. Y., Omar, H. A., Huang, K. Y., Tsao, C. C., Chiu, C. C., et al. (2015). Reactive oxygen species mediate Terbufos-induced apoptosis in mouse testicular cell lines via the modulation of cell cycle and pro-apoptotic proteins. *Environ. Toxicol.* 31, 1888–1898. doi: 10.1002/tox.22190

Kim, G., Kim, T.-H., Hwang, E.-H., Chang, K.-T., Hong, J.-J., and Park, J.-H. (2017). Withaferin A inhibits the proliferation of gastric cancer cells by inducing G2/M cell cycle arrest and apoptosis. *Oncol. Lett.* 14, 416–422. doi: 10.3892/ol.2017.6169

Lee, J. C., Hou, M. F., Huang, H. W., Chang, F. R., Yeh, C. C., Tang, J. Y., et al. (2013). Marine algal natural products with anti-oxidative, anti-inflammatory, and anti-cancer properties. *Cancer Cell Int.* 13:55. doi: 10.1186/1475-2867-13-55

Lee, J., Hahm, E. R., Marcus, A. I., and Singh, S. V. (2015). Withaferin A inhibits experimental epithelial-mesenchymal transition in MCF10A cells and suppresses vimentin protein level *in vivo* in breast tumors. *Mol. Carcinog.* 54, 417–429. doi: 10.1002/mc.22110

Lee, T. J., Um, H. J., Min do, S., Park, J. W., Choi, K. S., and Kwon, T. K., et al. (2009). induced apoptosis through reactive oxygen species-mediated up-regulation of death receptor 5 and down-regulation of c-FLIP. *Free Radic. Biol. Med.* 46, 1639–1649. doi: 10.1016/j.freeradbiomed.2009.03.022

Li, A. X., Sun, M., and Li, X. (2017). Withaferin-A induces apoptosis in osteosarcoma U2OS cell line via generation of ROS and disruption of mitochondrial membrane potential. *Eur. Rev. Med. Pharmacol. Sci.* 21, 1368–1374. Available online at: http://www.europeanreview.org/wp/wp-content/uploads/1368-1374-Withaferin-A-induces-apoptosis-in-osteosarcoma-U2OS-cell-line.pdf

Li, J. J., Tang, Q., Li, Y., Hu, B. R., Ming, Z. Y., Fu, Q., et al. (2006). Role of oxidative stress in the apoptosis of hepatocellular carcinoma induced by combination of arsenic trioxide and ascorbic acid. *Acta Pharmacol. Sin.* 27, 1078–1084. doi: 10.1111/j.1745-7254.2006.00345.x

Li, X., Zhu, F., Jiang, J., Sun, C., Wang, X., Shen, M., et al. (2015). Synergistic antitumor activity of withaferin A combined with oxaliplatin triggers reactive oxygen species-mediated inactivation of the PI3K/AKT pathway in human pancreatic cancer cells. *Cancer Lett.* 357, 219–230. doi: 10.1016/j.canlet.2014.11.026

Lien, J. C., Lin, M. W., Chang, S. J., Lai, K. C., Huang, A. C., Yu, F. S., et al. (2017). Tetrandrine induces programmed cell death in human oral cancer CAL27 cells through the reactive oxygen species production and caspase-dependent pathways and associated with beclin-1-induced cell autophagy. *Environ. Toxicol.* 32, 329–343. doi: 10.1002/tox.22238

Liu, X., Chen, L., Liang, T., Tian, X. D., Liu, Y., and Zhang, T., et al. (2017). Withaferin induces mitochondrial-dependent apoptosis in non-small cell lung cancer cells via generation of reactive oxygen species. *J. BUON* 22, 244–250. Available online at: https://www.jbuon.com/pdfs/244-250.pdf

Lv, T. Z., and Wang, G. S. (2015). Antiproliferation potential of withaferin A on human osteosarcoma cells via the inhibition of G2/M checkpoint proteins. *Exp. Ther. Med.* 10, 323–329. doi: 10.3892/etm.2015.2480

Malik, F., Kumar, A., Bhushan, S., Khan, S., Bhatia, A., Suri, K. A., et al. (2007). Reactive oxygen species generation and mitochondrial dysfunction in the apoptotic cell death of human myeloid leukemia HL-60 cells by a dietary compound withaferin A with concomitant protection by N-acetyl cysteine. *Apoptosis* 12, 2115–2133. doi: 10.1007/s10495-007-0129-x

Mayola, E., Gallerne, C., Esposti, D. D., Martel, C., Pervaiz, S., Larue, L., et al. (2011). Withaferin A induces apoptosis in human melanoma cells through generation of reactive oxygen species and down-regulation of Bcl-2. *Apoptosis* 16, 1014–1027. doi: 10.1007/s10495-011-0625-x

Miao, E. A., Rajan, J. V., and Aderem, A. (2011). Caspase-1-induced pyroptotic cell death. *Immunol. Rev.* 243, 206–214. doi: 10.1111/j.1600-065X.2011.01044.x

Munagala, R., Kausar, H., Munjal, C., and Gupta, R. C. (2011). Withaferin A induces p53-dependent apoptosis by repression of HPV oncogenes and upregulation of tumor suppressor proteins in human cervical cancer cells. *Carcinogenesis* 32, 1697–1705. doi: 10.1093/carcin/bgr192

Nicco, C., Laurent, A., Chereau, C., Weill, B., and Batteux, F. (2005). Differential modulation of normal and tumor cell proliferation by reactive oxygen species. *Biomed. Pharmacother.* 59, 169–174. doi: 10.1016/j.biopha.2005.03.009

Norbury, C. J., and Zhivotovsky, B. D. N. A. (2004). Damage-induced apoptosis. *Oncogene* 23, 2797–2808. doi: 10.1038/sj.onc.1207532

Oh, S. H., and Lim, S. C. A. (2006). Rapid and transient ROS generation by cadmium triggers apoptosis via caspase-dependent pathway in HepG2 cells and this is inhibited through N-acetylcysteine-mediated catalase upregulation. *Toxicol. Appl. Pharmacol.* 212, 212–223. doi: 10.1016/j.taap.2005.07.018

Pollack, I. F., Erff, M., and Ashkenazi, A. (2001). Direct stimulation of apoptotic signaling by soluble Apo2l/tumor necrosis factor-related apoptosis-inducing ligand leads to selective killing of glioma cells. *Clin. Cancer Res.* 7, 1362–1369. Available online at: http://clincancerres.aacrjournals.org/content/7/5/1362.full-text.pdf

Real, P. J., Cao, Y., Wang, R., Nikolovska-Coleska, Z., Sanz-Ortiz, J., Wang, S., et al. (2004). Breast cancer cells can evade apoptosis-mediated selective killing by a novel small molecule inhibitor of Bcl-2. *Cancer Res.* 64, 7947–7953. doi: 10.1158/0008-5472.CAN-04-0945

Reed, J. C. (1999). Mechanisms of apoptosis avoidance in cancer. *Curr. Opin. Oncol.* 11, 68–75. doi: 10.1097/00001622-199901000-00014

Roos, W. P., and Kaina, B. D. N. A. (2006). Damage-induced cell death by apoptosis. *Trends Mol. Med.* 12, 440–450. doi: 10.1016/j.molmed.2006.07.007

Roy, R. V., Suman, S., Das, T. P., Luevano, J. E., Damodaran, C., and Withaferin, A. (2013). A steroidal lactone from *Withania somnifera*, induces mitotic catastrophe and growth arrest in prostate cancer cells. *J. Nat. Prod.* 76, 1909–1915. doi: 10.1021/np400441f

Samhan-Arias, A. K., Martin-Romero, F. J., and Gutierrez-Merino, C. (2004). Kaempferol blocks oxidative stress in cerebellar granule cells and reveals a key role for reactive oxygen species production at the plasma membrane in the commitment to apoptosis. *Free Radic. Biol. Med.* 37, 48–61. doi: 10.1016/j.freeradbiomed.2004.04.002

Scully, C., Field, J. K., and Tanzawa, H. (2000). Genetic aberrations in oral or head and neck squamous cell carcinoma (SCCHN): 1. Carcinogen metabolism, DNA repair and cell cycle control. *Oral Oncol.* 36, 256–263. doi: 10.1016/S1368-8375(00)00007-5

Shieh, J. M., Shen, C. J., Chang, W. C., Cheng, H. C., Chan, Y. Y., Huang, W. C., et al. (2014). An increase in reactive oxygen species by deregulation of ARNT enhances chemotherapeutic drug-induced cancer cell death. *PLoS ONE* 9:e99242. doi: 10.1371/journal.pone.0099242

Shih, H. C., El-Shazly, M., Juan, Y. S., Chang, C. Y., Su, J. H., Chen, Y. C., et al. (2014). Cracking the cytotoxicity code: apoptotic induction of 10-acetylirciformonin B is mediated through ROS generation and mitochondrial dysfunction. *Mar. Drugs* 12, 3072–3090. doi: 10.3390/md12053072

Sollberger, G., Strittmatter, G. E., Garstkiewicz, M., Sand, J., and Beer, H. D. (2014). Caspase-1: the inflammasome and beyond. *Innate Immun.* 20, 115–125. doi: 10.1177/1753425913484374

Trachootham, D., Zhou, Y., Zhang, H., Demizu, Y., Chen, Z., Pelicano, H., et al. (2006). Selective killing of oncogenically transformed cells through a ROS mediated mechanism by beta-phenylethyl isothiocyanate. *Cancer Cell* 10, 241–252. doi: 10.1016/j.ccr.2006.08.009

Uma Devi, P., Utsumiz, H., Takata, M., and Takeda, S. (2008). Enhancement of radiation induced cell death in chicken B lymphocytes by withaferin A. *Indian J. Exp. Biol.* 46, 437–442. Available online at: http://nopr.niscair.res.in/bitstream/123456789/4497/1/IJEB%2046(6)%20437-442.pdf

Vanden Berghe, W., Sabbe, L., Kaileh, M., Haegeman, G., and Heyninck, K. (2012). Molecular insight in the multifunctional activities of Withaferin A. *Biochem. Pharmacol.* 84, 1282–1291. doi: 10.1016/j.bcp.2012.08.027

Vignon, C., Debeissat, C., Georget, M. T., Bouscary, D., Gyan, E., Rosset, P., et al. (2013). Flow cytometric quantification of all phases of the cell cycle and apoptosis in a two-color fluorescence plot. *PLoS ONE* 8:e68425. doi: 10.1371/journal.pone.0068425

Vyas, A. R., and Singh, S. V. (2014). Molecular targets and mechanisms of cancer prevention and treatment by withaferin A, a naturally occurring steroidal lactone. *AAPS J.* 16, 1–10. doi: 10.1208/s12248-013-9531-1

Widodo, N., Priyandoko, D., Shah, N., Wadhwa, R., and Kaul, S. C. (2010). Selective killing of cancer cells by Ashwagandha leaf extract and its component Withanone involves ROS signaling. *PLoS ONE* 5:e13536. doi: 10.1371/journal.pone.0013536

Wilkes, M. C., Murphy, S. J., Garamszegi, N., and Leof, E. B. (2003). Cell-type-specific activation of PAK2 by transforming growth factor beta independent of Smad2 and Smad3. *Mol. Cell Biol.* 23, 8878–8889. doi: 10.1128/MCB.23.23.8878-8889.2003

Woo, S. M., Min, K. J., Kim, S., Park, J. W., Kim, D. E., Kim, S. H., et al. (2014). Axl is a novel target of withaferin A in the induction of apoptosis and the suppression of invasion. *Biochem. Biophys. Res. Commun.* 451, 455–460. doi: 10.1016/j.bbrc.2014.08.018

Wu, X. J., and Hua, X. (2007). Targeting ROS selective killing of cancer cells by a cruciferous vegetable derived pro-oxidant compound. *Cancer Biol Ther.* 6, 646–647. doi: 10.4161/cbt.6.5.4092

Yang, J. I., Yeh, C. C., Lee, J. C., Yi, S. C., Huang, H. W., Tseng, C. N., et al. (2012). Aqueous extracts of the edible Gracilaria tenuistipitata are protective against H_2O_2-induced DNA damage, growth inhibition, and cell cycle arrest. *Molecules* 17, 7241–7254. doi: 10.3390/molecules17067241

Yeh, C. C., Tseng, C. N., Yang, J. I., Huang, H. W., Fang, Y., Tang, J. Y., et al. (2012). Antiproliferation and induction of apoptosis in Ca9-22 oral cancer cells by ethanolic extract of *Gracilaria tenuistipitata*. *Molecules* 17, 10916–10927. doi: 10.3390/molecules170910916

Yen, C. Y., Chiu, C. C., Haung, R. W., Yeh, C. C., Huang, K. J., Chang, K. F., et al. (2012). Antiproliferative effects of goniothalamin on Ca9-22 oral cancer cells through apoptosis, DNA damage and ROS induction. *Mutat. Res.* 747, 253–258. doi: 10.1016/j.mrgentox.2012.06.003

Zhang, X., Mukerji, R., Samadi, A. K., and Cohen, M. S. (2011). Down-regulation of estrogen receptor-alpha and rearranged during transfection tyrosine kinase is associated with withaferin A-induced apoptosis in MCF-7 breast cancer cells. *BMC Complement. Altern. Med.* 11:84. doi: 10.1186/1472-6882-11-84

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The Effect of *Calendula officinalis* on Oxidative Stress and Bone Loss in Experimental Periodontitis

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Periodontitis is associated with reduced antioxidant capacity and increased oxidative damage. Oxidative stress induces inflammation and bone loss contributing to the pathological progression of periodontal disease. *Calendula officinalis* (CLO) has demonstrated anti-inflammatory and anti-oxidant activities. Therefore, the aim of this study was to evaluate the effect of CLO on oxidative stress and bone loss in rats subjected to experimental periodontitis (EP). For this, 72 male Wistar rats were divided into groups: Naïve, Saline (SAL) and CLO. Rats received SAL or CLO (90 mg/kg) 30 min before ligature and daily until the 11th day. Naïve group experienced no manipulation. After 11 days, the animals were euthanized and left maxillae collected for macroscopic analysis of alveolar bone loss (ABL). Periodontium was analyzed by macroscopy, scanning electron microscopy; confocal and light polarized microscopy. Immunohistochemical examination of DKK1, WNT 10b and β-catenin was performed. The gingival tissue was collected to reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) analyses. The 11 days of ligature induced bone loss, breakdown of collagen fibers, increased the immunostaining DKK-1 while reduced WNT 10b and β-catenin expressions. Periodontitis reduced GSH, SOD, CAT and increase MDA. All findings were reversed by 90 mg/kg of CLO. In summary our findings demonstrated that CLO reduced oxidative stress and bone loss and preserved collagen fibers in rats with EP, with participation of WNT signaling pathway.

Keywords: ***Calendula officinalis*, periodontitis, bone loss, oxidative stress, rats**

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INTRODUCTION

Periodontitis is a high prevalent infect-inflammatory disease (Petersen and Ogawa, 2005). It is considered the second more important cause of tooth loss on the population (Kayal, 2013). Its etiology is multifactorial, where dental biofilm stimulates immunoinflammatory host response resulting in tissue destruction (Redlich and Smolen, 2012).

During periodontitis, the inflammatory process is marked by neutrophils that invade periodontium and induces the release of proteolytic enzymes and production of reactive oxygen species (ROS) (D'Aiuto et al., 2010). An increased generation of ROS initiates a chain reaction of degradation steps, termed lipid peroxidation, that can ultimately decompose into secondary

products such as malondialdehyde (MDA) (Busch and Binder, 2016). In the other hand, the body produces a variety of defense mechanisms to combat the excess of ROS, among these there are the antioxidants (AOs) (Palwankar et al., 2015). Superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) peroxidase are important AOs that enzymatically eliminate, hydrogen peroxide (H_2O_2), oxygen superoxide (O_2) or the hydroxyl radical (OH), highly reactive oxygen radicals, which are responsible for most oxidative stress in cells (Galli et al., 2011).

The continuous exacerbation of inflammation culminates with collagen fiber destruction and bone resorption (Zheng et al., 2015). Literature has described the role of RANK-RANKL-OPG axis on bone loss (Graves et al., 2011). However, recently another signaling pathway has stood out as a regulator of bone homeostasis, known as the canonical WNT pathway (Kobayashi et al., 2016). Briefly, on bone tissue, WNT proteins, mainly WNT10b, interacts with its receptor (LPR5/6) and induce cytoplasmatic inhibition of GSK3β, which stabilizes β-catenin, which in turn, gain access to the nucleus activating transcriptors factors that promote osteoblast differentiation (Baron and Kneissel, 2013). DKK acts as a regulator of this pathway blocking the interaction between WNT and its receptor, with consequent β-catenin degradation and lack of osteoblast differentiation (Baron and Kneissel, 2013). The expression of WNT pathway inhibitors seems to be induced by inflammatory mediators (Rauner et al., 2008). In this context, knowing that inflammation plays an important role on bone disorders, seems interesting to study the effect of pharmacological agents that may modulate the inflammatory process.

CLO is annual herb well known by its anti-inflammatory activity (Parente et al., 2012). More than 100 constituents have already been identified from the crude extract of this plant, but the flavonoid quercetin is considered of great importance due to its anti-inflammatory and antioxidant effects (Li et al., 2016). It has been reported that CLO extract inhibited significantly the paw edema and inflammation, lowered LPS-induced IL-1, and -6, TNF-alpha, interferon and acute-phase proteins levels (Preethi et al., 2009). In periodontal diseases, as a gel or mouthwash, CLO has shown positive effect on gingivitis treatment (Lauten et al., 2005; Machado, 2010). Specifically in periodontitis, this is the first time that the effect of CLO is studied. Therefore, considering that inflammation and oxidative stress can lead to bone loss, which is the main cause of periodontitis, and that CLO has an important anti-inflammatory and antioxidant effects, it seems interesting to evaluate the effect of CLO in rats subjected to experimental periodontitis.

METHODS

Animals

Seventy-two male Wistar rats (±200 g) (*Rattus novergicus*), from our own facilities, were used in this study. The animals were kept in cages with temperature-controlled rooms, with food and water *ad libitum* throughout the experiment. In accordance to the Ethical Principles for Animal Research, all efforts were done in order to reduce pain or discomfort to the animals. All procedures and animal treatments were conducted after approval by the institutional Ethical Committee for Animal Research from Federal University of Ceará (UFC) (number 38/15).

Experimental Periodontitis

For the induction of experimental periodontitis (EP) it was used the model of ligature-induced periodontitis (Goes et al., 2010). The animals were anesthetized with ketamine (70 mg/kg administered i.p., 10% Quetamina, Vetnil, São Paulo, SP, Brazil) and xylazine (10 mg/kg administered i.p., 2% Calmium, São Paulo, SP, Brazil). Later, a sterilized nylon (000) thread ligature was placed around the cervix of the second left upper molar. The ligature was then knotted on the vestibular side of the referred tooth. Eleven days after ligature placement the animals were euthanized with 20 mg/kg thiopental (0.5 g Thiopentax, Cristália, São Paulo, SP, Brazil). The ligatures were blinded to the group.

Experimental Groups

The animals ($n = 6$ in each group) were randomly assigned into 3 groups: SAL, CLO and Naïve. The rats received, according to the group, either 0.9% of Saline solution (SAL) at the dose of 2 ml/kg or 90 mg/kg of aqueous flower extract of *Calendula officinalis* (CLO) (Batch number PROD004257) purchased from Mapric Cosmetic and Pharmaceutical Products, Brazil (chemical abstract service—CAS – number 64-17-5; 7732-18-5; 99-76-3), by gavage, 30 min before EP and daily for 10 days. The Naïve group experienced no manipulation.

Macroscopic Analysis of Alveolar Bone

On the 11th day, animals were euthanized under anesthesia and had their maxillae removed and fixed in 10% neutral formalin for 24 h. Following, maxillae were divided in half, dissected and stained with 1% methylene blue (Goes et al., 2010). For the measurement of ABL, hemi-maxillae were placed in microscope slides and photographed with a digital camera (Nikon® D40, Melville, NY, USA). The acquired image was analyzed using the IMAGE J® software (ImageJ 1.32j; National Institute of Health, Bethesda, MD, USA), according to the methodology described by Goes et al. (2010).

Scanning Electron Microscopy (SEM) of Alveolar Bone

Two additional groups of 6 animals submitted to EP, which received SAL or CLO (90 mg/kg), were euthanized and had their maxillae removed. The specimens were fixed in Karnovisky for at least 6 h, then they were held in a Cacodylate buffer. The maxillae were cut in a diamond blade cutter, in a mesial-distal plane, to obtain the maxilla fragment (0.5 × 0.2 cm and 0.5 mm thick). The fragment was placed in an eppendorf tube and left in the desiccator drying for 24 h. The fragments were assembled into stubs for metallization with gold powder (Quorum Metallizer QT150ES, Quorum Technologies, Laughton, England) for the analysis by a scanning electron microscopy (SEM inspect-50, FEI, Hillsboro, Oregon, USA). It was evaluated the bone topography of the interproximal region between the first and second maxillary left molars (Lu et al., 2014).

In addition, it was used the Energy Dispersive Spectroscopy (EDS), which is a detector (Oxford Instruments, Abingdon,

Oxfordshire, UK) installed in the vacuum chamber of the MEV. The elemental and chemical analysis of the sample were performed using the manufacturer's software (AztecEnergy, Oxford Instruments, Abingdon, Oxfordshire, UK)

Analysis of Collagen Fibers in Periodontium

Two additional groups of 6 animals submitted to EP, which received SAL or CLO (90 mg/kg), were euthanized and had their maxillae removed. The specimens were fixed in 10% neutral buffered formalin for 24 h, and then demineralized in 10% EDTA for 30 days. Following this, the specimens were dehydrated, embedded in paraffin and sectioned (4 μm) along the molars in a mesiodistal plane for H&E staining (Goes et al., 2012). Considering that collagen is a structural protein that presents a natural phenomena of self-fluorescence, the images were obtained using the Confocal LSM 710 microscope (Zeiss, Jena, Germany) and analyzed by manufacture's software (Zen 2.1 lite black, 64-bit version, 758 MB, Zeiss, Jena, Germany). In order to evaluate the presence and organization of collagen fibers in periodontium, between the first and second left upper molar, it was used 488 nm wavelength laser and emission channel for FITC-green fluorescence (Gonçalves et al., 2014)

In order to identify the presence and type of fibrilar collagen, histological sections (4 μm) were obtained from the previously prepared paraffin blocks, and were stained with Picrosirius Red. The slides were evaluated under a polarized light filter. Collagen birefringence, as yellow-red for type I collagen and green for type III collagen (Junqueira et al., 1979), was evaluated between the first and second maxillary left molars.

Immunohistochemical Analyses of DKK1, WNT 10b and β-Catenin

The streptavidin–biotin–peroxidase method was used for Immunohistochemistry assay, in paraffin-embedded tissue sections 4 μm thick. Sections of the excised maxillae, demineralized in a 10% EDTA solution were used for DKK1 (antibody rabbit policlonal IgG, Santa Cruz Biotechnology), WNT 10b (antibody rabbit policlonal IgG, Abcam), and β-catenin (antibody goat policlonal IgG, Santa Cruz Biotechnology) (Sousa et al., 2016). Five microscopic fields (400x) were used to count osteoblasts exhibiting cytoplasmic positivity for DKK1, WNT 10b and β-catenin (de Barros Silva et al., 2016).

Gingival Levels of Reduced Glutathione, Enzyme Catalase, Enzyme Superoxide Dismutase and Malondialdehyde

Reduced glutathione (GSH), catalase (CAT) superoxide dismutase (SOD) and malondialdehyde (MDA) were performed to evaluate oxidative stress. For this, the gingival tissue was removed 11 days after EP, then stored at −80°C. The level of GSH in gingival tissue was estimated according to the methods described previously (Sedlak and Lindsay, 1968). The GSH concentration was expressed as micrometers of GSH per gram of wet tissue.

Superoxide dismutase (SOD) activity was assayed as described previously (Beauchamp and Fridovich, 1971). In a dark room, the gingival samples were homogenized in 20 μl of ice-cold phosphate buffer at 15,000 G for 20 min. The supernatants were mixed with a solution comprised of phosphate buffer (50 nM), EDTA (100 nM) and L-methionine (19.5 mM) in a pH of 7.8. Then, 150 ml of a solution of riboflavin (10 nM) and NBT (750 nM) as added and the mixture was exposed to light (20 W) for 15 min. The absorbance of the samples was measured at 560 nm. The results are expressed as grams of SOD per ml.

CAT activity has as principle the measurement of O_2 production rate and H_2O in proportion of H_2O_2 (Maehly and Chance, 1954). Briefly, 20 μl of gingival homogenate was mixed with a solution comprised of 3% H_2O_2 and Tris-HCl EDTA buffer (5 nM, pH 8.0). The absorbance at a 230 nm wavelength was measured immediately and 6 min after preparing the samples.

Malondialdehyde (MDA) indicates lipid peroxidation based on the reaction of this substance with thiobarbituric acid, in the gingival tissue of rats. Briefly, 250 μl of 10% homogenate of gingival tissue were mixed with 1.5 ml of 1% H3PO4 and 0.5 ml of 0.6% thiobarbituric acid aqueous solution and the mixture was stirred and heated in boiling water for 45 min. After cooling, 2 ml of n-butanol were added and the mixture was homogenized. The butanol layer was separated, and the difference between the optical densities at 535 was used for calculating the MDA concentrations, which were expressed as nanomol of MDA per gram of gingival tissue (Mihara and Uchivam, 1978).

Statistical Analysis

Data are presented as mean ± SEM. In order to compare means it was used Analysis of variance (ANOVA) followed by Bonferroni test. $P < 0.05$ was set to indicate significant differences among groups. All analyses were performed using GraphPad Prism 6 software, San Diego, CA, USA.

RESULTS

Effect of CLO on Alveolar Bone Loss

Morphometric study demonstrated that the experimental periodontitis caused intense bone resorption (**Figure 1D**) compared to the normal hemi-maxillae from Naïve group (**Figure 1A**). Rats from SAL group presented intense alveolar resorption, root exposure and furcation lesion (**Figure 1B**). Ninety mg/kg of CLO prevented bone loss (**Figure 1C**), by 42.8%, when compared to SAL ($p < 0.05$). The treated animals showed greater preservation of bone tissue.

Effect of CLO on Bone Topography and Mineral Distribution

Experimental Periodontitis (**Figure 2B**) caused important destruction of bone tissue when compared to the normal tissue from Naïve group (**Figure 2A**). CLO, at 90 mg/kg, prevented bone loss (**Figure 2C**). In 800x magnification, it was possible the observe that the bone tissue from an animal of SAL group (**Figure 2E**) presented an irregular topography, when compared to the bone tissue of an animal from Naïve

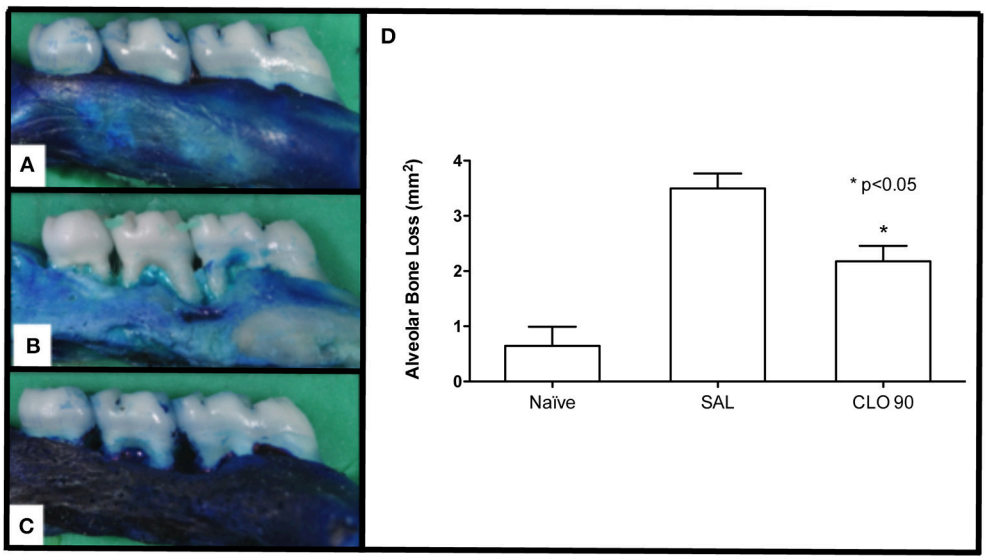

FIGURE 1 | Effect of CLO on ABL in rats with EP. Naoïve hemimaxilla **(A)**, Hemimaxilla from SAL group **(B)**, Hemimaxilla tretated with CLO 90 mg/kg **(C)**, Macroscopic Analysis **(D)**. Bars represent the mean ± SEM of 6 animals per group. *$P < 0.05$ was considered to be significantly different compared with SAL (ANOVA followed by the Bonferroni test). (**A–C**, 4x magnification).

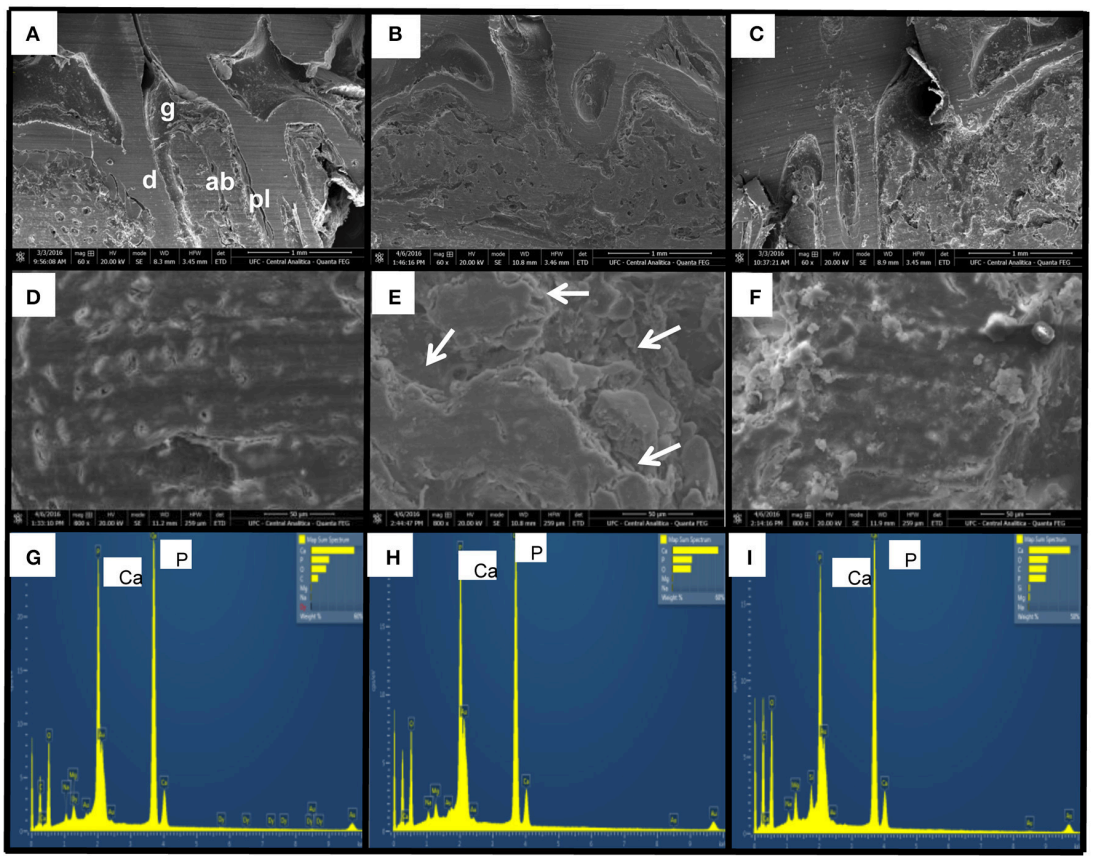

FIGURE 2 | Effect of CLO on topography and mineral distribution of alveolar bone of rats with EP. Naïve **(A,D,G)**, SAL **(B,E,H)**, CLO **(C,F,I)**. Dentin (d); Alveolar bone (ab); Periodontal ligament (pl); Gingiva (g). Arrows indicate irregularity on bone tissue. (Magnification 60x **A–C**; Magnification 800x **D–F**).

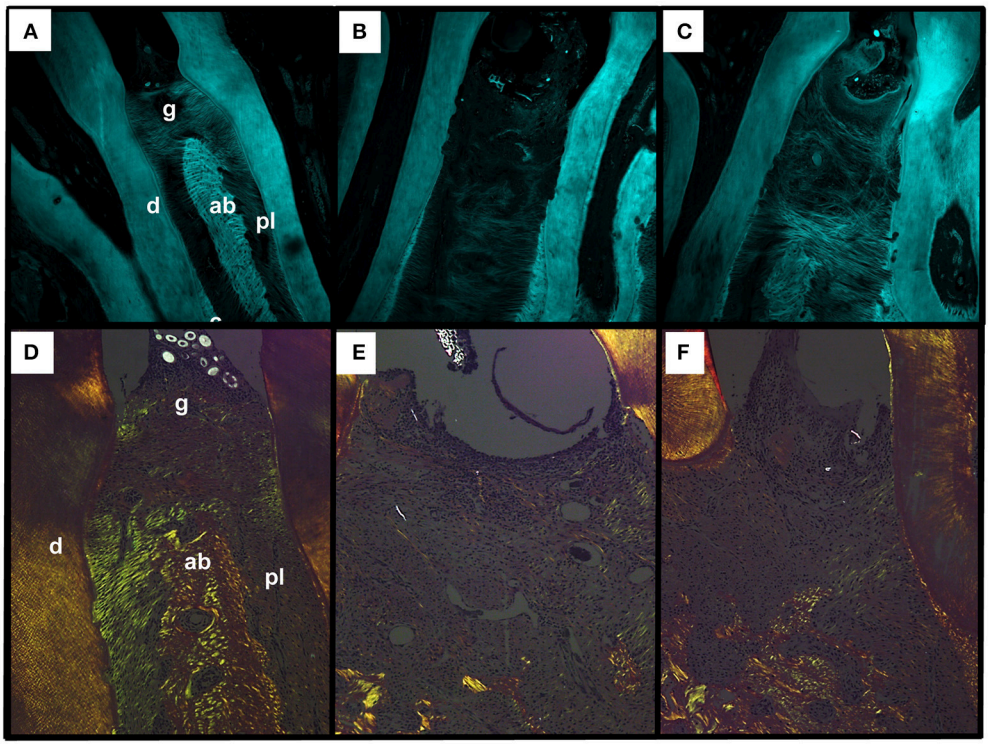

FIGURE 3 | Effect of CLO on collagen fibers of alveolar bone of rats with EP. Naïve **(A,D)**, SAL **(B,E)**, CLO **(C,F)**. Dentin (d); Alveolar bone (ab); Periodontal ligament (pl); Gingiva (g). (Magnification 40x).

group (**Figure 2D**). The treatment with CLO (**Figure 2F**) kept the tissue topography more regular than the one seen on SAL group.

The EDS analysis allowed the immediate identification of minerals and the chemical elements distribution on the samples (**Figures 2G–I**). It was possible to see elevated peaks of calcium and phosphorus on bone tissue, without any difference between groups.

Effect of CLO on Collagen Fiber of Periodontium

The confocal analysis of periodontium of animals from SAL group (**Figure 3B**) demonstrated great destruction and derangement of collagen fibers in periodontal ligament compared to the Naïve group (**Figure 3A**). The treatment with 90 mg/kg CLO (**Figure 3C**) preserved these collagen fibers.

Using picrosirius red staining under polarized light filter, it was possible to observe that the majority of collagen fibers from periodontal ligament from animals of Naïve group showed green color (**Figure 3E**) The collagen fiber of periodontium of animals from SAL group presented reduction on the birefringencewhen compared to Naïve (**Figure 3D**) The treatment with CLO caused a slight increase on birefringence when compared to SAL, showing fibers in greenish color (**Figure 3F**).

Effect of CLO on Immunolabeling of WNT10b, DKK-1 and Beta-Catenin

Considering the canonical WNT pathway, the animals submitted to EP that received SAL showed reduction on the amount of immunopositive cells to WNT 10b (8.50 ± 1.25 positive cells/mm^2) (**Figures 4A**, **5C**) ($p < 0.05$), β-catenin (12.80 ± 1.20) (**Figures 4B**, **5G**) ($p > 0.05$), and an increase to DKK-1 (58.00 ± 11.03) (**Figures 4C**, **5K**) ($p < 0.05$) when compared to Naïve [WNT 10b = 22.44 ± 2.82 (**Figure 5B**); β–catenin = 15.80 ± 1.77 (**Figure 5F**); DKK-1 = 17.00 ± 3.21 (**Figure 5J**)]. The treatment with CLO 90 mg/kg increased the count of immunopositive cells for WNT 10b (23.89 ± 2.41) (**Figure 5D**) and β-catenin (22.25 ± 3.16) (**Figure 5H**) ($P < 0.05$) when compared to SAL (WNT 10b = 8.50 ± 1.25 and β-catenin = 12.80 ± 1.20). There was a significant decrease on the count of immunopositive cells for DKK-1 in CLO group (22.20 ± 5.55) (**Figure 5L**), compared to SAL (58.00 ± 11.02). The negative controls for WNT 10b, β-catenin, and DKK-1 can be seen on **Figures 5A,E,I**, respectively.

Effect of CLO on Gingival GSH, SOD, CAT, and MDA Levels

The experimental periodontitis caused significant reduction of the GSH (**Figure 6A**), SOD (**Figure 6B**) and CAT (**Figure 6C**), as well as an increase on MDA (**Figure 6D**) gingival levels ($P < 0.05$) when compared to the Naïve group, suggesting that oxidative stress is observed in gingival tissue subjected to periodontitis.

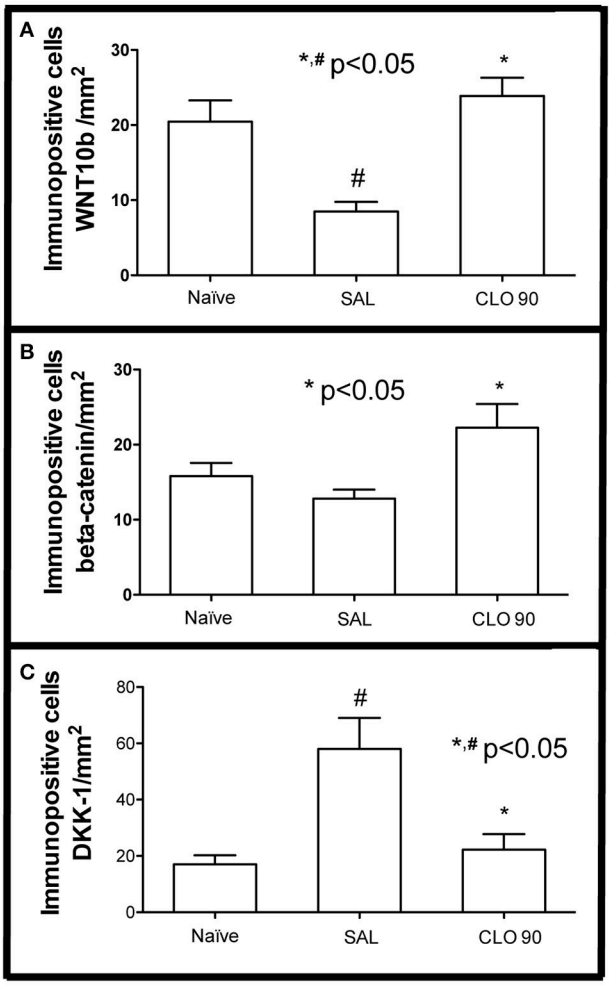

FIGURE 4 | Effect of CLO on quantification of immunopositive cells for markers of WNT pathway. WNT 10b **(A)**, beta-catenin **(B)**, DKK-1 **(C)**. Bars represent the mean ± SEM of 6 animals per group. $^{\#}P < 0.05$ was considered to be significantly different compared with Naïve. $^{*}P < 0.05$ was considered to be significantly different compared with SAL (ANOVA followed by the Bonferroni test).

Administration of 90 mg/kg CLO increased gingival GSH, SOD and CAT concentration, and reduced MDA levels compared with SAL group.

DISCUSSION

In order to study periodontitis, it has been well described on literature the use of animal models, among them the one of ligature-induced model in rats. Several studies have shown that this model is able to reproduce the main characteristics seen on human periodontitis, such as bone loss and destruction of periodontal ligament (Goes et al., 2010, 2016; Sousa et al., 2016) increase on oxidative stress (Di Paola et al., 2004), and exacerbation of inflammatory process (Leitão et al., 2005; Goes et al., 2012, 2016; Sousa et al., 2016), which confirm our findings.

Therefore, considering the important role of inflammation and oxidative stress on bone resorption, it seems interesting the use of pharmacological agents that present anti-inflammatory and antioxidant actions showing low incidence of adverse effects and of low-cost, as observed in several natural products. So *Calendula officinalis* (CLO) stands out as a modulator of inflammation that can be used as adjuvant to the treatment of periodontitis.

On this study it was seen that CLO presented antirresorptive effect preventing bone loss and preserving its topography. Despite the lack of studies evaluating the effect of the crude extract of CLO on bone tissue, it has been reported that queretin, the main flavonoid found on CLO extract, is the one responsible, in great part, of the pharmacological effects of this plant (Saini et al., 2012). *In vitro*, quercetin significantly increased osteoblast differentiation (Zhou et al., 2015) and induced mRNA expression of sialoprotein and osteocalcin in osteoblast culture (Satué et al., 2013). *In vivo*, quercetin inhibited bone loss in periodontitis models in rats (Napimoga et al., 2013), increased serum osteocalcin and the activity of alkaline phosphatase (Liang et al., 2011), contributing to bone tissue preservation.

By occasion of SEM analysis, we performed the EDS evaluation that can analyze the chemical elements compounds, showing its distribution on the sample surface (Newbury and Ritchie, 2015). Our results showed no difference on the chemical compounds of bone tissue considering the experimental groups, what indicates that CLO does not affect the type and/or amount of minerals when compared to the normal tissue.

Considering WNT pathway, our results showed, for the first time, the effect of CLO on this signaling pathway, by the increase of WNT10b and beta-catenin, while it reduced DKK-1 immunoexpressions. Among the several types of well-known WNT proteins in mammals (Kikuchi, 2009) WNT10b stands out as a positive modulator of bone formation (Stevens et al., 2010). This pathway is regulated by DKK and Sclerostin (SOST), two extracellular antagonists, which have their expression increased during inflammatory conditions (Wang et al., 2011). So the effect of CLO on WNT pathway may be related to the anti-inflammatory effect of this plant (Preethi et al., 2009).

In addition, it has been described a relationship between oxidative stress and WNT pathway. WNT proteins activate the Frizzled/LRP5 or LRP6 receptor complex preventing β-catenin degradation (Clevers and Nusse, 2012), allowing its association with the T cell factor (TCF) lymphoid-enhancer binding factor (LEF) family of transcription factors which regulates the expression of WNT-target genes, such as Osterix1 in bone-producing osteoblasts (Rodda and McMahon, 2006). However, during stress conditions, the high levels of reactive oxygen species (ROS) promotes the activation of the transcription fator, Forkhead box O (FOXO) (Iyer et al., 2013). The binding of β-catenin to FOXOs diverts β-catenin from Wnt/TCF- to FOXO-mediated transcription decreasing osteoblastogenesis *in vitro* (Almeida et al., 2007). These findings highlight the beneficial anti-inflammatory effect of CLO on bone metabolism.

This study performed analyses of collagen fibers. CLO preserved collagen confirming others studies which already showed that CLO extract reduced collagen breakdown (Millán et al., 2016), and increased collagen concentration (Aro et al., 2015). In gingival fibroblasts, CLO inhibited matrix

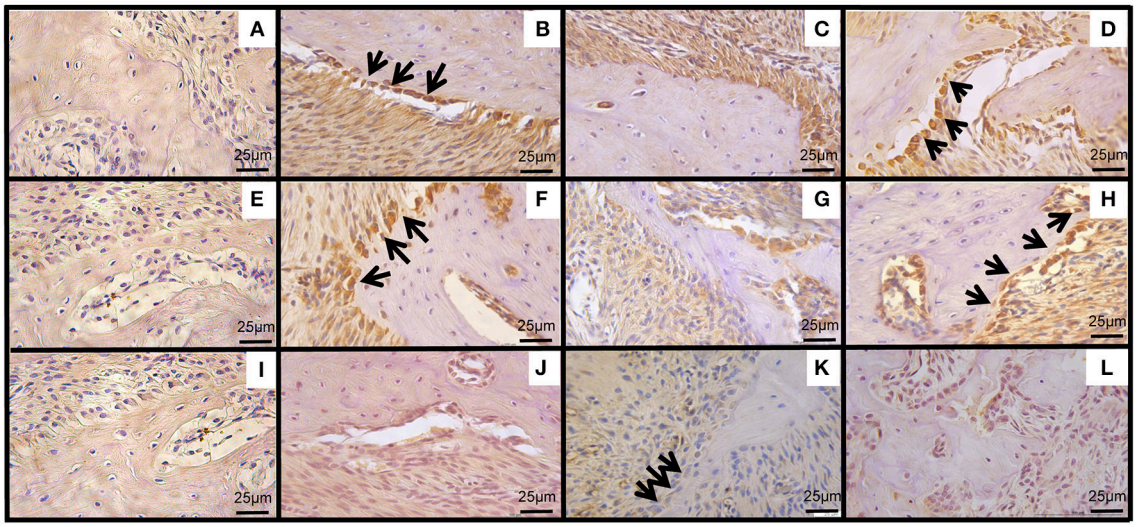

FIGURE 5 | Effect of CLO on immunoexpression of WNT pathway in periodontium of rats with. WNT10b **(B–D)**, β-catenin **(F–H)** and DKK-1 **(J–L)**, between first and second molar of a periodontium from Naïve group **(B,F,J)**, periodontium from SAL group **(C,G,K)**, periodontium of animals treated with CLO 90 mg/kg **(D,H,L)**. Negative controls of WNT10b **(A)**, β-catenin **(E)**, DKK-1 **(I)**. (Magnification 400x). (→) indicate immunopositive osteoblasts. Bar = 25 μm.

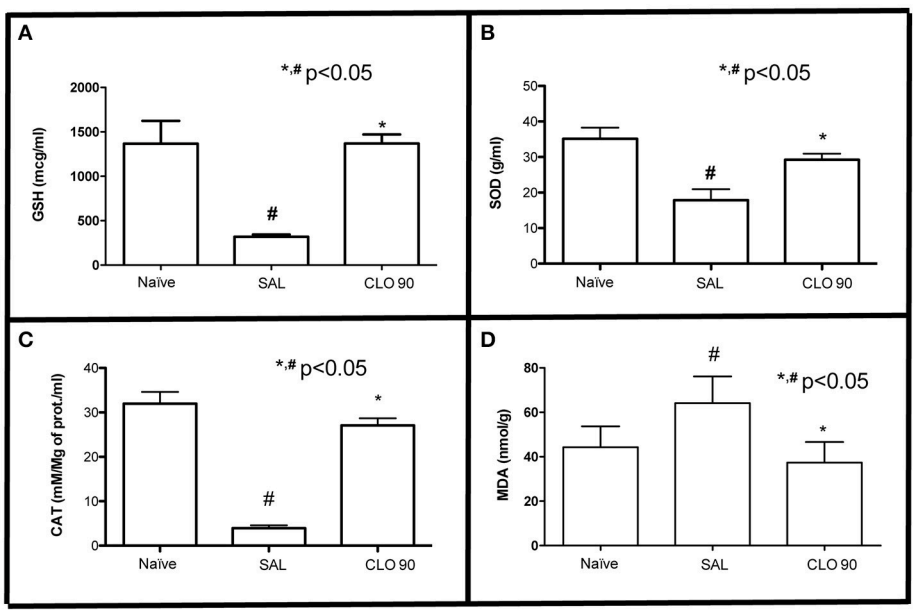

FIGURE 6 | Effect of CLO on oxidative stress markers in gingival tissue of rats with EP. GSH **(A)**, SOD **(B)**, CAT **(C)**, MDA **(D)**. Bars represent the mean ± SEM. $^{\#}P <$ 0.05 was considered to be significantly different compared with Naïve. $^{*}P < 0.05$ was considered to be significantly different compared with SAL (ANOVA followed by the Bonferroni test).

metalloproteinases (MMP)-2 (Saini et al., 2012). Collagen is the main constituent of periodontal ligament and plays a key role in the architecture of the periodontium (Kaku and Yamauchi, 2014). Therefore, collagen breakdown is understood as the main marker of periodontal disease progression (de Almeida et al., 2015). Moreover, evaluating its birefringence, in CLO group the collagen fibers were, in its majority, green (Type III collagen). Type III collagen is observed on the initial phases of healing (Li and Sae-Lim, 2007), and it is considered essential to further production of type I collagen (Liu et al., 1997).

The oxidative stress is a characteristic of inflammatory process, and it is considered an important factor on periodontal pathogenesis (Chapple and Matthews, 2007). CLO reduced the periodontal oxidative stress, reestablishing GSH, SOD and

CAT, important antioxidant enzymes, and reducing MDA levels on periodontal tissue, a marker of tissue destruction, as in accordance to other studies (Tanideh et al., 2016; Verma et al., 2016). Quercetin seems to play a role on this effect, since it has exhibited antioxidant activity (Fonseca et al., 2010). According to Heijnen et al. (2002) this effect is attributed to the presence of two antioxidant pharmacophores within the molecule of quercetin that have the optimal configuration for free radical scavenging.

In summary the present findings showed that CLO exhibited antiresorptive effect, preserved collagen fibers and presented antioxidant activity with participation of WNT signaling pathway. Therefore, this natural product deserves further investigation as pharmacological tool for preventing periodontal bone loss.

AUTHOR CONTRIBUTIONS

ML and AL induced periodontitis, treated the animals, performed all the assays. CM performed immunohistochemical assay, GB performed microscopy analysis. VC performed SEM assay and analysis, PG was the supervisor of the study.

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REFERENCES

Almeida, M., Han, L., Martin-Millan, M., O'Brien, C. A., and Manolagas, S. C. (2007). Oxidative stress antagonizes Wnt signaling in osteoblast precursors by diverting beta-catenin from T cell factor- to forkhead box O-mediated transcription. *J. Biol. Chem.* 282, 27298–27305. doi: 10.1074/jbc.M702811200

Aro, A. A., Perez, M. O., Vieira, C. P., Esquisatto, M. A., Rodrigues, R. A., Gomes, L., et al. (2015). Effect of *Calendula officinalis* cream on achilles tendon healing. *Anat. Rec.* 298, 428–435. doi: 10.1002/ar.23057

Baron, R., and Kneissel, M. (2013). WNT signaling in bone homeostasis and disease: from human mutations to treatments. *Nat. Med.* 19, 179–192. doi: 10.1038/nm.3074

Beauchamp, C., and Fridovich, I. (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* 44, 276–287. doi: 10.1016/0003-2697(71)90370-8

Busch, C. J., and Binder, C. J. (2016). Malondialdehyde epitopes as mediators of sterile inflammation. *Biochim. Biophys. Acta* S1388–S1981, 30168. doi: 10.1016/j.bbalip.2016.06.016

Chapple, I. L., and Matthews, J. B. (2007). The role of reactive oxygen and antioxidant species in periodontal tissue destruction. *Periodontol.* 2000:43, 160–232. doi: 10.1111/j.1600-0757.2006.00178.x

Clevers, H., and Nusse, R. (2012). Wnt/β-catenin signaling and disease. *Cell* 149, 1192–1205. doi: 10.1016/j.cell.2012.05.012

D'Aiuto, F., Nibali, L., Parkar, M., Patel, K., Suvan, J., and Donos, N. (2010). Oxidative stress, systemic inflammation, and severe periodontitis. *J. Dent. Res.* 89, 1241–1246. doi: 10.1177/0022034510375830

de Almeida, L. F., Souza, S. E., Sampaio, A. A., Cavalcanti, Y. W., da Silva, W. J., Del Bel Cur, A. A., et al. (2015). Effect of collagen matrix saturation on the surface free energy of dentin using different agents. *J. Contemp. Dent. Pract.* 16, 531–536. doi: 10.5005/jp-journals-10024-1717

de Barros Silva, P. G., de Oliveira, C. C., Brizeno, L., Wong, D., Lima Jr., R., Gonçalves, R. P., et al. (2016). Immune cellular profile of bisphosphonate-related osteonecrosis of the jaw. *Oral Dis.* 22, 649–657. doi: 10.1111/odi.12513

Di Paola, R., Marzocco, S., Mazzon, E., Dattola, F., Rotondo, F., Britti, D., et al. (2004). Effect of aminoguanidine in ligature-induced eriodontitis in rats. *J. Dent. Res.* 83, 343–348. doi: 10.1177/154405910408300414

Fonseca, Y. M., Catini, C. D., Vicentini, F. T., Nomizo, A., Gerlach, R. F., and Fonseca, M. J. (2010). Protective effect of *Calendula officinalis* extract against UVB-induced oxidative stress in skin: evaluation of reduced glutathione levels and matrix metalloproteinase secretion. *J. Ethnopharmacol.* 127, 596–601. doi: 10.1016/j.jep.2009.12.019

Galli, C., Passeri, G., and Macaluso, G. M. (2011). FoxOs, Wnts and oxidative stress-induced bone loss: new players in the periodontitis arena? *J. Periodont. Res.* 46, 397–406. doi: 10.1111/j.1600-0765.2011.01354.x

Goes, P., Lima, A. P., Melo, I. M., Rêgo, R. O., and Lima, V. (2010). Effect of Atorvastatin in radiographic density on alveolar bone loss in wistar rats. *Braz. Dent. J.* 21, 193–198. doi: 10.1590/S0103-64402010000300003

Goes, P., Lima, N. A., Rodrigues, J. A., Benevides, N. M., Brito, G. A., and Lima, V. (2016). Anti-inflammatory and anti-resorptive effects of atorvastatin on alveolar bone loss in wistar rats. *Braz. Dent. J.* 27, 267–272. doi: 10.1590/0103-6440201600600

Goes, P., Melo, I. M., Dutra, C. S., Lima, A. P., and Lima, V. (2012). Effect of alendronate on bone-specific alkaline phosphatase on periodontal bone loss in rats. *Arch. Oral Biol.* 57, 1537–1544. doi: 10.1016/j.archoralbio.2012.07.007

Gonçalves, D. C., Evangelista, R. C., da Silva, R. R., Santos, M. J., Silva, F. S. Jr., Aragão, K. S., et al. (2014). Infliximab attenuates inflammatory osteolysis in a model of periodontitis in Wistar rats. *Exp. Biol. Med.* 239, 442–453. doi: 10.1177/1535370213520114

Graves, D. T., Oates, T., and Garlet, G. P. (2011). Review of osteoimmunology and the host response in endodontic and periodontal lesions. *J. Oral Microbiol.* 3. doi: 10.3402/jom.v3i0.5304

Heijnen, C. G., Haenen, G. R., Oostveen, R. M., Stalpers, E. M., and Bast, A. (2002). Protection of flavonoids against lipid peroxidation: the structure activity relationship revisited. *Free Radic. Res.* 36, 575–581. doi: 10.1080/10715760290025951

Iyer, S., Ambrogini, E., Bartell, S. M., Han, L., Roberson, P. K., de Cabo, R., et al. (2013). FOXOs attenuate bone formation by suppressing Wnt signaling. *J. Clin. Invest.* 123, 3409–34019. doi: 10.1172/JCI68049

Junqueira, L. C., Bignolas, G., and Brentani, R. R. (1979). Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. *Histochem. J.* 11, 447–455. doi: 10.1007/BF01002772

Kaku, M., and Yamauchi, M. (2014). Mechano-regulation of collagen biosynthesis in periodontal ligament. *J. Prosthodont. Res.* 58, 193–207. doi: 10.1016/j.jpor.2014.08.003

Kayal, R. A. (2013). The role of osteoimmunology in periodontal disease. *Biomed Res. Int.* 2013:639368. doi: 10.1155/2013/639368

Kikuchi, A. (2009). Wnt signaling; its abnormalities and diseases. *Seikagaku* 81, 780–792.

Kobayashi, Y., Uehara, S., Udagawa, N., and Takahashi, N. (2016). Regulation of bone metabolism by Wnt signals. *J. Biochem.* 159, 387–392. doi: 10.1093/jb/mvv124

Lauten, J. D., Boyd, L., Hanson, M. B., Lillie, D., Gullion, C., and Madden, T. E. (2005). A clinical study: Melaleuca, Manuka, Calendula and green tea mouth rinse. *Phytother. Res.* 19, 951–957. doi: 10.1002/ptr.1763

Leitão, R. F., Ribeiro, R. A., Chaves, H. V., Rocha, F. A., Lima, V., and Brito, G. A. (2005). Nitric oxide synthase inhibition prevents alveolar bone resorption in experimental periodontitis in rats. *J. Periodontol.* 76, 956–963. doi: 10.1902/jop.2005.76.6.956

Li, Y., Yao, J., Han, C., Yang, J., Chaudhry, M. T., Wang, S., et al. (2016). Quercetin, Inflammation and Immunity. *Nutrients* 8:167. doi: 10.3390/nu8030167

Li, Z., and Sae-Lim, V. (2007). Comparison of acidic fibroblast growth factor on collagen carrier with calcium hydroxide as pulp capping agents in monkeys. *Dent. Traumatol.* 23, 278–286. doi: 10.1111/j.1600-9657.2006.00459.x

Liang, W., Luo, Z., Ge, S., Li, M., Du, J., Yang, M., et al. (2011). Oral administration of quercetin inhibits bone loss in rat model of diabetic osteopenia. *Eur. J. Pharmacol.* 670, 317–324. doi: 10.1016/j.ejphar.2011.08.014

Liu, X., Wu, H., Byrne, M., Krane, S., and Jaenisch, R. (1997). Type III collagen is crucial for collagen I fibrillogenesis and for normal

cardiovascular development. *Proc. Natl. Acad. Sci. U.S.A.* 94, 1852–1856. doi: 10.1073/pnas.94.5.1852

Lu, H., Yuan, G., Yin, Z., Dai, S., Jia, R., Xu, J., et al. (2014). Effects of subchronic exposure to lead acetate and cadmium chloride on rat's bone: Ca and Pi contents, bone density, and histopathological evaluation. *Int. J. Clin. Exp. Pathol.* 7, 640–647.

Machado, M. A. (2010). Management of two cases of desquamative gingivitis with clobetasol and *Calendula officinalis* gel. *Biomed. Pap. Med. Fac. Univ. Palacky Olomouc Czech. Repub.* 154, 335–338. doi: 10.5507/bp.2010.050

Maehly, A. C., and Chance, B. (1954). The assay of catalases and peroxidases. *Methods Biochem. Anal.* 1, 357–424. doi: 10.1002/9780470110171.ch14

Mihara, M., and Uchivam, M. (1978). Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Anal. Biochem.* 86, 271–278. doi: 10.1016/0003-2697(78)90342-1

Millán, D., Jiménez, R. A., Nieto, L. E., Linero, I., Laverde, M., and Fontanilla, M. R. (2016). Preclinical evaluation of collagen type I scaffolds, including gelatin-collagen microparticles and loaded with a hydroglycolic *Calendula officinalis* extract in a lagomorph model of full-thickness skin wound. *Drug Deliv. Transl. Res.* 6, 57–66. doi: 10.1007/s13346-015-0265-8

Napimoga, M. H., Clemente-Napimoga, J. T., Macedo, C. G., Freitas, F. F., Stipp, R. N., Pinho-Ribeiro, F. A., et al. (2013). Quercetin inhibits inflammatory bone resorption in a mouse periodontitis model. *J. Nat. Prod.* 76, 2316–2321. doi: 10.1021/np400691n

Newbury, D. E., and Ritchie, N. W. (2015). Performing elemental microanalysis with high accuracy and high precision by scanning electron microscopy/silicon drift detector energy-dispersive X-ray spectrometry (SEM/SDD-EDS). *J. Mater Sci.* 50, 493–518. doi: 10.1007/s10853-014-8685-2

Palwankar, P., Rana, M., Arora, K., and Deepthy, C. (2015). Evaluation of non-surgical therapy on glutathione levels in chronic periodontitis. *Eur. J. Dent.* 9, 415–422. doi: 10.4103/1305-7456.163226

Parente, L. M., Lino Júnior, R., Tresvenzol, L. M., Vinaud, M. C., de Paula, J. R., and Paulo, N. M. (2012). Wound Healing and Anti-Inflammatory Effect in Animal Models of *Calendula officinalis* L. Growing in Brazil. *Evid. Based Complement. Alternat. Med.* 2012:375671. doi: 10.1155/2012/375671

Petersen, P. E., and Ogawa, H. (2005). Strengthening the prevention of periodontal disease: the WHO approach. *J. Periodontol.* 76, 2187–2193. doi: 10.1902/jop.2005.76.12.2187

Preethi, K. C., Kuttan, G., and Kuttan, R. (2009). Anti-inflammatory activity of flower extract of *Calendula officinalis* Linn. and its possible mechanism of action. *Indian J. Exp. Biol.* 47, 113–120.

Rauner, M., Sipos, W., and Pietschmann, P. (2008). Age-dependent Wnt gene expression in bone and during the course of osteoblast differentiation. *Age* 30, 273–282. doi: 10.1007/s11357-008-9069-9

Redlich, K., and Smolen, J. S. (2012). Inflammatory bone loss: pathogenesis and therapeutic intervention. *Nat. Rev. Drug Discov.* 11, 234–250. doi: 10.1038/nrd3669

Rodda, S. J., and McMahon, A. P. (2006). Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. *Development* 133, 3231–3244. doi: 10.1242/dev.02480

Saini, P., Al-Shibani, N., Sun, J., Zhang, W., Song, F., Gregson, K. S., et al. (2012). Effects of *Calendula officinalis* on human gingival fibroblasts. *Homeopathy* 101, 92–98. doi: 10.1016/j.homp.2012.02.003

Satué, M., Arriero, M., del, M., Monjo, M., and Ramis, J. M. (2013). Quercitrin and taxifolin stimulate osteoblast differentiation in MC3T3-E1 cells and inhibit osteoclastogenesis in RAW 264.7 cells. *Biochem. Pharmacol.* 86, 1476–1486. doi: 10.1016/j.bcp.2013.09.009

Sedlak, J., and Lindsay, R. H. (1968). Estimation of total protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal. Biochem.* 25, 1192–1205. doi: 10.1016/0003-2697(68)90092-4

Sousa, L. H., Linhares, E. V., Alexandre, J. T., Lisboa, M. R., Furlaneto, F., Freitas, R., et al. (2016). Effects of atorvastatin on periodontitis of rats subjected to glucocorticoid-induced osteoporosis. *J. Periodontol.* 87, 1206–1216. doi: 10.1902/jop.2016.160075

Stevens, J. R., Miranda-Carboni, G. A., Singer, M. A., Brugger, S. M., Lyons, K. M., and Lane, T. F. (2010). Wnt10b deficiency results in age-dependent loss of bone mass and progressive reduction of mesenchymal progenitor cells. *J. Bone Miner. Res.* 2010, 2138–2147. doi: 10.1002/jbmr.118

Tanideh, N., Jamshidzadeh, A., Sepehrimanesh, M., Hosseinzadeh, M., Koohi-Hosseinabadi, O., Najibi, A., et al. (2016). Healing acceleration of acetic acid-induced colitis by marigold (*Calendula officinalis*) in male rats. *Saudi J. Gastroenterol.* 22, 50–56. doi: 10.4103/1319-3767.173759

Verma, P. K., Raina, R., Sultana, M., Singh, M., and Kumar, P. (2016). Total antioxidant and oxidant status of plasma and renal tissue of cisplatin-induced nephrotoxic rats: protection by floral extracts of *Calendula officinalis* Linn. *Ren. Fail.* 38, 142–150. doi: 10.3109/0886022X.2015.1103585

Wang, S. Y., Liu, Y. Y., Ye, H., Guo, J. P., Li, R., Liu, X., et al. (2011). Circulating Dickkopf-1 is correlated with bone erosion and inflammation in rheumatoid arthritis. *J. Rheumatol.* 38, 821–827. doi: 10.3899/jrheum.100089

Zheng, W., Wang, S., Wang, J., and Jin, F. (2015). Periodontitis promotes the proliferation and suppresses the differentiation potential of human periodontal ligament stem cells. *Int. J. Mol. Med.* 36, 915–922. doi: 10.3892/ijmm.2015.2314

Zhou, Y., Wu, Y., Jiang, X., Zhang, X., Xia, L., Lin, K., et al. (2015). The effect of quercetin on the osteogenesis differentiation and angiogenic factor expression of bone marrow-derived mesenchymal stem cells. *PLoS ONE* 10:e0129605. doi: 10.1371/journal.pone.0129605

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frontiers in Physiology

REVIEW
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The Role of Reactive Oxygen Species and Autophagy in Periodontitis and Their Potential Linkage

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Periodontitis is a chronic inflammatory disease that causes damage to periodontal tissues, which include the gingiva, periodontal ligament, and alveolar bone. The major cause of periodontal tissue destruction is an inappropriate host response to microorganisms and their products. Specifically, a homeostatic imbalance between reactive oxygen species (ROS) and antioxidant defense systems has been implicated in the pathogenesis of periodontitis. Elevated levels of ROS acting as intracellular signal transducers result in autophagy, which plays a dual role in periodontitis by promoting cell death or blocking apoptosis in infected cells. Autophagy can also regulate ROS generation and scavenging. Investigations are ongoing to elucidate the crosstalk mechanisms between ROS and autophagy. Here, we review the physiological and pathological roles of ROS and autophagy in periodontal tissues. The redox-sensitive pathways related to autophagy, such as mTORC1, Beclin 1, and the Atg12-Atg5 complex, are explored in depth to provide a comprehensive overview of the crosstalk between ROS and autophagy. Based on the current evidence, we suggest that a potential linkage between ROS and autophagy is involved in the pathogenesis of periodontitis.

Keywords: periodontitis, reactive oxygen species, autophagy, NF-κB, JNK, mTORC1, Beclin 1, Atg12-Atg5 complex

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INTRODUCTION

Periodontitis is an inflammatory disease that compromises the integrity of the tooth-supporting tissues through the interplay of periodontal pathogens and the host immune response (Kinane et al., 2008; Dumitrescu, 2016). A new model of the pathogenesis of periodontitis showed that pathogens alone are necessary but insufficient for the development of periodontal lesions *per se*. The majority of periodontal tissue damage is caused by the subversion of host immune responses, with the involvement of leukocytes, complement and reactive oxygen species (ROS) (Hajishengallis, 2015). ROS are short-lived, highly reactive reduced products of oxygen, such as superoxide ($O_2^-\cdot$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($\cdot OH$), and singlet oxygen ($^1O_2$) (Di Meo et al., 2016). The close relationship between ROS and periodontitis has long been appreciated, beginning with the pioneering studies of the early 1970s (Shapira et al., 1991; Marquis, 1995; Chapple, 1997). The role of ROS in periodontitis has been comprehensively reviewed (Chapple and Matthews, 2007; Nibali and Donos, 2013). In brief, ROS at the cellular level are essential for physiologic

processes of eukaryotic cells, including cellular signaling transduction, cellular differentiation, and apoptosis (McClean et al., 2015; Di Meo et al., 2016). Moreover, ROS contribute to the oxidative killing of pathogens (Roos et al., 2003). For instance, a clinical study found that levels of serum reactive oxygen metabolites were positively correlated with immunoglobulin G antibodies to specific periodontal pathogens, including *Porphyromonas gingivalis* (*P. gingivalis*), *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*), and *Prevotella intermedia* (*P. intermedia*) (Tamaki et al., 2014). However, a homeostatic imbalance between ROS and antioxidant defense systems can trigger an oxidative stress response, which is believed to be related to periodontal destruction (Waddington et al., 2000; Baltacioglu et al., 2014b). Clinically, there are strong positive correlations between periodontal parameters and malondialdehyde (MDA) and total oxidant status (TOS) levels (Akalin et al., 2007; Baltacioglu et al., 2014b). Further evidence has been derived from *in vitro* and animal model studies. Decreased ROS levels downregulated the expression of osteoclast differentiation marker genes and attenuated bone loss (Kanzaki et al., 2013). ROS can also evoke immune responses through redox-sensitive gene transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Gan et al., 2016). In addition, ROS can induce cellular apoptosis via c-Jun N-terminal kinase (JNK) activation (Liu et al., 2015).

Accumulating evidence has indicated a close connection between ROS and autophagy. A series of discoveries related to autophagy won Yoshinori Ohsumi the Nobel Prize for Physiology and Medicine in 2016 (Levine and Klionsky, 2016; Tooze and Dikic, 2016). Autophagy is a lysosomal degradation pathway of self-digestion (Klionsky and Emr, 2000; Yang and Klionsky, 2010; Levine and Klionsky, 2016). This process is thought to have evolved as a stress response that allows organisms to survive harsh conditions (Mizushima et al., 1998; Netea-Maier et al., 2016). There is a complex, reciprocal relationship between the autophagy pathway and ROS. Studies suggest that ROS influence autophagy and that autophagy reciprocally regulates ROS (He Y. et al., 2017; Wang et al., 2017). The most typical example of their interaction has been elucidated in cancer development (Zhao et al., 2016). Autophagy consists of five sequential steps: induction, elongation, maturation, transport to lysosomes, and degradation (Levine and Kroemer, 2008). Thus, the function of autophagy is step dependent. The regulation of autophagy by ROS appears to be complicated, involving various autophagic signaling pathways and autophagy-related genes (Atgs). Studies have clearly demonstrated that the regulation of autophagy by ROS plays both a cytoprotective and cytotoxic role in cancer development (Chen et al., 2016; Zhong et al., 2016). Recently, autophagy has been proposed to be involved in the pathogenesis of periodontitis through bacterial elimination, facilitating the internalization of specific periodontal pathogens, suppressing the immune response, and inhibiting periodontal cell apoptosis (Tsuda et al., 2010; An et al., 2016; Tan et al., 2016; Park et al., 2017).

ROS and autophagy are closely interconnected, and many key molecules are shared by the two processes. However, the available data suggest that the intricate interactions between ROS and autophagy in periodontitis remain unknown. Moreover, the mechanisms underlying how ROS participate in regulating autophagy remain to be elucidated. To contribute to the understanding of this issue, the present review focuses on redox-sensitive pathways and transcription factors related to autophagy and summarizes the physiologic and pathologic roles of oxidative stress and autophagy in periodontal tissues.

ROS HOMEOSTASIS

In general, ROS include $O_2^-\cdot$, H_2O_2, ·OH, and $^1O_2$ (Di Meo et al., 2016). These species are endogenously generated by peroxisomes; the endoplasmic reticulum (ER); enzymes such as NADPH oxidases (NOXs), xanthine oxidases (XOs), cyclooxygenases (COXs) and lipoxygenases (LOXs); and the mitochondrial electron transport chain (Mito-ETC) (Zhang L. et al., 2015). The amount of intracellular ROS produced daily reaches ∼1 billion molecules in every single cell. It is commonly accepted that the Mito-ETC is the major source of ROS (Filomeni et al., 2015). In the ETC, electrons are transferred from electron donors (e.g., NADH) to electron acceptors (e.g., O_2) via redox reactions, resulting in the synthesis of adenosine triphosphate (ATP). In mitochondria, premature electrons leakage to O_2 can occur, generating $O_2^-\cdot$ as a by-product of the ETC. Dismutation of $O_2^-\cdot$ by superoxide dismutase 1 (SOD1, also known as CuZn-SOD) in the intermembrane space, or by superoxide dismutase 2 (SOD2, also known as MnSOD) in the matrix, produces H_2O_2. In turn, H_2O_2 is reduced to H_2O by glutathione peroxidase (GPX) or catalase (CAT) (Perrone et al., 2016). H_2O_2 is partially degraded to ·OH in the Fenton and Haber-Weiss reactions (Turrens, 2003). The components of the ETC are organized into four complexes. ROS generation by the ETC is primarily dependent on complex I (also known as NADH-coenzyme Q reductase or NADH dehydrogenase) and complex III (also known as coenzyme Q reductase) (Lismont et al., 2015).

The generation of ROS occurs in equilibrium with a wealth of ROS scavengers, including enzymes (e.g., SOD, GPX, and CAT), small molecules [e.g., vitamin C and glutathione (GSH)], and glutaredoxin and thioredoxin systems, to maintain redox homeostasis (Venditti et al., 2013; Netto and Antunes, 2016). The balance between the generation and elimination of ROS is critical for human health. Excessive production of ROS, low levels of antioxidants, or inhibition of antioxidant enzymes causes oxidative stress and may lead to indiscriminate damage to biological macromolecules (lipids, proteins, and DNA). Increasing evidence has shown an association between ROS and a variety of diseases, including cancer, periodontitis, cardiovascular diseases, and diabetes (Di Meo et al., 2016).

ROS IN PERIODONTITIS

ROS are considered to be a double-edged sword in periodontal diseases (Nibali and Donos, 2013). At low concentrations, ROS stimulate the proliferation and differentiation of human periodontal ligament fibroblasts (hPDLFs) in culture, while at higher concentrations, they may have cytotoxic effects on

periodontal tissues and participate in pathogen killing (Chapple and Matthews, 2007; D'aiuto et al., 2010; Galli et al., 2011; Saita et al., 2016).

The Physiologic Role of ROS in Periodontal Tissues

Periodontitis is a disease caused by oral infection associated with polymicrobial dysbiosis and the activation of host immunity (Hajishengallis, 2014). Keystone or keystone-like pathogens, such as *P. gingivalis* and *Tannerella forsythia* (*T. forsythia*), can drive the disruption of periodontal tissue homeostasis and lead to inflammation (Wright et al., 2014; Lamont and Hajishengallis, 2015). Keystone or keystone-like pathogens of periodontitis, predominantly Gram-negative anaerobic or facultative bacteria, are appreciably sensitive to changes in the oxidative environment (Lamont and Hajishengallis, 2015). ROS can disturb the cellular oxidative environment and participate in the killing of keystone pathogens. For instance, a marked increase in ROS generation was observed when leukocytes were treated with *P. gingivalis* lipopolysaccharide (LPS) or *Fusobacterium nucleatum* (*F. nucleatum*) *in vitro* (Sheikhi et al., 2000; Zhu et al., 2016). Fascinatingly, it has very recently been reported that H_2O_2 is a central determinant of oral polymicrobial synergy (Lamont, 2016). However, several lines of evidence have suggested that periodontal pathogens such as *Treponema denticola* (*T. denticola*) have evolved strategies to suppress the induction of ROS (Shin et al., 2013).

Conversely, at basal levels, ROS serve as second messenger particulates in regulating signal transduction, cellular homeostasis, and cell death. For instance, H_2O_2 can trigger defensive inflammatory responses to environmental cues in periodontal tissues through mitogen-activated protein kinase (MAPK) and NF-κB as well as inflammasome activation (Almerich-Silla et al., 2015). Moreover, glucose oxidase, which continuously generates H_2O_2 at relatively low concentrations, could stimulate the proliferation and osteoblastic differentiation of hPDLFs through the upregulation of runt-related transcription factor-2 (Runx2) and osterix (Choe et al., 2012). H_2O_2could also increase the levels of gelatinolytic matrix metalloproteinases (MMPs), enhancing hPDLF migration in an MMP-dependent manner (Cavalla et al., 2015). These findings suggest that ROS participate in the proliferation and differentiation of hPDLFs. However, many studies have reported that H_2O_2 acts predominantly as an inhibitory mediator of cell proliferation and differentiation (Choi et al., 2009). A possible explanation for these contradictory results is that cellular responses to H_2O_2 can differ depending on the concentration of H_2O_2 and the type of cells. For example, Burdon et al. reported that exposure to 1 μM H_2O_2 promoted the proliferation of BHK-21 fibroblasts, while H_2O_2 at 0.5 and 1 mM caused apoptotic cell death (Burdon et al., 1996).

ROS in Periodontal Pathogenesis

ROS have multifaceted effects, and the function of ROS is determined by the redox state (Zhao et al., 2016). Oxidative stress is induced when ROS are produced in excess of the capacity of the antioxidant system to efficiently counteract their actions, resulting in cytotoxic effects and enhancing periodontal destruction (Nibali and Donos, 2013). The involvement of ROS in the pathogenesis of periodontal diseases is highlighted by the existence of a disturbed redox balance in periodontitis cases. The results of recently published relevant studies have been summarized in **Table 1**. Data from a few cross-sectional studies have demonstrated low plasma and serum total antioxidant (TAOC) concentrations in periodontitis patients relative to healthy controls (Chapple et al., 2002; Brock et al., 2004; D'aiuto et al., 2010; Baltacioglu et al., 2014a; Thomas et al., 2014; Baser et al., 2015; Patil et al., 2016). Saliva is well recognized as containing a pool of markers for periodontitis (Zhang et al., 2016). Studies have also found similar results regarding salivary TAOC. The salivary TAOC was significantly lower in patients with chronic periodontitis compared with healthy controls (Diab-Ladki et al., 2003; Baltacioglu et al., 2014a; Miricescu et al., 2014; Baser et al., 2015; Zhang T. et al., 2015). Moreover, higher levels of reactive oxygen metabolites and total oxidant status (TOS) were observed in the serum, saliva, and gingival crevicular fluid (GCF) of patients with periodontitis compared with controls (Akalin et al., 2007; D'aiuto et al., 2010; Wei et al., 2010; Baltacioglu et al., 2014a,b). Furthermore, there is a strong negative correlation between salivary TAOC and clinical attachment loss (CAL) in periodontitis patients (Baser et al., 2015). Significant positive correlations were also observed between malondialdehyde (MDA), an LPO product, and TOS levels and periodontal parameters (Akalin et al., 2007). Collectively, these results suggested that reduced TAOC and increased ROS may be risk factors for periodontitis or may be caused by periodontal inflammation. However, it is very difficult to determine whether the change in redox status is the cause or a result of periodontitis. In addition, as summarized in **Table 1**, decreased levels of specific antioxidants, such as SOD, CAT, and GPX, were observed in periodontitis patients compared with healthy controls (Panjamurthy et al., 2005; Wei et al., 2010; Trivedi et al., 2014; Patil et al., 2016).

Comprehensive reviews on tissue damage caused by ROS have been published (Waddington et al., 2000; Chapple and Matthews, 2007; Nibali and Donos, 2013). To summarize, periodontal tissue damage may arise directly from oxidative stress and indirectly via the activation of cell signaling pathways related to inflammation, apoptosis, and other factors. It has been demonstrated that direct tissue damage caused by ROS can be mediated by (1) the induction of lipid peroxidation and cell membrane destruction (Mashayekhi et al., 2005; Panjamurthy et al., 2005; Tsai et al., 2005; Pradeep et al., 2013), which results in (2) protein denaturation and enzyme deactivation (Nibali and Donos, 2013; Trivedi et al., 2014; Nguyen et al., 2016; Patil et al., 2016), leading to (3) nucleic acid damage (e.g., strand breaks and base pair mutations) and chromosome disruption (Takane et al., 2002) and causing (4) mitochondrial injury and ROS bursts (Battino et al., 1999). Tissue destruction can be assessed by measuring the levels of markers for lipid peroxidation, protein damage, and DNA damage, such as MDA, protein carbonylation and 8-hydroxy-2-deoxyguanosine (8-OHdG) (Sawamoto et al., 2005; Takane et al., 2005; Canakci et al., 2009; Su et al., 2009; Mai et al., 2012; Sezer et al., 2012; Dede et al., 2013; Hendek

TABLE 1 | Levels of oxidative stress and antioxidant parameters in periodontitis patients compared with healthy ones.

Reactive oxygen species	Total oxidant levels	Diacron reactive oxygen metabolites (D-ROM)	Increase in serum (D'aiuto et al., 2010)
		Total oxidant status (TOS)	Increase in serum, saliva, and GCF (Akalin et al., 2007; Wei et al., 2010; Baltacioglu et al., 2014a,b)
Antioxidants	Total antioxidant levels	3-ethylbenzothiazoline 6-sulfonate (ABTS) reduction assays	Decrease in saliva (Diab-Ladki et al., 2003; Miricescu et al., 2014; Zhang T. et al., 2015)
		Plasma biological antioxidant potential (BAP) assay	Decrease in serum (D'aiuto et al., 2010)
		Ferric reducing antioxidant power (FRAP) assay	Decrease in serum and saliva (Baltacioglu et al., 2014b)
		Enhanced chemiluminescent (ECL) assay	Decrease in plasma (Chapple et al., 2002; Brock et al., 2004) and GCF (Chapple et al., 2002)
		Total blood antioxidant capacity (NBT test)	Decrease in serum (Thomas et al., 2014)
	Specific antioxidants	Superoxide dismutase (SOD)	Decrease in RBC lysate and saliva (Trivedi et al., 2014; Patil et al., 2016) Increase in serum, saliva, and GCF (Wei et al., 2010) Increase in plasma and tissue (Panjamurthy et al., 2005)
		Catalase (CAT) activity	Decrease in RBC lysate and saliva (Trivedi et al., 2014; Patil et al., 2016) Increase in plasma and tissue (Panjamurthy et al., 2005)
		Reduced and oxidized glutathione (GSH and GSSG)	Decrease in saliva (Tsai et al., 2005), GCF (Chapple et al., 2002), blood (Panjamurthy et al., 2005), and tissue (Panjamurthy et al., 2005)
		Glutathione peroxidase (GPX)	Decrease in RBC lysate (Trivedi et al., 2014) and saliva (Miricescu et al., 2014; Trivedi et al., 2014) No significant change in saliva (Tsai et al., 2005)
		Vitamin C	Decrease in plasma (Panjamurthy et al., 2005; Patil et al., 2016) and tissue (Panjamurthy et al., 2005)
		Vitamin E	Decrease in plasma and tissue (Panjamurthy et al., 2005)

et al., 2015). The results of relevant published studies have been summarized in **Table 2**.

A more complex question is how ROS result in periodontal tissue damage by regulating signal transduction and gene transcription, which is described in **Figure 1**. There are at least four pathways relevant to this topic. First, ROS are able to activate NF-κB, initiating a signaling cascade that regulates inflammatory and immune responses (Morgan and Liu, 2011). Second, ROS are involved in inducing JNK activation, resulting in cell apoptosis (Nakano et al., 2006). Third, ROS are associated with inflammasome activation, leading to pyroptic cell death (Zhou et al., 2010). Fourth, ROS play a critical role in autophagy (Filomeni et al., 2015). This section will focus on evidence for the mechanisms of ROS-mediated activation of NF-κB, JNK, and inflammasomes in periodontitis. The relationship between ROS and autophagy in periodontitis will be described later.

ROS have been reported to both activate and repress NF-κB signaling in studies of different cells and different upstream pathways, and ROS-mediated activation of NF-κB signaling results in the expression of pro-inflammatory cytokines and chemokines (Ozcan et al., 2016). The expression of these cytokines leads to periodontal destruction by triggering inflammatory responses and osteoclastic differentiation (Hans and Hans, 2011; Souza and Lerner, 2013). This effect can be inhibited by exogenously added antioxidants. For example, when intracellular ROS were scavenged during receptor activator for nuclear factor-κB ligand (RANKL)-stimulated osteoclastogenesis, the RANKL-induced activation of NF-κB was abrogated (Nikhil et al., 2015). Furthermore, Thummuri et al. demonstrated that thymoquinone, an antioxidant, could inhibit inflammation-induced ROS generation and the activation of NF-κB in osteoclast precursors (Thummuri et al., 2015).

ROS can also trigger JNK signaling during periodontitis (Wang et al., 2015; Lee et al., 2016). A recent study showed that ROS induced the activation of JNK signaling, which disrupted the periodontal junctional epithelium through the dissociation of E-cadherin (Wang et al., 2015; Lee et al., 2016). Consistent with this result, nicotine-induced ROS generation induced JNK phosphorylation in human gingival fibroblasts (HGFs). Furthermore, constitutive activation of JNK initiated the apoptosis cascade via the caspase-3-dependent pathway (Kang et al., 2011). In contrast to the pro-apoptotic function of JNK in HGFs, another study demonstrated an anti-apoptotic role of JNK in response to bacterial invasion (Wang et al., 2015). This study reported that JNK activation could induce the expression of genes that counter oxidative stress (Cat, Sod2, Prdx3) and apoptosis (Bcl-6) via the activation of the transcription factor forkhead box protein O1 (FoxO1) (Wang et al., 2015). Collectively, these results suggest that the activation of JNK in periodontal cells mediates cell survival, and this function may be condition and cell type dependent.

Another mechanism by which ROS are involved in periodontal pathogenesis is via the activation of inflammasomes. As previously reported, ROS induced the activation of NLRP3 by causing thioredoxin (TRX)-interacting protein (TXNIP) to dissociate from thioredoxin, which may be associated with periodontitis (Schroder et al., 2010; Zhou et al., 2010). Increasing

TABLE 2 | Levels of markers of oxidative stress damage in periodontitis patients compared with healthy ones.

Types	Markers	Expression levels
Lipid damage	Lipid peroxidation (TBARS assay)	Increase in saliva (Mashayekhi et al., 2005; Tsai et al., 2005), plasma (Panjamurthy et al., 2005) and tissue (Panjamurthy et al., 2005)
Protein damage	Malondialdehyde (MDA)	Increase in plasma (Trivedi et al., 2014), RBC lysate (Patil et al., 2016), saliva (Akalin et al., 2007; Khalili and Biloklytska, 2008; Baltacioglu et al., 2014a; Miricescu et al., 2014; Trivedi et al., 2014; Nguyen et al., 2016), and GCF (Akalin et al., 2007; Wei et al., 2010) No significant change in serum (Akalin et al., 2007; Baltacioglu et al., 2014b)
	Protein carbonylation	Increase in saliva (Su et al., 2009; Nguyen et al., 2016)
DNA damage	8-hydroxy-2-deoxyguanosine (8-OHdG)	Increase in saliva (Takane et al., 2002, 2005; Sawamoto et al., 2005; Canakci et al., 2009; Su et al., 2009; Sezer et al., 2012; Miricescu et al., 2014; Nguyen et al., 2016) and GCF (Hendek et al., 2015) No significant change in saliva (Dede et al., 2013)
	Leukocyte telomere lengths (LTL)	LTL was negatively correlated with oxidative stress ($P = 0.008$); and severity of periodontitis ($P = 0.003$; $R = -0.2$) (Masi et al., 2011)

clinical data support this point. Bostanci et al. first reported significantly high levels of NLRP3 as well as enhanced release of pro-inflammatory cytokines (IL-1β and IL-18) in patients with periodontitis compared with healthy controls (Bostanci et al., 2009). Elevated IL-1β and IL-18 levels could contribute to the triggering of periodontal destruction. Consistent with this study, Xue et al. and Huang et al. demonstrated increased levels of NLRP3 in the gingival tissues of periodontitis patients compared with healthy individuals via real-time PCR and immunohistochemistry (Huang et al., 2015; Xue et al., 2015). The activation of NLRP3 inflammasomes via ROS can lead to IL-1β secretion and pyroptosis.

Emerging evidence has indicated that nuclear factor erythroid 2-related factor 2 (Nrf2) plays an important cytoprotective role in oxidative-stress-associated periodontal damage. As a redox-sensitive factor, Nrf2 protects cells against cytotoxic ROS. As illustrated in **Figure 1**, oxidative stress disrupts critical cysteine residues in Kelch like-ECH-associated protein 1 (Keap1). When Nrf2 is not ubiquitinated, it dissociates from Keap1, translocates into the nucleus and binds to antioxidant response elements (AREs) to initiate the transcription of antioxidant genes, such as heme oxygenase-1 (HO-1), SOD, and CAT (Ma, 2013). Nrf2 knockout exacerbated the loss of periodontal tissues in a mouse model of periodontitis. In addition, an analysis of oral polymorphonuclear neutrophils (oPMNs) and blood PMNs revealed that Nrf2 expression was significantly decreased in patients with severe chronic periodontitis compared with periodontally healthy controls (Sima et al., 2016). Moreover, compared with blood PMNs, thirty Nrf2 pathway-related genes were differentially expressed in oPMNs from chronic periodontitis patients (Sima et al., 2016). These results indicated that Nrf2 and its downstream genes may be involved in the pathological process of periodontitis via their antioxidative effects.

Sirtuins (silent information regulator, Sir2) belong to a conserved family of nicotinamide adenine dinucleotide (NAD)-dependent protein deacylases. There are seven human Sir2 homologs, SIRT1 through SIRT7 (Chalkiadaki and Guarente, 2015). Increasing evidence has indicated that SIRT activation suppresses oxidative stress (Kumar et al., 2017), and the expression of SIRT1 was shown to be increased at the gene and protein levels in LPS-treated human periodontal ligament cells (Park et al., 2012). However, the direct linkage between SIRT and oxidative stress in periodontitis has not been elucidated.

AUTOPHAGY ACTIVATION IN PERIODONTITIS

Autophagy is an evolutionarily conserved intracellular degradation system that delivers damaged or superfluous cytoplasmic material (e.g., damaged organelles, denatured proteins, and bacteria) to the lysosome and recycles degradation products for new synthesis or energy production (Filomeni et al., 2015). In a broad sense, there are four different forms of autophagy: macroautophagy, microautophagy, chaperone-mediated autophagy (CMA) and non-canonical autophagy. Among these forms, macroautophagy (hereafter referred to as autophagy) is the most widely investigated type (Kabat et al., 2016). Unlike other intracellular degradation pathways, autophagy sequesters intracellular material inside a double-membrane vesicle called the autophagosome. Subsequently, the autophagosome fuses with lysosomes, resulting in the degradation of the vesicle (Shibutani et al., 2015). The complete autophagy process can be divided into five highly regulated stages, including induction, elongation, maturation, transport to lysosomes, and degradation (Tooze and Dikic, 2016). Periodontitis is a multifactorial inflammatory disease (Dumitrescu, 2015). Periodontal pathogens residing in dental plaques and the periodontal pocket are believed to be the primary etiology of periodontitis (Hajishengallis, 2015). In previous studies, depending on context, the induction of autophagy has been shown to have both protective and pathological effect in periodontitis. *Song* et al. have comprehensively reviewed the role of autophagy in periodontitis (Song et al., 2016). In summary, autophagy may participate in periodontitis via the following mechanisms: (1) regulating periodontal pathogen invasion; (2) regulating immune

signaling, resulting in inflammatory disorders and periodontal tissue damage; and (3) protecting periodontal cells from apoptosis.

Autophagy in Periodontal Pathogen Invasion

Abundant evidence has demonstrated that periodontitis is highly associated with microbial infection. As an intracellular innate immune defense pathway, autophagy is usually enhanced in infected cells, contributing to antimicrobial defense mechanisms. Autophagy can eliminate intracellular pathogens such as *Mycobacterium tuberculosis* (*M. tuberculosis*) (Kim et al., 2012). To avoid lysosomal killing, many pathogens, including *Legionella pneumophila* (*L. pneumophila*), have developed strategies to suppress cellular autophagy. However, findings obtained via *in vitro* experiments in which cultured cells were exposed to bacteria have suggested that periodontal pathogens such as *P. gingivalis* participate in the induction of autophagy. Belanger et al. found that *P. gingivalis* trafficked quickly from phagosomes to autophagosomes in human coronary artery endothelial cells (Belanger et al., 2006). This result is consistent with the finding that ROS generated by *P. gingivalis* contribute to increased levels of LC3 proteins and promoting the conversion of LC3-I to LC3-II (Park et al., 2017). Taken together, these results strongly suggest that the induction of autophagy can facilitate specific periodontal bacterial survival by replication within an autophagosome-like compartment. However, no LC3 lipidation was found when cells were infected with *A. actinomycetemcomitans* (Blasi et al., 2016), suggesting that the activity of cellular autophagy in response to infection is associated with periodontal bacterial species.

Autophagy in the Periodontal Immune Response and Inflammation

The relationship between autophagy and immunity has been systematically reviewed in the published literature (Levine et al., 2011; Deretic et al., 2013; Shibutani et al., 2015). Here, we mainly focus on the potential immune consequences of autophagy for periodontitis. Autophagy functions as a modulator of classical pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), and Nod-like receptors (NLRs), regulating the periodontal innate immune response (Deretic et al., 2013; Oh and Lee, 2014). Furthermore, autophagy can suppress the periodontal immune response by inhibiting cytokine secretion. First, autophagy plays a negative role in inflammasome activation and secretion of IL-1β and IL-18. Animal studies have shown that mice lacking LC3B produced higher levels of caspase-1-dependent cytokines than wild-type mice. Similar results were found in LC3B-deficient macrophages (Nakahira et al., 2011). LC3B is a ubiquitin-like protein that participates in autophagosome formation and maturation (Anton et al., 2016). Second, autophagy negatively regulates the secretion of IL-1α. Castillo et al. found that mice lacking Atg5 produced more IL-1α via a ROS-calpain pro-inflammatory pathway (Castillo et al., 2012). It therefore seems reasonable to consider that autophagy might influence periodontal inflammation by regulating both inflammasome-dependent and inflammasome-independent inflammation.

Autophagy Protects Periodontal Cells from Apoptosis

Studies have shown that the inhibition of autophagy in HGFs treated with *P. gingivalis* LPS induced apoptosis, suggesting a protective role of autophagy (Bullon et al., 2012). To further explore the role of autophagy in periodontitis, a recent study measured the expression of LC3 and observed autophagic vacuoles in periodontal ligament (PDL) tissues from individuals with and without periodontitis. The results showed increased LC3 expression and autophagosome production in inflammatory PDL tissues (An et al., 2016). In addition, co-localization of LC3 and melanoregulin (MREG) was found in gingival epithelial cells isolated from severe periodontal disease-affected individuals, while this effect was absent in cells from healthy or moderately affected individuals (Blasi et al., 2016). As multiple studies have proposed that autophagy may antagonize apoptosis, these results suggest a potential protective role of autophagy in periodontal tissues. However, whether the blocking of autophagy induces apoptosis in periodontal tissues remains unknown.

REDOX REGULATION OF AUTOPHAGY IN PERIODONTITIS

Mitochondrial ROS have been identified as important signaling molecules in regulating autophagy. Moreover, bacterial infection induces the generation of ROS (Golz et al., 2014). Elevated ROS can regulate autophagy activity by targeting autophagy-related genes (Atgs) and/or upstream signaling pathways, including mammalian target of rapamycin complex 1 (mTORC1), Beclin 1, and the Atg12-Atg5 complex, as outlined in **Figure 2**. Emerging evidence has suggested the involvement of ROS-autophagy reciprocity in periodontitis. The expression of autophagy-related genes (Atg12 and LC3) was shown to be positively correlated with mitochondrial ROS production in peripheral blood mononuclear cells from patients with periodontitis (Bullon et al., 2012). Furthermore, a reduction of mitochondrial ROS induced a decrease in autophagy (Bullon et al., 2012).

ROS Disrupts Autophagy Induction by Interfering with mTORC1

The activity of mTORC1 is regulated by numerous input signals, such as rapamycin, insulin, and oxidative stress. Studies have found that ROS could influence mTORC1 activity through the tuberous sclerosis complex 1/2 (TSC1/TSC2) heterodimer. Increased levels of ROS activate AMP-activated protein kinase (AMPK), which causes TSC2 phosphorylation and activates the TSC1/TSC2 complex, thus inhibiting mTORC1 and stimulating ULK (an important initiator of the autophagy complex) to induce autophagy (Yu et al., 2010; Zhang et al., 2013; Zhang J. et al., 2015). Conversely, ROS can activate the phosphoinositide-3-kinase (PI3K)-protein kinase B (Akt)-mTORC1 signaling pathway by directly activating PI3K or by regulating the phosphorylation state of Akt, thus inhibiting

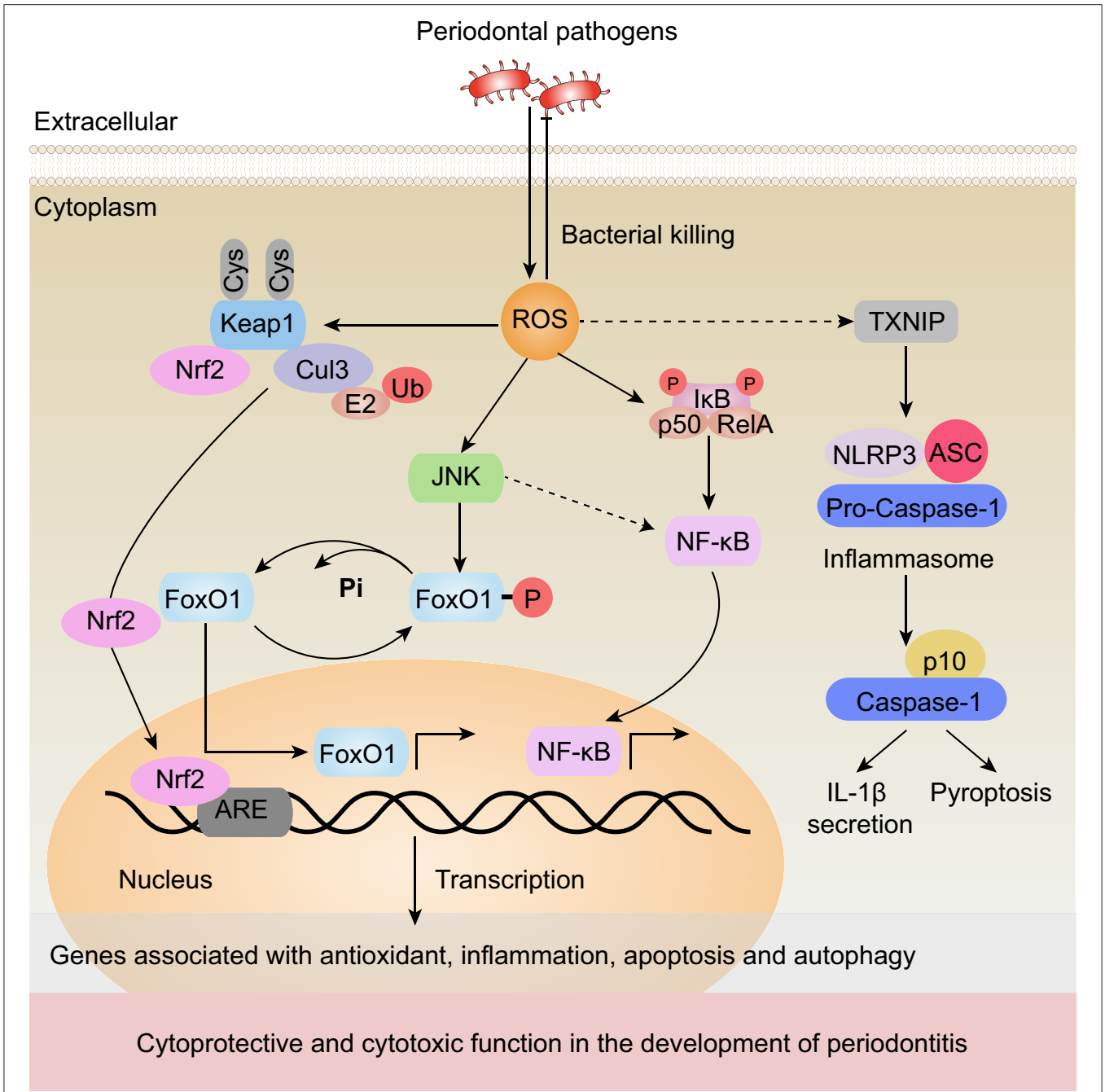

FIGURE 1 | Underlying signaling pathways of ROS regulation in periodontitis. Periodontal pathogen infection can promote ROS generation. In turn, ROS can contribute to the oxidative killing of the pathogens. ROS generated from mitochondria activate the transcription of genes associated with inflammation, apoptosis and autophagy through JNK, NF-κB, and inflammasome-dependent signaling pathways, which leads to cytoprotective and cytotoxic effects in the development of periodontitis. (1) ROS activate JNK, which results in the dephosphorylation of FoxO1. (2) ROS have been shown to activate NF-κB in periodontitis. (3) ROS promote excessive inflammation by activating TXNIP, which subsequently activates the NLRP3 inflammasome, elevates the secretion of its substrates, such as IL-1β, and induces pyroptosis. (4) Meanwhile, ROS interact with cysteine residues in Keap1, disrupting the Keap1-Cul3 ubiquitination system and leading to the release of Nrf2 to the nucleus. In the nucleus, Nrf2 binds to AREs to initiate the transcription of a number of antioxidant genes. Black arrows (↑) and perpendicular lines (⊥) denote activation and suppression, respectively. Dashed lines denote regulatory relationships that need to be confirmed in periodontitis.

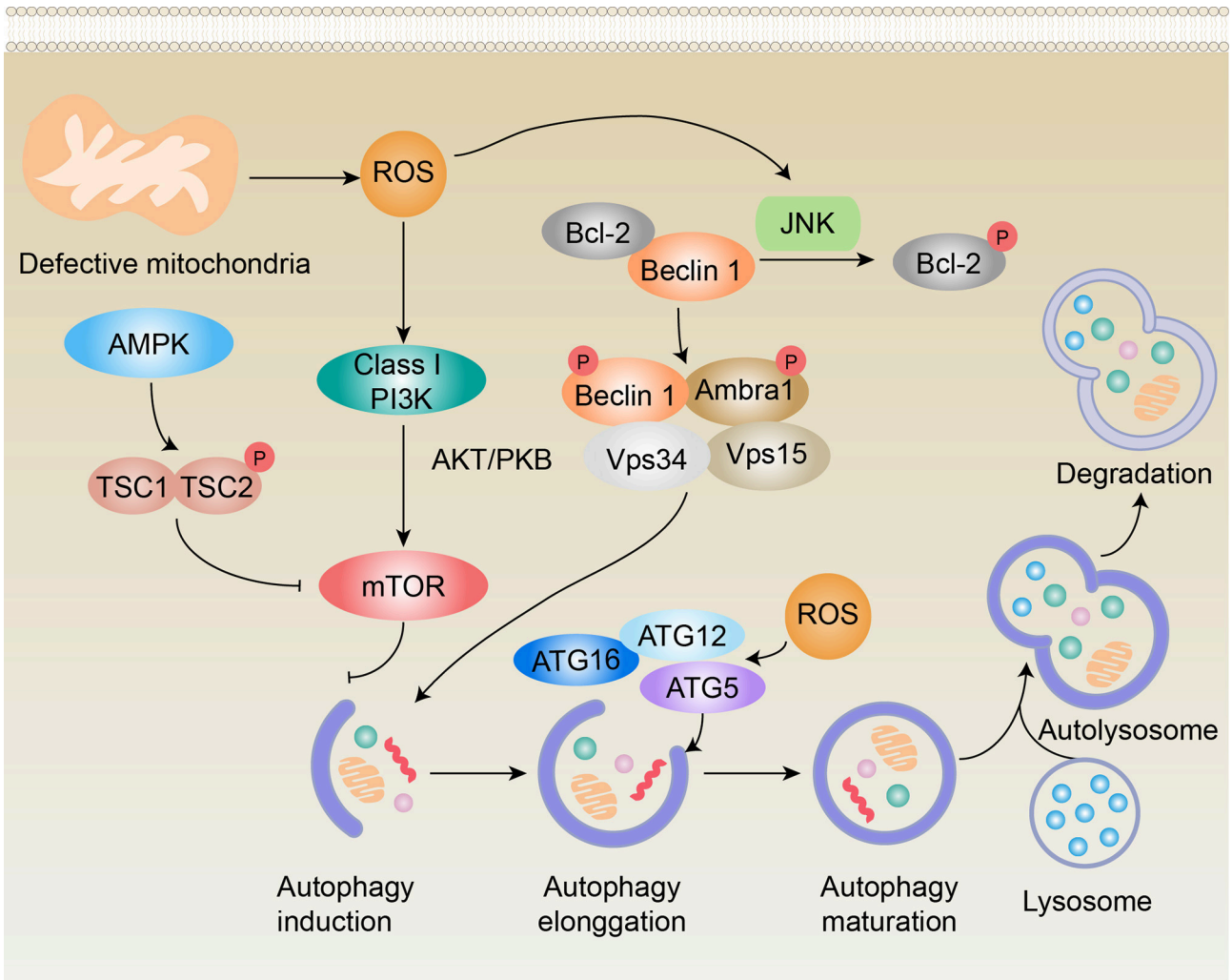

FIGURE 2 | Schematic representation of potential pathways of redox regulation of autophagy in periodontitis. ROS regulate autophagy via at least four different mechanisms, including (1) the phosphorylation of Bcl-2 by JNK in a ROS-dependent manner that leads to Beclin 1 dissociation and autophagy induction; (2) initiation of the PI3K-AKT pathway, resulting in the activation of mTOR, which functions as an inhibitor of autophagy induction; (3) inhibition of TORC1 activity in an AMPK-dependent manner, contributing to the activation of autophagy; and (4) activation of the Atg12-Atg5 complex, which promotes autophagy elongation. Black arrows (↑) and perpendicular lines (⊥) denote activation and suppression, respectively.

autophagy induction (Dermit et al., 2016; Su et al., 2017). Stafford *et al.* found that invasion of *P. gingivalis* inhibited the mTOR pathway in oral epithelial cells, which was the first reported evidence to suggest a potential role for mTORC1 in periodontitis (Stafford et al., 2013).

ROS Inhibits Autophagic Flux by Targeting Beclin 1

As noted above, increased levels of ROS can activate NF-κB, which may result in the upregulation of Atgs, including Beclin 1 (He Z. J. et al., 2017). Moreover, the activation of JNK signaling during oxidative stress leads to the phosphorylation of Bcl-2, which causes Beclin 1 to dissociate from the Vps34 complex and results in the activation of autophagy (Ni et al., 2014). Several studies have shown the relevance of these Atgs in periodontitis. Specifically, there were higher protein expression levels of LC3II/I and Beclin 1, as well as increased transcriptional levels of LC3, Beclin-1, Atg7, and Atg12, in periodontal ligament stem cells isolated from patients with periodontitis compared with healthy individuals (An et al., 2016).

ROS Induces Autophagy by Activating the Atg12-Atg5 Complex

The Atg12-Atg5 conjugate is a ubiquitin-like protein complex that is essential for autophagophore elongation in autophagy (Otomo et al., 2013). A number of studies have provided evidence of the fine-tuning of Atg12-Atg5 in relation to the intracellular redox state (Mai et al., 2012). Pei et al. reported that the levels of Atg12-Atg5 were upregulated in a preodontoblast cell line (mDPC6T cells) after treatment with LPS for 6 h and 12 h but were downregulated after treatment with LPS for 24 h (Pei et al., 2015).

These findings show that autophagy can be induced in response to ROS through two master regulators of autophagosome biogenesis (mTORC1 and Beclin-1) and the Atg5-Atg12 complex, which also plays important roles in autophagosome biogenesis (**Figure 2**).

Autophagy is also crucial in mitochondrial ROS generation and scavenging, which is predominantly achieved by the release and activation of Nrf2 (Komatsu et al., 2010). Emerging evidence has indicated that Nrf2 and its target genes are crucial for maintaining cellular redox homeostasis in the attenuation of oxidative stress-associated periodontal destruction (Tamaki et al., 2014; Kataoka et al., 2016; Sima et al., 2016).

Collectively, progress in the field of redox regulation in autophagy has provided increasing details of the crosstalk mechanisms between ROS and autophagy. However, there is still no direct evidence demonstrating that the activation/inactivation of autophagy is triggered by redox regulation signaling in periodontitis. Hence, the precise process in periodontal tissues still needs to be elucidated. Whether ROS is an upstream signal of autophagy in periodontitis also requires further investigation.

CONCLUSIONS

The physiologic and pathologic roles of ROS in the initiation and development of periodontitis have been studied for decades (Battino et al., 1999; Patil et al., 2016). Accumulating evidence has demonstrated that although low levels of ROS can be beneficial, excessive generation of ROS and/or antioxidant deficiency results in tissue destruction in periodontal diseases (Di Meo et al., 2016). More importantly, studies have indicated that ROS function as upstream modulators of autophagy (Bhattacharya and Eissa, 2015). In turn, autophagy can regulate ROS through the Nrf2 signaling pathway (Komatsu et al., 2010). Furthermore, several lines of evidence suggest that autophagy is involved in the development of periodontitis (Tan et al., 2016). The relationship between ROS and autophagy has also been shown to be associated with processes of other diseases, such as cancer (He Z. J. et al., 2017). Based on the accumulated evidence, we speculate that redox regulation of autophagy may play an important role in the initiation and development of periodontitis. As a form of cytotoxic signaling, excessive generation of ROS can trigger aggravated inflammation, apoptosis, and dysregulated autophagy activity that induces periodontal dysfunction. Conversely, redox regulation of autophagy is an effective measure for antibacterial responses and is also associated with protecting periodontal cells from apoptosis. As there is insufficient evidence concerning the interplay between ROS and autophagy in periodontal dysfunction, it is very difficult to generalize the role of redox regulation in periodontitis-related autophagy. However, previous studies have suggested a dual role for the redox regulation of autophagy. These studies have demonstrated that ROS may play a crucial role in determining cell fate by inducing autophagy or apoptosis. Therefore, further studies are required to clarify the role and mechanism of redox regulation of autophagy in periodontitis, which may be particularly beneficial for developing new therapeutic strategies for periodontal disease.

AUTHOR CONTRIBUTIONS

CL drafted the manuscript and prepared the figures. LM, YN, XL, and XZ drafted parts of the manuscript and prepared the tables. XX reviewed, edited, and approved the final version of the manuscript.

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REFERENCES

Akalin, F. A., Baltacioglu, E., Alver, A., and 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. doi: 10.1111/j.1600-051X.2007.01091.x

Almerich-Silla, J. M., Montiel-Company, J. M., Pastor, S., Serrano, F., Puig-Silla, M., and Dasi, F. (2015). Oxidative stress parameters in saliva and its association with periodontal disease and types of bacteria. *Dis. Markers* 2015:653537. doi: 10.1155/2015/653537

An, Y., Liu, W., Xue, P., Zhang, Y., Wang, Q., and Jin, Y. (2016). Increased autophagy is required to protect periodontal ligament stem cells from apoptosis in inflammatory microenvironment. *J. Clin. Periodontol.* 43, 618–625. doi: 10.1111/jcpe.12549

Anton, Z., Landajuela, A., Hervas, J. H., Montes, L. R., Hernandez-Tiedra, S., Velasco, G., et al. (2016). Human Atg8-cardiolipin interactions in mitophagy: specific properties of LC3B, GABARAPL2 and GABARAP. *Autophagy* 12, 2386–2403. doi: 10.1080/15548627.2016.1240856

Baltacioglu, E., Kehribar, M. A., Yuva, P., Alver, A., Atagun, O. S., Karabulut, E., et al. (2014a). Total oxidant status and bone resorption biomarkers in serum and gingival crevicular fluid of patients with periodontitis. *J. Periodontol.* 85, 317–326. doi: 10.1902/jop.2013.130012

Baltacioglu, E., Yuva, P., Aydin, G., Alver, A., Kahraman, C., Karabulut, E., et al. (2014b). Lipid peroxidation levels and total oxidant/antioxidant status in serum and saliva from patients with chronic and aggressive periodontitis. Oxidative stress index: a new biomarker for periodontal disease? *J. Periodontol.* 85, 1432–1441. doi: 10.1902/jop.2014.130654

Baser, U., Gamsiz-Isik, H., Cifcibasi, E., Ademoglu, E., and Yalcin, F. (2015). Plasma and salivary total antioxidant capacity in healthy controls compared with aggressive and chronic periodontitis patients. *Saudi Med. J.* 36, 856–861. doi: 10.15537/smj.2015.7.11954

Battino, M., Bullon, P., Wilson, M., and 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. doi: 10.1177/10454411990100040301

Belanger, M., Rodrigues, P. H., Dunn, W. A. Jr., and Progulske-Fox, A. (2006). Autophagy: a highway for *Porphyromonas gingivalis* in endothelial cells. *Autophagy* 2, 165–170. doi: 10.4161/auto.2828

Bhattacharya, A., and Eissa, N. T. (2015). Autophagy as a stress response pathway in the immune system. *Int. Rev. Immunol.* 34, 382–402. doi: 10.3109/08830185.2014.999156

Blasi, I., Korostoff, J., Dhingra, A., Reyes-Reveles, J., Shenker, B. J., Shahabuddin, N., et al. (2016). Variants of *Porphyromonas gingivalis* lipopolysaccharide alter lipidation of autophagic protein, microtubule-associated protein 1 light chain 3, LC3. *Mol. Oral Microbiol.* 31, 486–500. doi: 10.1111/omi.12141

Bostanci, N., Emingil, G., Saygan, B., Turkoglu, O., Atilla, G., Curtis, M. A., et al. (2009). Expression and regulation of the NALP3 inflammasome complex in periodontal diseases. *Clin. Exp. Immunol.* 157, 415–422. doi: 10.1111/j.1365-2249.2009.03972.x

Brock, G. R., Butterworth, C. J., Matthews, J. B., and Chapple, I. L. (2004). Local and systemic total antioxidant capacity in periodontitis and health. *J. Clin. Periodontol.* 31, 515–521. doi: 10.1111/j.1600-051X.2004.00509.x

Bullon, P., Cordero, M. D., Quiles, J. L., Ramirez-Tortosa Mdel, C., Gonzalez-Alonso, A., Alfonsi, S., et al. (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

Burdon, R. H., Gill, V., and Alliangana, D. (1996). Hydrogen peroxide in relation to proliferation and apoptosis in BHK-21 hamster fibroblasts. *Free Radic. Res.* 24, 81–93. doi: 10.3109/10715769609088004

Canakci, C. F., Canakci, V., Tatar, A., Eltas, A., Sezer, U., Cicek, Y., et al. (2009). Increased salivary level of 8-hydroxydeoxyguanosine is a marker of premature oxidative mitochondrial DNA damage in gingival tissue of patients with periodontitis. *Arch. Immunol. Ther. Exp. (Warsz).* 57, 205–211. doi: 10.1007/s00005-009-0026-9

Castillo, E. F., Dekonenko, A., Arko-Mensah, J., Mandell, M. A., Dupont, N., Jiang, S., et al. (2012). Autophagy protects against active tuberculosis by suppressing bacterial burden and inflammation. *Proc. Natl. Acad. Sci. U.S.A.* 109, E3168–E3176. doi: 10.1073/pnas.1210500109

Cavalla, F., Osorio, C., Paredes, R., Valenzuela, M. A., Garcia-Sesnich, J., Sorsa, T., et al. (2015). Matrix metalloproteinases regulate extracellular levels of SDF-1/CXCL12, IL-6 and VEGF in hydrogen peroxide-stimulated human periodontal ligament fibroblasts. *Cytokine* 73, 114–121. doi: 10.1016/j.cyto.2015.02.001

Chalkiadaki, A., and Guarente, L. (2015). The multifaceted functions of sirtuins in cancer. *Nat. Rev. Cancer* 15, 608–624. doi: 10.1038/nrc3985

Chapple, I. L. (1997). Reactive oxygen species and antioxidants in inflammatory diseases. *J. Clin. Periodontol.* 24, 287–296. doi: 10.1111/j.1600-051X.1997.tb00760.x

Chapple, I. L., Brock, G., Eftimiadi, C., and Matthews, J. B. (2002). Glutathione in gingival crevicular fluid and its relation to local antioxidant capacity in periodontal health and disease. *Mol. Pathol.* 55, 367–373. doi: 10.1136/mp.55.6.367

Chapple, I. L., and Matthews, J. B. (2007). The role of reactive oxygen and antioxidant species in periodontal tissue destruction. *Periodontol. 2000* 43, 160–232. doi: 10.1111/j.1600-0757.2006.00178.x

Chen, Y., Zhang, H., Zhou, H. J., Ji, W., and Min, W. (2016). Mitochondrial redox signaling and tumor progression. *Cancers (Basel)* 8:40. doi: 10.3390/cancers8040040

Choe, Y., Yu, J. Y., Son, Y. O., Park, S. M., Kim, J. G., Shi, X., et al. (2012). Continuously generated H_2O_2 stimulates the proliferation and osteoblastic differentiation of human periodontal ligament fibroblasts. *J. Cell. Biochem.* 113, 1426–1436. doi: 10.1002/jcb.24017

Choi, E. M., Kim, G. H., and Lee, Y. S. (2009). Atractylodes japonica root extract protects osteoblastic MC3T3-E1 cells against hydrogen peroxide-induced inhibition of osteoblastic differentiation. *Phytother. Res.* 23, 1537–1542. doi: 10.1002/ptr.2813

D'aiuto, F., Nibali, L., Parkar, M., Patel, K., Suvan, J., and Donos, N. (2010). Oxidative stress, systemic inflammation, and severe periodontitis. *J. Dent. Res.* 89, 1241–1246. doi: 10.1177/0022034510375830

Dede, F. O., Ozden, F. O., and Avci, B. (2013). 8-hydroxy-deoxyguanosine levels in gingival crevicular fluid and saliva in patients with chronic periodontitis after initial periodontal treatment. *J. Periodontol.* 84, 821–828. doi: 10.1902/jop.2012.120195

Deretic, V., Saitoh, T., and Akira, S. (2013). Autophagy in infection, inflammation and immunity. *Nat. Rev. Immunol.* 13, 722–737. doi: 10.1038/nri3532

Dermit, M., Casado, P., Rajeeve, V., Wilkes, E. H., Foxler, D. E., Campbell, H., et al. (2016). Oxidative stress downstream of mTORC1 but not AKT causes a proliferative defect in cancer cells resistant to PI3K inhibition. *Oncogene.* 36, 2762–2774. doi: 10.1038/onc.2016.435

Diab-Ladki, R., Pellat, B., and 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

Di Meo, S., Reed, T. T., Venditti, P., and Victor, V. M. (2016). Role of ROS and RNS sources in physiological and pathological conditions. *Oxid. Med. Cell. Longev.* 2016:1245049. doi: 10.1155/2016/1245049

Dumitrescu, A. L. (2015). Editorial: periodontal disease - a public health problem. *Front. Public Health* 3:278. doi: 10.3389/fpubh.2015.00278

Dumitrescu, A. L. (2016). Depression and inflammatory periodontal disease considerations-an interdisciplinary approach. *Front. Psychol.* 7:347. doi: 10.3389/fpsyg.2016.00347

Filomeni, G., De Zio, D., and Cecconi, F. (2015). Oxidative stress and autophagy: the clash between damage and metabolic needs. *Cell Death Differ.* 22, 377–388. doi: 10.1038/cdd.2014.150

Galli, C., Passeri, G., and Macaluso, G. M. (2011). FoxOs, Wnts and oxidative stress-induced bone loss: new players in the periodontitis arena? *J. Periodont. Res.* 46, 397–406. doi: 10.1111/j.1600-0765.2011.01354.x

Gan, P., Gao, Z., Zhao, X., and Qi, G. (2016). Surfactin inducing mitochondria-dependent ROS to activate MAPKs, NF-kappaB and inflammasomes in macrophages for adjuvant activity. *Sci. Rep.* 6:39303. doi: 10.1038/srep39303

Golz, L., Memmert, S., Rath-Deschner, B., Jager, A., Appel, T., Baumgarten, G., et al. (2014). LPS from *P. gingivalis* and hypoxia increases oxidative stress in periodontal ligament fibroblasts and contributes to periodontitis. *Mediators Inflamm.* 2014:986264. doi: 10.1155/2014/986264

Hajishengallis, G. (2014). Immunomicrobial pathogenesis of periodontitis: keystones, pathobionts, and host response. *Trends Immunol.* 35, 3–11. doi: 10.1016/j.it.2013.09.001

Hajishengallis, G. (2015). Periodontitis: from microbial immune subversion to systemic inflammation. *Nat. Rev. Immunol.* 15, 30–44. doi: 10.1038/nri3785

Hans, M., and Hans, V. M. (2011). Toll-like receptors and their dual role in periodontitis: a review. *J. Oral Sci.* 53, 263–271. doi: 10.2334/josnusd.53.263

He, Y., Li, S., Zhang, W., Dai, W., Cui, T., Wang, G., et al. (2017). Dysregulated autophagy increased melanocyte sensitivity to H_2O_2-induced oxidative stress in vitiligo. *Sci. Rep.* 7:42394. doi: 10.1038/srep42394

He, Z. J., Zhu, F. Y., Li, S. S., Zhong, L., Tan, H. Y., and Wang, K. (2017). Inhibiting ROS-NF-kappaB-dependent autophagy enhanced brazilin-induced apoptosis in head and neck squamous cell carcinoma. *Food Chem. Toxicol.* 101, 55–66. doi: 10.1016/j.fct.2017.01.002

Hendek, M. K., Erdemir, E. O., Kisa, U., and Ozcan, G. (2015). Effect of initial periodontal therapy on oxidative stress markers in gingival crevicular fluid, saliva, and serum in smokers and non-smokers with chronic periodontitis. *J. Periodontol.* 86, 273–282. doi: 10.1902/jop.2014.140338

Huang, X., Yang, X., Ni, J., Xie, B., Liu, Y., Xuan, D., et al. (2015). Hyperglucose contributes to periodontitis: involvement of the NLRP3 pathway by engaging the innate immunity of oral gingival epithelium. *J. Periodontol.* 86, 327–335. doi: 10.1902/jop.2014.140403

Kabat, A. M., Pott, J., and Maloy, K. J. (2016). The mucosal immune system and its regulation by autophagy. *Front. Immunol.* 7:240. doi: 10.3389/fimmu.2016.00240

Kang, S. W., Park, H. J., Ban, J. Y., Chung, J. H., Chun, G. S., and Cho, J. O. (2011). Effects of nicotine on apoptosis in human gingival fibroblasts. *Arch. Oral Biol.* 56, 1091–1097. doi: 10.1016/j.archoralbio.2011.03.016

Kanzaki, H., Shinohara, F., Kajiya, M., and Kodama, T. (2013). The Keap1/Nrf2 protein axis plays a role in osteoclast differentiation by regulating intracellular reactive oxygen species signaling. *J. Biol. Chem.* 288, 23009–23020. doi: 10.1074/jbc.M113.478545

Kataoka, K., Ekuni, D., Tomofuji, T., Irie, K., Kunitomo, M., Uchida, Y., et al. (2016). Visualization of oxidative stress induced by experimental periodontitis

in keap1-dependent oxidative stress detector-luciferase mice. *Int. J. Mol. Sci.* 17:1907. doi: 10.3390/ijms17111907

Khalili, J., and Biloklytska, H. F. (2008). Salivary malondialdehyde levels in clinically healthy and periodontal diseased individuals. *Oral Dis.* 14, 754–760. doi: 10.1111/j.1601-0825.2008.01464.x

Kim, J. J., Lee, H. M., Shin, D. M., Kim, W., Yuk, J. M., Jin, H. S., et al. (2012). Host cell autophagy activated by antibiotics is required for their effective antimycobacterial drug action. *Cell Host Microbe* 11, 457–468. doi: 10.1016/j.chom.2012.03.008

Kinane, D., Bouchard, P., and Group E of European Workshop on Periodontology (2008). Periodontal diseases and health: Consensus Report of the Sixth European Workshop on Periodontology. *J. Clin. Periodontol.* 35, 333–337. doi: 10.1111/j.1600-051X.2008.01278.x

Klionsky, D. J., and Emr, S. D. (2000). Autophagy as a regulated pathway of cellular degradation. *Science* 290, 1717–1721. doi: 10.1126/science.290.5497.1717

Komatsu, M., Kurokawa, H., Waguri, S., Taguchi, K., Kobayashi, A., Ichimura, Y., et al. (2010). The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. *Nat. Cell Biol.* 12, 213–223. doi: 10.1038/ncb2021

Kumar, S., Kim, Y. R., Vikram, A., Naqvi, A., Li, Q., Kassan, M., et al. (2017). Sirtuin1-regulated lysine acetylation of p66Shc governs diabetes-induced vascular oxidative stress and endothelial dysfunction. *Proc. Natl. Acad. Sci. U.S.A.* 114, 1714–1719. doi: 10.1073/pnas.1614112114

Lamont, R. J. (2016). Hydrogen peroxide is a central determinant of oral polymicrobial synergy. *Environ. Microbiol.* 18, 3609–3611. doi: 10.1111/1462-2920.13537

Lamont, R. J., and Hajishengallis, G. (2015). Polymicrobial synergy and dysbiosis in inflammatory disease. *Trends Mol. Med.* 21, 172–183. doi: 10.1016/j.molmed.2014.11.004

Lee, G., Kim, H. J., and Kim, H. M. (2016). RhoA-JNK regulates the E-cadherin junctions of human gingival epithelial cells. *J. Dent. Res.* 95, 284–291. doi: 10.1177/0022034515619375

Levine, B., and Klionsky, D. J. (2016). Autophagy wins the 2016 Nobel Prize in Physiology or Medicine: Breakthroughs in baker's yeast fuel advances in biomedical research. *Proc. Natl. Acad. Sci. U.S.A.* 114, 201–205. doi: 10.1073/pnas.1619876114

Levine, B., and Kroemer, G. (2008). Autophagy in the pathogenesis of disease. *Cell* 132, 27–42. doi: 10.1016/j.cell.2007.12.018

Levine, B., Mizushima, N., and Virgin, H. W. (2011). Autophagy in immunity and inflammation. *Nature* 469, 323–335. doi: 10.1038/nature09782

Lismont, C., Nordgren, M., Van Veldhoven, P. P., and Fransen, M. (2015). Redox interplay between mitochondria and peroxisomes. *Front. Cell Dev. Biol.* 3:35. doi: 10.3389/fcell.2015.00035

Liu, W., Gu, J., Qi, J., Zeng, X. N., Ji, J., Chen, Z. Z., et al. (2015). Lentinan exerts synergistic apoptotic effects with paclitaxel in A549 cells via activating ROS-TXNIP-NLRP3 inflammasome. *J. Cell. Mol. Med.* 19, 1949–1955. doi: 10.1111/jcmm.12570

Ma, Q. (2013). Role of nrf2 in oxidative stress and toxicity. *Annu. Rev. Pharmacol. Toxicol.* 53, 401–426. doi: 10.1146/annurev-pharmtox-011112-140320

Mai, S., Muster, B., Bereiter-Hahn, J., and Jendrach, M. (2012). Autophagy proteins LC3B, ATG5 and ATG12 participate in quality control after mitochondrial damage and influence lifespan. *Autophagy* 8, 47–62. doi: 10.4161/auto.8.1.18174

Marquis, R. E. (1995). Oxygen metabolism, oxidative stress and acid-base physiology of dental plaque biofilms. *J. Ind. Microbiol.* 15, 198–207. doi: 10.1007/BF01569826

Mashayekhi, F., Aghahoseini, F., Rezaie, A., Zamani, M. J., Khorasani, R., and Abdollahi, M. (2005). Alteration of cyclic nucleotides levels and oxidative stress in saliva of human subjects with periodontitis. *J. Contemp. Dent. Pract.* 6, 46–53.

Masi, S., Salpea, K. D., Li, K., 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, 730–735. doi: 10.1016/j.freeradbiomed.2010.12.031

McClean, C., Harris, R. A., Brown, M., Brown, J. C., and Davison, G. W. (2015). Effects of exercise intensity on postexercise endothelial function and oxidative stress. *Oxid. Med. Cell. Longev.* 2015:723679. doi: 10.1155/2015/723679

Miricescu, D., Totan, A., Calenic, B., Mocanu, B., Didilescu, A., Mohora, M., et al. (2014). Salivary biomarkers: relationship between oxidative stress and alveolar bone loss in chronic periodontitis. *Acta Odontol. Scand.* 72, 42–47. doi: 10.3109/00016357.2013.795659

Mizushima, N., Noda, T., Yoshimori, T., Tanaka, Y., Ishii, T., George, M. D., et al. (1998). A protein conjugation system essential for autophagy. *Nature* 395, 395–398. doi: 10.1038/26506

Morgan, M. J., and Liu, Z. G. (2011). Crosstalk of reactive oxygen species and NF-kappaB signaling. *Cell Res.* 21, 103–115. doi: 10.1038/cr.2010.178

Nakahira, K., Haspel, J. A., Rathinam, V. A., Lee, S. J., Dolinay, T., Lam, H. C., et al. (2011). Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. *Nat. Immunol.* 12, 222–230. doi: 10.1038/ni.1980

Nakano, H., Nakajima, A., Sakon-Komazawa, S., Piao, J. H., Xue, X., and Okumura, K. (2006). Reactive oxygen species mediate crosstalk between NF-kappaB and JNK. *Cell Death Differ.* 13, 730–737. doi: 10.1038/sj.cdd.4401830

Netea-Maier, R. T., Plantinga, T. S., Van De Veerdonk, F. L., Smit, J. W., and Netea, M. G. (2016). Modulation of inflammation by autophagy: consequences for human disease. *Autophagy* 12, 245–260. doi: 10.1080/15548627.2015.1071759

Netto, L. E., and Antunes, F. (2016). The roles of peroxiredoxin and thioredoxin in hydrogen peroxide sensing and in signal transduction. *Mol. Cells* 39, 65–71. doi: 10.14348/molcells.2016.2349

Nguyen, T. T., Ngo, L. Q., Promsudthi, A., and Surarit, R. (2016). Salivary oxidative stress biomarkers in chronic periodontitis and acute coronary syndrome. *Clin. Oral Investig.* doi: 10.1007/s00784-016-2029-3. [Epub ahead of print].

Ni, Z., Wang, B., Dai, X., Ding, W., Yang, T., Li, X., et al. (2014). HCC cells with high levels of Bcl-2 are resistant to ABT-737 via activation of the ROS-JNK-autophagy pathway. *Free Radic. Biol. Med.* 70, 194–203. doi: 10.1016/j.freeradbiomed.2014.02.012

Nibali, L., and Donos, N. (2013). Periodontitis and redox status: a review. *Curr. Pharm. Des.* 19, 2687–2697. doi: 10.2174/1381612811319150003

Nikhil, K., Sharan, S., and Roy, P. (2015). A pterostilbene derivative suppresses osteoclastogenesis by regulating RANKL-mediated NFkappaB and MAPK signaling in RAW264.7 cells. *Pharmacol. Rep.* 67, 1264–1272. doi: 10.1016/j.pharep.2015.05.009

Oh, J. E., and Lee, H. K. (2014). Pattern recognition receptors and autophagy. *Front. Immunol.* 5:300. doi: 10.3389/fimmu.2014.00300

Otomo, C., Metlagel, Z., Takaesu, G., and Otomo, T. (2013). Structure of the human ATG12∼ATG5 conjugate required for LC3 lipidation in autophagy. *Nat. Struct. Mol. Biol.* 20, 59–66. doi: 10.1038/nsmb.2431

Ozcan, E., Saygun, N. I., Ilikci, R., Karslioglu, Y., Musabak, U., and Yesillik, S. (2016). Increased visfatin expression is associated with nuclear factor-kappa B and phosphatidylinositol 3-kinase in periodontal inflammation. *Clin. Oral Investig.* 21, 1113–1121. doi: 10.1007/s00784-016-1871-7

Panjamurthy, K., Manoharan, S., and Ramachandran, C. R. (2005). Lipid peroxidation and antioxidant status in patients with periodontitis. *Cell. Mol. Biol. Lett.* 10, 255–264.

Park, M. H., Jeong, S. Y., Na, H. S., and Chung, J. (2017). *Porphyromonas gingivalis* induces autophagy in THP-1-derived macrophages. *Mol. Oral Microbiol.* 32, 48–59. doi: 10.1111/omi.12153

Park, Y. D., Kim, Y. S., Jung, Y. M., Lee, S. I., Lee, Y. M., Bang, J. B., et al. (2012). *Porphyromonas gingivalis* lipopolysaccharide regulates interleukin (IL)-17 and IL-23 expression via SIRT1 modulation in human periodontal ligament cells. *Cytokine* 60, 284–293. doi: 10.1016/j.cyto.2012.05.021

Patil, V. S., Patil, V. P., Gokhale, N., Acharya, A., and Kangokar, P. (2016). Chronic periodontitis in type 2 diabetes mellitus: oxidative stress as a common factor in periodontal tissue injury. *J. Clin. Diagn. Res.* 10, BC12–BC16. doi: 10.7860/jcdr/2016/17350.7542

Pei, F., Lin, H., Liu, H., Li, L., Zhang, L., and Chen, Z. (2015). Dual role of autophagy in lipopolysaccharide-induced preodontoblastic cells. *J. Dent. Res.* 94, 175–182. doi: 10.1177/0022034514553815

Perrone, S., Bracciali, C., Di Virgilio, N., and Buonocore, G. (2016). Oxygen use in neonatal care: a two-edged sword. *Front Pediatr* 4:143. doi: 10.3389/fped.2016.00143

Pradeep, A. R., Rao, N. S., Bajaj, P., and Agarwal, E. (2013). 8-Isoprostane: a lipid peroxidation product in gingival crevicular fluid in healthy, gingivitis and chronic periodontitis subjects. *Arch. Oral Biol.* 58, 500–504. doi: 10.1016/j.archoralbio.2013.01.011

Roos, D., Van Bruggen, R., and Meischl, C. (2003). Oxidative killing of microbes by neutrophils. *Microbes Infect.* 5, 1307–1315. doi: 10.1016/j.micinf.2003.09.009

Saita, M., Kaneko, J., Sato, T., Takahashi, S. S., Wada-Takahashi, S., Kawamata, R., et al. (2016). Novel antioxidative nanotherapeutics in a rat periodontitis model: reactive oxygen species scavenging by redox injectable gel suppresses alveolar bone resorption. *Biomaterials* 76, 292–301. doi: 10.1016/j.biomaterials.2015.10.077

Sawamoto, Y., Sugano, N., Tanaka, H., and Ito, K. (2005). Detection of periodontopathic bacteria and an oxidative stress marker in saliva from periodontitis patients. *Oral Microbiol. Immunol.* 20, 216–220. doi: 10.1111/j.1399-302X.2005.00215.x

Schroder, K., Zhou, R., and Tschopp, J. (2010). The NLRP3 inflammasome: a sensor for metabolic danger? *Science* 327, 296–300. doi: 10.1126/science.1184003

Sezer, U., Cicek, Y., and Canakci, C. F. (2012). Increased salivary levels of 8-hydroxydeoxyguanosine may be a marker for disease activity for periodontitis. *Dis. Markers* 32, 165–172. doi: 10.1155/2012/215430

Shapira, L., Borinski, R., Sela, M. N., and Soskolne, A. (1991). Superoxide formation and chemiluminescence of peripheral polymorphonuclear leukocytes in rapidly progressive periodontitis patients. *J. Clin. Periodontol.* 18, 44–48. doi: 10.1111/j.1600-051X.1991.tb01118.x

Sheikhi, M., Gustafsson, A., and Jarstrand, C. (2000). Cytokine, elastase and oxygen radical release by Fusobacterium nucleatum-activated leukocytes: a possible pathogenic factor in periodontitis. *J. Clin. Periodontol.* 27, 758–762. doi: 10.1034/j.1600-051x.2000.027010758.x

Shibutani, S. T., Saitoh, T., Nowag, H., Munz, C., and Yoshimori, T. (2015). Autophagy and autophagy-related proteins in the immune system. *Nat. Immunol.* 16, 1014–1024. doi: 10.1038/ni.3273

Shin, J. E., Baek, K. J., Choi, Y. S., and Choi, Y. (2013). A periodontal pathogen Treponema denticola hijacks the Fusobacterium nucleatum-driven host response. *Immunol. Cell Biol.* 91, 503–510. doi: 10.1038/icb.2013.35

Sima, C., Aboodi, G. M., Lakschevitz, F. S., Sun, C., Goldberg, M. B., and Glogauer, M. (2016). Nuclear factor erythroid 2-related factor 2 down-regulation in oral neutrophils is associated with periodontal oxidative damage and severe chronic periodontitis. *Am. J. Pathol.* 186, 1417–1426. doi: 10.1016/j.ajpath.2016.01.013

Song, B., Zhou, T., Yang, W. L., Liu, J., and Shao, L. Q. (2016). Programmed cell death in periodontitis: recent advances and future perspectives. *Oral Dis.* 23, 609–619. doi: 10.1111/odi.12574

Souza, P. P., and Lerner, U. H. (2013). The role of cytokines in inflammatory bone loss. *Immunol. Invest.* 42, 555–622. doi: 10.3109/08820139.2013.822766

Stafford, P., Higham, J., Pinnock, A., Murdoch, C., Douglas, C. W., Stafford, G. P., et al. (2013). Gingipain-dependent degradation of mammalian target of rapamycin pathway proteins by the periodontal pathogen *Porphyromonas gingivalis* during invasion. *Mol. Oral Microbiol.* 28, 366–378. doi: 10.1111/omi.12030

Su, H., Gornitsky, M., Velly, A. M., Yu, H., Benarroch, M., and Schipper, H. M. (2009). Salivary DNA, lipid, and protein oxidation in nonsmokers with periodontal disease. *Free Radic. Biol. Med.* 46, 914–921. doi: 10.1016/j.freeradbiomed.2009.01.008

Su, R., Jin, X., Zhang, W., Li, Z., Liu, X., and Ren, J. (2017). Particulate matter exposure induces the autophagy of macrophages via oxidative stress-mediated PI3K/AKT/mTOR pathway. *Chemosphere* 167, 444–453. doi: 10.1016/j.chemosphere.2016.10.024

Takane, M., Sugano, N., Ezawa, T., Uchiyama, T., and Ito, K. (2005). A marker of oxidative stress in saliva: association with periodontally-involved teeth of a hopeless prognosis. *J. Oral Sci.* 47, 53–57. doi: 10.2334/josnusd.47.53

Takane, M., Sugano, N., Iwasaki, H., Iwano, Y., Shimizu, N., and 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. doi: 10.1902/jop.2002.73.5.551

Tamaki, N., Hayashida, H., Fukui, M., Kitamura, M., Kawasaki, K., Nakazato, M., et al. (2014). Oxidative stress and antibody levels to periodontal bacteria in adults: the Nagasaki Islands study. *Oral Dis* 20, e49–e56. doi: 10.1111/odi.12127

Tan, Y. Q., Zhang, J., and Zhou, G. (2016). Autophagy and its implication in human oral diseases. *Autophagy* 13, 225–236. doi: 10.1080/15548627.2016.1234563

Thomas, B., Madani, S. M., Prasad, B. R., and Kumari, S. (2014). Comparative evaluation of serum antioxidant levels in periodontally diseased patients: an interventional study. *Contemp. Clin. Dent.* 5, 340–344. doi: 10.4103/0976-237X.137938

Thummuri, D., Jeengar, M. K., Shrivastava, S., Nemani, H., Ramavat, R. N., Chaudhari, P., et al. (2015). Thymoquinone prevents RANKL-induced osteoclastogenesis activation and osteolysis in an *in vivo* model of inflammation by suppressing NF-KB and MAPK Signalling. *Pharmacol. Res.* 99, 63–73. doi: 10.1016/j.phrs.2015.05.006

Tooze, S. A., and Dikic, I. (2016). Autophagy captures the nobel prize. *Cell* 167, 1433–1435. doi: 10.1016/j.cell.2016.11.023

Trivedi, S., Lal, N., Mahdi, A. A., Mittal, M., Singh, B., and Pandey, S. (2014). Evaluation of antioxidant enzymes activity and malondialdehyde levels in patients with chronic periodontitis and diabetes mellitus. *J. Periodontol.* 85, 713–720. doi: 10.1902/jop.2013.130066

Tsai, C. C., Chen, H. S., Chen, S. L., Ho, Y. P., Ho, K. Y., Wu, Y. M., et al. (2005). Lipid peroxidation: a possible role in the induction and progression of chronic periodontitis. *J. Periodontal. Res.* 40, 378–384. doi: 10.1111/j.1600-0765.2005.00818.x

Tsuda, H., Ochiai, K., Suzuki, N., and Otsuka, K. (2010). Butyrate, a bacterial metabolite, induces apoptosis and autophagic cell death in gingival epithelial cells. *J. Periodont. Res.* 45, 626–634. doi: 10.1111/j.1600-0765.2010.01277.x

Turrens, J. F. (2003). Mitochondrial formation of reactive oxygen species. *J. Physiol. (Lond).* 552, 335–344. doi: 10.1113/jphysiol.2003.049478

Venditti, P., Di Stefano, L., and Di Meo, S. (2013). Mitochondrial metabolism of reactive oxygen species. *Mitochondrion* 13, 71–82. doi: 10.1016/j.mito.2013.01.008

Waddington, R. J., Moseley, R., and Embery, G. (2000). Reactive oxygen species: a potential role in the pathogenesis of periodontal diseases. *Oral Dis.* 6, 138–151. doi: 10.1111/j.1601-0825.2000.tb00325.x

Wang, G., Zhang, T., Sun, W., Wang, H., Yin, F., Wang, Z., et al. (2017). Arsenic Sulfide induces apoptosis and autophagy through the activation of ROS/JNK and suppression of Akt/mTOR signaling pathways in osteosarcoma. *Free Radic. Biol. Med.* 106, 24–37. doi: 10.1016/j.freeradbiomed.2017.02.015

Wang, Q., Sztukowska, M., Ojo, A., Scott, D. A., Wang, H., and Lamont, R. J. (2015). FOXO responses to *Porphyromonas gingivalis* in epithelial cells. *Cell. Microbiol.* 17, 1605–1617. doi: 10.1111/cmi.12459

Wei, D., Zhang, X. L., Wang, Y. Z., Yang, C. X., and 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. doi: 10.1111/j.1834-7819.2009.01123.x

Wright, C. J., Xue, P., Hirano, T., Liu, C., Whitmore, S. E., Hackett, M., et al. (2014). Characterization of a bacterial tyrosine kinase in *Porphyromonas gingivalis* involved in polymicrobial synergy. *Microbiologyopen* 3, 383–394. doi: 10.1002/mbo3.177

Xue, F., Shu, R., and Xie, Y. (2015). The expression of NLRP3, NLRP1 and AIM2 in the gingival tissue of periodontitis patients: RT-PCR study and immunohistochemistry. *Arch. Oral Biol.* 60, 948–958. doi: 10.1016/j.archoralbio.2015.03.005

Yang, Z., and Klionsky, D. J. (2010). Eaten alive: a history of macroautophagy. *Nat. Cell Biol.* 12, 814–822. doi: 10.1038/ncb0910-814

Yu, L., McPhee, C. K., Zheng, L., Mardones, G. A., Rong, Y., Peng, J., et al. (2010). Termination of autophagy and reformation of lysosomes regulated by mTOR. *Nature* 465, 942–946. doi: 10.1038/nature09076

Zhang, C. Z., Cheng, X. Q., Li, J. Y., Zhang, P., Yi, P., Xu, X., et al. (2016). Saliva in the diagnosis of diseases. *Int. J. Oral Sci.* 8, 133–137. doi: 10.1038/ijos.2016.38

Zhang, J., Kim, J., Alexander, A., Cai, S., Tripathi, D. N., Dere, R., et al. (2013). A tuberous sclerosis complex signalling node at the peroxisome regulates mTORC1 and autophagy in response to ROS. *Nat. Cell Biol.* 15, 1186–1196. doi: 10.1038/ncb2822

Zhang, J., Tripathi, D. N., Jing, J., Alexander, A., Kim, J., Powell, R. T., et al. (2015). ATM functions at the peroxisome to induce pexophagy in response to ROS. *Nat. Cell Biol.* 17, 1259–1269. doi: 10.1038/ncb3230

Zhang, L., Wang, K., Lei, Y., Li, Q., Nice, E. C., and Huang, C. (2015). Redox signaling: potential arbitrator of autophagy and apoptosis in therapeutic response. *Free Radic. Biol. Med.* 89, 452–465. doi: 10.1016/j.freeradbiomed.2015.08.030

Zhang, T., Andrukhov, O., Haririan, H., Muller-Kern, M., Liu, S., Liu, Z., et al. (2015). Total antioxidant capacity and total oxidant status in saliva of

periodontitis patients in relation to bacterial load. *Front. Cell. Infect. Microbiol.* 5:97. doi: 10.3389/fcimb.2015.00097

Zhao, Y., Qu, T., Wang, P., Li, X., Qiang, J., Xia, Z., et al. (2016). Unravelling the relationship between macroautophagy and mitochondrial ROS in cancer therapy. *Apoptosis* 21, 517–531. doi: 10.1007/s10495-016-1236-3

Zhong, Z., Sanchez-Lopez, E., and Karin, M. (2016). Autophagy, inflammation, and immunity: a troika governing cancer and its treatment. *Cell* 166, 288–298. doi: 10.1016/j.cell.2016.05.051

Zhou, R., Tardivel, A., Thorens, B., Choi, I., and Tschopp, J. (2010). Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *Nat. Immunol.* 11, 136–140. doi: 10.1038/ni.1831

Zhu, X. Q., Lu, W., Chen, Y., Cheng, X. F., Qiu, J. Y., Xu, Y., et al. (2016). Effects of *Porphyromonas gingivalis* lipopolysaccharidetolerized monocytes on inflammatory responses in neutrophils. *PLoS ONE* 11:e0161482. doi: 10.1371/journal.pone.0161482

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The Redox Balance in Erythrocytes, Plasma, and Periosteum of Patients with Titanium Fixation of the Jaw

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Titanium miniplates and screws are commonly used for fixation of jaw fractured or osteotomies. Despite the opinion of their biocompatibility, in clinical practice symptoms of chronic inflammation around the fixation develop in some patients, even many years after the application of miniplates and screws. The cause of these complications is still an unanswered question. Taking into account that oxidative stress is one of the toxic action of titanium, we have evaluated the antioxidant barrier as well as oxidative stress in the erythrocytes, plasma and periosteum covering the titanium fixation of the jaw. The study group was composed of 32 patients aged 20–30 with inserted miniplates and screws. The antioxidant defense: catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase-1 (SOD1), uric acid (UA), total antioxidant capacity (TAC), as well as oxidative damage products: advanced oxidation protein products (AOPP), advanced glycation end products (AGE), dityrosine, kynurenine, N-formylkynurenine, tryptophan, malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), total oxidant status (TOS), and oxidative status index (OSI) were evaluated. SOD1 activity (↓37%), and tryptophan levels (↓34%) showed a significant decrease while AOPP (↑25%), TOS (↑80%) and OSI (↑101%) were significantly elevated in maxillary periosteum of patients who underwent bimaxillary osteotomies as compared to the control group. SOD-1 (↓55%), TAC (↓58.6%), AGE (↓60%) and N-formylkynurenine (↓34%) was statistically reduced while AOPP (↑38%), MDA (↑29%), 4-HNE (↑114%), TOS (↑99%), and OSI (↑381%) were significantly higher in the mandibular periosteum covering miniplates/screw compared with the control tissues. There were no correlations between antioxidants and oxidative stress markers in the periosteum of all patients and the blood. As exposure to the Ti6Al4V titanium alloy leads to disturbances of redox balance in the periosteum surrounding titanium implants of the maxilla and the mandible so antioxidant supplementation should be recommended to the patients undergoing

treatment of dentofacial deformities with the use of titanium implants. The results we obtained may also indicate a need to improve the quality of titanium jaw fixations through increase of TiO_2 passivation layer thickness or to develop new, the most highly biodegradable materials for their production.

Keywords: antioxidants, dentofacial deformities, miniplates and screws, oxidative stress, titanium fixation

INTRODUCTION

Titanium and its alloys are commonly used for the production of medical implants. They have a broad range of applications in oral surgery and orthopedics as a bone fixation (plates, screws, rods, stabilizers, and wires), joint prostheses, dental implants and other devices used in reconstructive surgery (Borys et al., 2004). A common use of implants composed of titanium and its alloys results from good mechanical properties, resistant to corrosion and biocompatibility. Their higher biocompatibility in human tissues as compared to other metallic materials results from the presence of an inactive layer of titanium dioxide (TiO_2) on the implant surface, which should reduce the corrosion potential of the metal (Tsaryk et al., 2007). Despite the opinion of their biocompatibility, in clinical practice symptoms of chronic inflammation around the fixation develop in some patients, even many years after the application of miniplates and screws (Peters et al., 2007; Olmedo et al., 2008; Lee et al., 2013). It is unknown what changes in the tissues surrounding the implant lead to these complications, however, it is believed that one of them may be an increased production of free radicals and reactive nitrogen species by exposure to titanium implants (Mentus, 2004; Peters et al., 2007; Tsaryk et al., 2007; Olmedo et al., 2008).

To date, it is still unclear whether titanium implants induce oxidative stress, which is a situation in which the antioxidant barrier is insufficient to quench the excess production of the reactive oxygen species (ROS). A failure to neutralize ROS leads to cellular metabolism disorders and oxidative damage to major biomolecules and cellular structures, including lipids, proteins, DNA and carbohydrates (Lushchak, 2014).

The aim of the study was to exam the influence of titanium implants on the antioxidant defense and oxidative stress in the serum, erythrocytes and periosteum covering jaw bone fixation locations in patients treated for dentofacial deformities.

MATERIALS AND METHODS

Patients

All procedures included in this study were approved by the Bioethics Committee of the Medical University of Bialystok (permission number R-I-002/3/2-16). After the explanation of the nature, purpose and potential risk of the study, a written informed consent was obtained from each patient.

The study and control patients were operated on at the Department of Maxillofacial and Plastic Surgery at the Medical University in Bialystok, Poland (from 28.01.2016 to 21.01.2017).

The study group was composed of 32 patients who had previously implants placed (21 women and 11 men aged 20–30, mean age–25 years, 8 months). In the study group, the osteotomy segments in the mandible were fixed with a 5-hole miniplate and 4 screws on the right and left side—in total 2 miniplates + 8 screws; while in the maxilla on the right and left side—with two 4-hole miniplates and 6 screws (in total 4 plates and 12 screws; MEDGAL Sp. z o.o., Bialystok, Poland). The Bioethics Committee gave us approval to carry out the research within 1 year only. We have not always been able to complete the tissue sampling in the allotted time period of 1 year, therefore the control and the study groups consist of different patients.

Jaw fixations were removed from 12 to 30 months after insertion of implants. The control group (C) consisted of 24 generally healthy patients (11 women and 13 men) aged 21–28 (mean age of 23 and 3 months), whose periosteum and blood were taken on the day of surgery, prior to insertion of titanium miniplates and screws. Both the control and study patients were operated on due to class III dentofacial deformities (underdevelopment of the maxilla and hypertrophy of the mandible).

The inclusion criteria for patients in the study group was the presence of maxillary bone fixations after surgical correction of dentofacial deformities. The inclusion criteria for patients in the study group and the healthy control were: absence of any former treatment for bone fractures or previous jaw osteotomies with the use of titanium fixations; a non-inflammation-induced healing process starting from the point of inserting fixations until their removal; age of patients 20–30. Patients and healthy controls had $18.5 \leq \text{BMI} \geq 24.5$, were non- smokers and did not have any illnesses or a history of gastrointestinal disorders, hypertension, hyperlipidemia, liver or renal disease, diabetes, thyroid diseases, immunological disorders or other general diseases as well as periodontitis, gingivitis and active odontogenic infection foci. Patients and the control declared abstinence from alcohol or intoxicating drugs during the previous 2 months. The patients and the controls had meet the correct values of blood parameters (WBC 4.6–8.3 $\times$ $10^3/\mu$L, RBC 4.2–5.3 $\times$ $10^6/\mu$L, HGB 12.4–15.1 g/L, PLT 143-278 $\times$ $10^3/\mu$L; electrolytes level—sodium (Na 136.8–144.2 mmol/L) and potassium (K 3.7–4.8 mmol/L); Activated Partial Thromboplastin Time (APTT 24.3–34.1 sek), Prothrombin Time (PT 11.8–14.2 sek), International Normalized Ratio (INR 0.84–1.2), CRP (0.1–4.6 mg/L). For 1 month before the surgical procedure until the implant was removed, patients in the control and the study group had been on a diet containing 2,000 kcal including 55% carbohydrates, 15.5% of protein and 29.5% of fat. The diet was determined by a dietitian, and for the duration of the experiment the patients were left under his control. Exclusion criteria were: age below 20 and above 30; inflammatory complications and jaw synostosis disorders after operations on bimaxillary osteotomies; present or resolved systemic inflammation or within the oral cavity and coexistent

systemic illnesses; treatment with antibiotics, corticosteroids during the previous 12 months; operations for other reasons in the year preceding the research; operation due to jaw fractures and/or subject to osteotomies of these bones in the past; addiction to alcohol and/or drugs. Patients taking antioxidants, vitamins, and dietary supplements were also excluded.

Surgical Procedure

All surgical procedures were performed by one qualified surgeon (JB). Jaw fixations (miniplates and screws) were removed under local anesthesia (2% lignocaine with noradrenalin) between the 12th and the 30th month (approximately 19 months on average). The research material consisted of a small fragments of periosteum (gray-pigmented) adhering to the titanium miniplate excised as a standard procedure during the removal of the maxillary bone fixations (Max1, Man1) or in the case of the control group- healthy periosteum taken separately from maxilla (Max C) and mandible (Man C) during bimaxillary osteotomy before implantation of the miniplates and screws. The gray-pigmented periosteum was aseptic (data not shown). Before the surgery in the fasting patients and controls, 10 mL of venous blood samples were collected in ethylenediaminetetracetic acid (EDTA) tubes and centrifuged 1500 × g at 4°C for 10 min to separate plasma and erythrocytes. Erythrocytes were washed three times in cold saline (0.9% NaCl) and hemolyzed by the addition of a nine-fold volume of cold phosphate buffer (50 mM, pH 7.4). In order to prevent sample oxidation and proteolysis, 10 μL 0.5 M BHT (butylated hydroxytoluene, BHT, Sigma-Aldrich, Germany) in acetonitrile and the protease inhibitor (1 tablet/10 mL of the buffer) (Complete Mini Roche, France) were added per 1 mL of plasma and erythrocytes and stored at −80°C in until assayed.

Preparation of Tissue Homogenates

The tissues were removed, immediately frozen in liquid nitrogen and stored at −80°C until use. They were rinsed in ice-cold PBS (0.02 mol L^{-1}, pH 7.0–7.2) to be cleaned from any remaining blood elements, weighted (laboratory weight KERN PLI 510-3M), placed in glass tubes, minced into small pieces, then diluted in ice-cold PBS (1:13) and homogenized with a homogenizer (Omni TH, Omni International, Kennesaw, GA, USA) on ice and sonicated with an ultrasonic cell disrupter (1800 J per sample, 20 s × 3 on ice, UP 400S; Hielscher, Teltow, Germany) for further cell membrane breakdown. To all samples the protease inhibitor (1 tablet/10 mL of the buffer) (Complete Mini Roche, France) as well as BHT (10 μL 0.5 M BHT in acetonitryle per 1 mL of the buffer; Sigma-Aldrich, Germany) were added. Homogenates were centrifuged for 10 min at 3,500 × g. The resulting supernatants were analyzed on the same day.

Biochemical Analysis

Determination of Enzymatic and Non-enzymatic Antioxidants

Catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GPx, EC 1.11.1.9), copper and zinc-containing superoxide dismutase 1 (SOD-1, E.C. 1.15.1.1) and protein were determined in the erythrocytes and tissue homogenates, uric acids (UA), total antioxidant capacity (TAC) and protein were analyzed in the plasma and tissue homogenates.

CAT activity was assessed colorimetrically measuring the decrease in absorbance at 240 nm as a consequence of hydrogen peroxide (H_2O_2) consumption (Aebi, 1984).

GPx activity was determined colorimetrically measuring the conversion of NADPH to NADP+ at 340 nm. One unit of GPx activity was defined as the amount of enzyme, which catalyzes the oxidation of 1 millimole NADPH/1 min (Paglia and Valentine, 1967).

SOD1 activity was estimated colorimetrically based on the ability of SOD to inhibit the autooxidation of epinephrine at pH 10.2. One unit of SOD activity was defined as the amount of enzyme, which inhibits epinephrine oxidation by 50% (Misra and Fridovich, 1972).

UA was measured colorimetrically using a commercial kit QuantiChromTM Uric Acid Assay Kit DIUA-250 (BioAssay Systems, Harward, CA, USA). This method is based on the formation of a blue complex with iron, which is determined at a wavelength 490 nm.

The concentration of TAC was estimated in triplicate samples by 2,2′-azinobis(3-ethylbenzo-thiazoline-6-sulfonate) ($ABTS^{\cdot+}$)-based colorimetric method described by (Erel, 2004). Changes in absorbance of the reaction solution was measured at 660 nm and the results were expressed as μmol Trolox equivalent/100 of the total protein.

All assays were performed in a duplicate samples, except for the TAC determination (see above) and converted to mg of the total protein.

Determination of Oxidative Stress Markers

Oxidative modifications products were assessed both in the plasma and tissue homogenates.

Advanced Oxidation Protein Products (AOPP) were estimated colorimetrically using a method Kalousová et al. (2002), which measures the total iodide ion oxidizing capacity of the samples. Absorbance at 340 nm was measured immediately by Infinite M200 PRO Multimode Microplate Reader, Tecan.

Advanced glycation end products (AGE) were estimated spectrofluorimetrically at the excitation and emission wavelengths of 350 and 440 nm using Infinite M200 PRO Multimode Microplate Reader, Tecan. Results were expressed as fluorescence/mg of the total protein.

The content of dityrosine, kynurenine, N-formylkynurenine and tryptophan was analyzed spectrofluorimetrically on 96-well microplates measuring the characteristic fluorescence at 330/415, 365/480, 325/434, and 95/340 nm respectively by Infinite M200 PRO Multimode Microplate Reader, Tecan. Results were expressed as fluorescence/mg of the total protein.

Lipid peroxidation was estimated colorimetrically using the Thiobarbituric Acid Reactive Substances (TBARS) method for measuring a malondialdehyde (MDA). 1,3,3,3 tetraethoxypropane was used as a standard (Buege and Aust, 1978).

The concentration of 4-hydroxynonenal (4-HNE) protein adducts was measured by commercial enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's

instructions (OxiSelect™ HNE Adduct Competitive ELISA Kit, Cell Biolabs, Inc. San Diego, CA, USA). The quantity of 4-HNE protein adducts was determined colorimetrically from a calibration curve for 4-HNE-BSA.

Total oxidant status (TOS) was measured colorimetrically based on the oxidation of ferrous ion (Fe^{2+}) to ferric ion (Fe^{3+}) in the presence of oxidants comprised in a sample (Erel, 2005). Changes in absorbance of the reaction solution were measured bichromatically (560/800 nm) in triplicate samples. The results were expressed as micromolar hydrogen peroxide (H_2O_2) equivalent per mg of the total protein (μmol H_2O_2 Equiv/mg of the total protein).

Oxidative stress index (OSI) was calculated according to the formula: OSI = TOS/TAC·100% (Knaś et al., 2016).

The total protein content was determined colorimetrically using the bicinchoninic acid assay (BCA assay) with bovine serum albumin (BSA) as a standard (Thermo Scientific PIERCE BCA Protein Assay Kit, Rockford, IL, USA).

All assays were performed in duplicate samples, except for the TOS determination (see above) and converted to mg of the total protein. Graphical representation of the experiment was presented on **Figure 1**.

Statistical Analysis

The data were reported as median, minimum and maximum. All analyses were performed using Statistica 12.0 (Statsoft, Cracow, Poland). The Kolmogorov-Smirnov test showed no normal distribution of the obtained results, which was the reason for using nonparametric methods. The control and Max1, Man1 groups were compared using the non-parametrical U Mann-Whitney test. The associations between the antioxidants, oxidative stress markers in the tissue homogenates and plasma antioxidants and oxidative stress markers concentrations as well as protein concentrations and the time elapsed since the surgery and removal of the miniplates/screw were analyzed using the Spearman Correlation Coefficient. Differences with $p \leq 0.05$ were considered significant.

RESULTS

Maxilla

The activity of SOD1 (↓37%) was significantly decreased in tissue homogenates (maxillary gray- pigmented periosteum covered miniplates or screws) of patients who undergo bimaxillary osteotomies compared to the control periosteum ($p = 0.0003$). The activity of CAT and GPx and concentrations of TAC, UA as well as protein concentration did not differ between gray-pigmented periosteum of Max1 and the periosteum of the maxilla of the control group (**Figure 2**).

Regarding oxidative stress markers, only the concentration of tryptophan were decreased in the gray- pigmented periosteum of Max1 group compared to the periosteum of the maxilla of control (↓34%, $p = 0.006$). The concentration of AOPP (↑25%), TOS (↑80%) as well as OSI (↑98%) were significantly elevated in homogenates of maxillary periosteum taken from region of miniplates/screw as compared to the periosteum of the maxilla of the control ($p = 0.035$, $p = 0.01$, and $p = 0.001$, respectively). All remaining parameters of oxidative stress were similar in periosteum of Max1 and the control patients (**Figure 3**).

Mandibula

As presented in **Figure 2**, with the exception of SOD1 and TAC, most of the examined parameters of antioxidant barrier, showed similar pattern in homogenates of mandibular periosteum

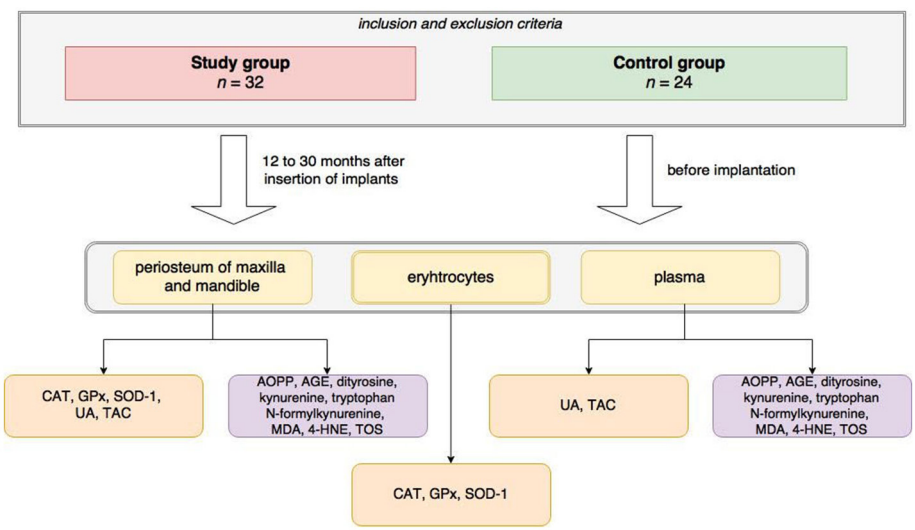

FIGURE 1 | Graphical representation of the experiment scheme. In the research material (periosteum of maxilla and mandible, erythrocytes and plasma) in the study and control group were evaluated: activity/concentrations of some antioxidants (SOD-1, superoxide dismutase-1; GPx, glutathione peroxidase; CAT, catalase; UA, uric acid; TAC, total antioxidant capacity) as well as oxidative modification products (AGE, advanced glycation end products; AOPP, advanced oxidation protein products, MDA, malondialdehyde, TOC, total oxidant capacity).

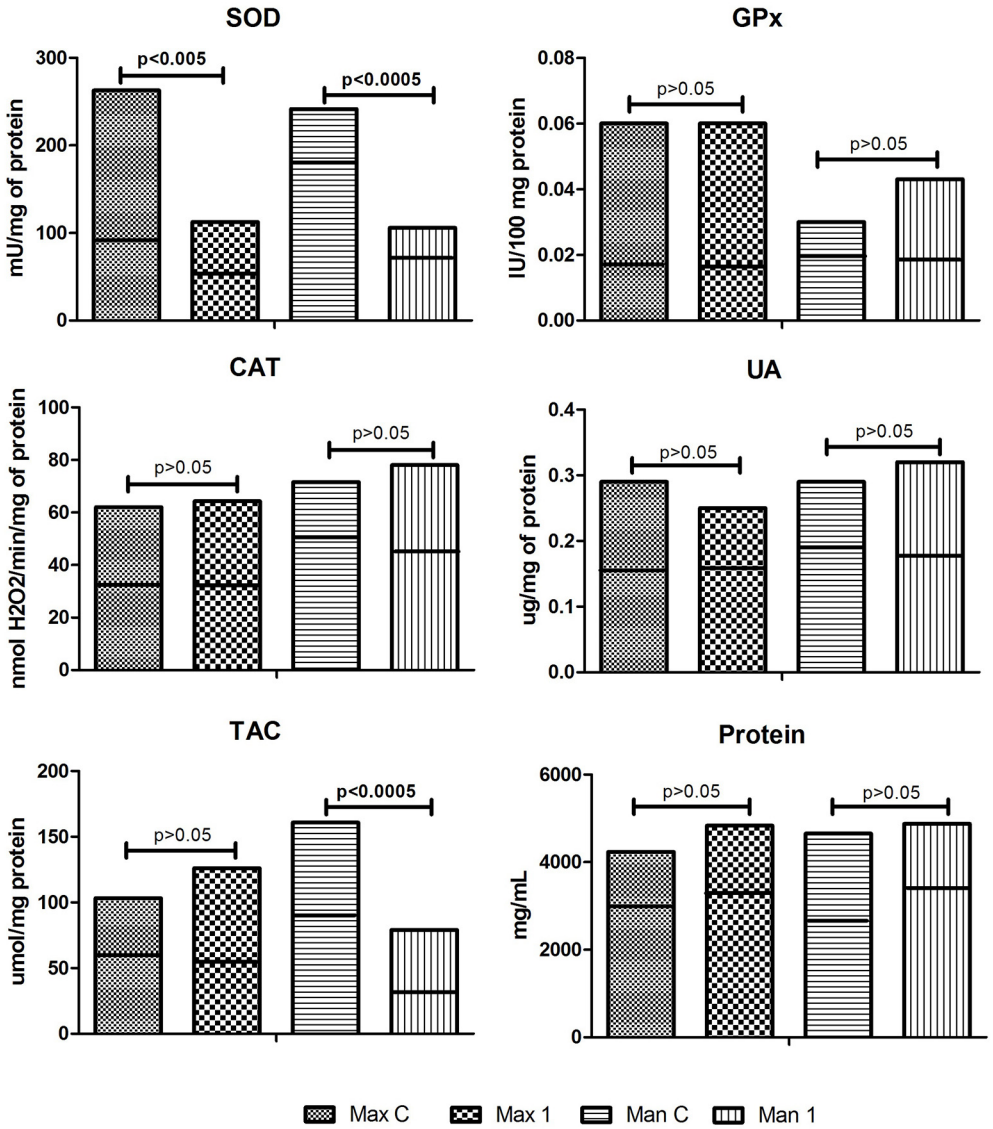

FIGURE 2 | Enzymatic and non-enzymatic antioxidants and protein concentration in the control and examined tissue homogenates. Max C, maxilla control; Man C, mandibula controll; Max 1, maxilla of the study group; Man 1, mandibula of the study group; SOD, superoxide dismutase, GPx, glutathione peroxidase, CAT, catalase, UA, uric acid, TAC, total antioxidant capacity. Horizontal line on the chart indicated the median.

covered miniplates/screw compared with the control tissue. Man1: GPx activity, UA, and protein concentration were comparable with the results of the control. SOD1 activity (↓55%) and TAC concentration (↓58.6%) in the homogenates of mandibular periosteum were significantly downregulated as compared to the control ($p = 0.0009$ and $p = 0.0007$, respectively).

Man1 group significantly decreased concentration of AGE (↓60%) and N-formylkynurenine (34%) when compared to the control group ($p = 0.008$ and $p = 0.04$, respectively). AOPP(↑38%), MDA (↑29%), 4-HNE protein adduct (↑114%), TOS (↑99%) concentrations as well as OSI (↑250%) were significantly elevated in the periosteum of Man1 patients as compared to the control group ($p = 0.03$, $p = 0.04$, $p = 0.0002$, $p = 0.0005$, $p = 0.0005$) (**Figure 3**).

Both Man1 and control groups showed similar tryptophan, kynurenine, ditirosine concentrations in their periosteum (**Figure 3**).

Blood Antioxidants Barrier and Oxidative Stress Markers

As presented in **Tables 1, 2**, with the exception of plasma AOPP in the study group, most of the examined parameters of antioxidant barrier and oxidative stress markers, showed a similar pattern in blood pellets and plasma of the control and experimental groups. We observed a significant decrease in

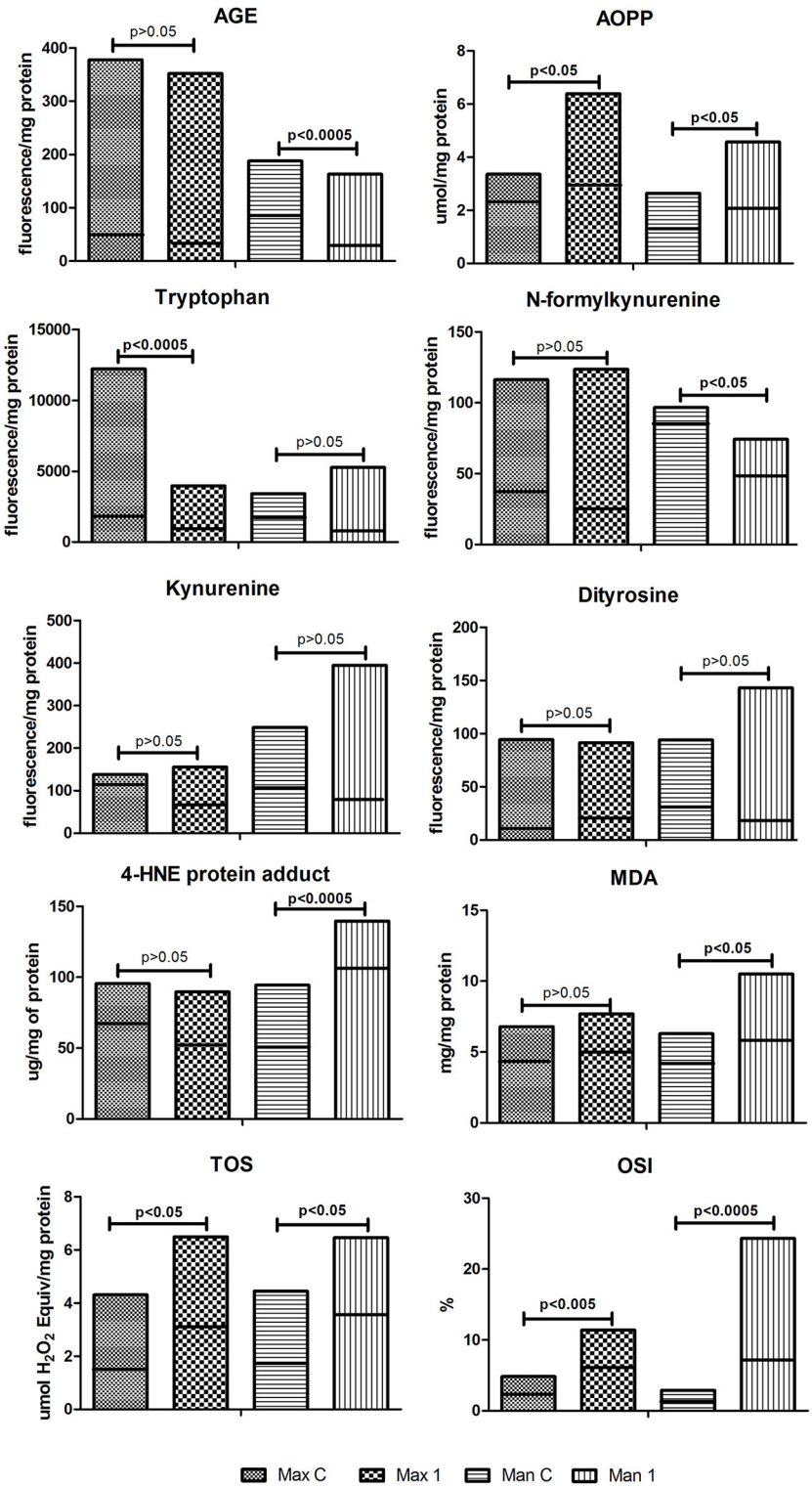

FIGURE 3 | Oxidative modification products and markers of oxidative stress in the control and examined tissue homogenates. Max C, maxilla control; Man C, mandibula control; Max 1, maxilla of the study group; Man 1, mandibula of the study group; AGE, advanced glycation end products; AOPP, advanced oxidation protein products; MDA, malondialdehyde; TOC, total oxidant capacity; OSI, oxidative status index. Horizontal line on the chart indicated the median.

TABLE 1 | Enzymatic, non-enzymatic antioxidants and protein concentration in blood cells or plasma of the control and examined patients.

	C $n = 24$, M(min–max)	Study group $n = 32$, M(min–max)
SOD mU/mg of protein	145.982 (102.037–209.085)	154.859 (98.854–211.821)
GPx IU/100 mg protein	0.013(0.011–0.018)	0.013(0.010–0.016)
CAT nmol H_2O_2/min/mg of protein	7.765(2.144–14.875)	6.951(1.095–17.758)
Plasma UA (μg/mg of protein)	0.169(0.071–0.256)	0.162(0.050–0.222)
Plasma TAC μmol/mg protein	79.97(63–109.06)	79.71(66.3–109.28)
Protein mg/mL	4,570.750 (3,576.900–5,514.300)	4,662.400 (3,596.800–5,317.600)

SOD, superoxide dismutase; GPx, glutathione peroxidase; CAT, catalase; UA, uric acid; TAC, total antioxidant capacity; BC, M (min-max), median (minimum-maximum); C, control group.

TABLE 2 | Oxidative modification products and markers of oxidative stress in the plasma of the control and examined patients.

	C $n = 24$, M(min–max)	Study group $n = 32$, M(min–max)
AGE fluorescence/mg protein	493.629 (311.700–690.682)	536.300 (324.161–734.959)
AOPP umol/mg protein	0.399(0.223–0.722)	0.356(0.185–0.546) $\downarrow^x$
Tryptofan fluorescence/mg protein	6,753.227 (5,412.820–8,678.744)	6,436.073 (5,063.058–8,234.360)
N-formylkynurenine fluorescence/mg protein	386.517 (239.529–582.396)	438.406 (205.043–644.803)
Kynurenine fluorescence/mg protein	697.819 (504.000–932.511)	690.890 (354.315–974.361)
Dityrosine fluorescence/mg protein	564.164 (357.300–754.105)	553.884 (285.143–794.734)
4-HNE protein adduct μg/mg protein	26.51(19.7–56.23)	28.51(27.17–46.03)
MDA mg/mg protein	4.836(2.559–9.270)	5.430(2.272–9.823)
TOC μmol H_2O_2 Equiv/mg protein	0.08(0.005–1.054)	0.079(0.01–0.57)
OSI %	0.093(0.006–1.2)	0.092(0.016–0.53)

AGE, advanced glycation end products; AOPP, advanced oxidation protein products; MDA, malondialdehyde; TOC, total oxidant capacity; OSI, oxidative status index; M (min–max), median (minimum-maximum); C, control group.
$\downarrow^x$- significant decrease, with $p < 0.05$ between C and study group.

plasma AOPP concentrations ($p = 0.01$) as compared to the control (**Table 2**).

Correlations

There were no correlations between antioxidants and oxidative stress markers in periosteum of Max1, Man1, and the plasma. There was no correlation between antioxidants and oxidative stress markers in periosteum of Max1, Man1, and age and BMI index of the patients. There were no correlations between antioxidants and oxidative stress markers in periosteum of Max1, Man1, and the time elapsed since the surgery and removal of the miniplates/screws. There was correlation between tryptophan and protein concentrations in periosteum of Max 1 group ($p = 0.01$, $r = -0.56$).

DISCUSSION

In the present study we showed that exposure to the Ti6Al4V titanium alloy leads to disrupted redox balance in the periosteum surrounding titanium implants of the maxilla and the mandible, however greater weakening of antioxidant systems, ROS generation and greater intensity of oxidative modifications were observed in the mandibular periosteum as opposed to the maxillary periosteum. We also proved that oxidative damage to the periosteum of the maxilla and mandible exposed to Ti6Al4V does not depend on the time elapsed since the surgery and removal of the miniplates/screw as well as that chronic exposition of the periosteum of the maxilla and mandible to Ti6Al4V does not influence the general redox balance.

The surface of titanium implants for numerous applications (joints prosthesis, bone fixation) is covered with a layer of titanium dioxide (titanium dioxide layer), which is responsible for the biocompatibility of the implant and reduces the corrosion potential of the metal (corrosion resistance and biocompatibility) (Tengvall et al., 1989; Tsaryk et al., 2007). However, in the human body, as a result of mechanical friction (metal on tissues, metal on metal) and electrochemical phenomena, the TiO_2 gets damaged, which leads to corrosion processes and the accumulation of wear debris in the region of implantation, plasma (Hallab et al., 2000), as well as in distant organs: lymph nodes, the spleen and liver (Case et al., 1994; Hallab et al., 2000; Urban et al., 2000). Titanium release is a result of the anodic corrosion process, taking place in the TiO_2 layer. The consequence of the accumulation of titanium ions is their phagocytosis by macrophages. Phagocytosis is linked to stimulation of respiratory processes and the formation of reactive oxygen species by activation of NADPH oxidase (Federico et al., 2007). Generated large quantities of superoxide anion $O_2^{\cdot-}$ enhance the secretion and the activity of IL6 (interleukin 6) as well as monocyte chemoattractant protein (MCP-1 protein), which promotes the infiltration of monocytes

and subsequent ROS formation (Sung et al., 2002). Moreover, the cathodic part of the corrosion of titanium implants, at physiological pH, reduces oxygen to hydrogen peroxide, which indeed is not a free radical, but has oxidizing ability of both proteins and lipids (Haliwell et al., 2000). Hydrogen peroxide may react with the TiO_2-layer producing highly reactive and the most dangerous reactive oxygen species- hydroxyl radical (Lee et al., 2005). Evidence showed that an increase in ROS formation may generate a situation in which the processes of bone formation are disturbed, and the processes responsible for bone resorption are enhanced. This also result in osteolysis around the implants, and consequently lead to an aseptic loss of the implant. However, it is still not known whether oxidative stress is induced under conditions of prolonged exposure to titanium (Hallab et al., 2000; Kinov et al., 2006; Tsaryk et al., 2007) in the periosteum of the mandible and the maxilla.

Among 32 patients undergoing double-stranded osteotomy, 18 had decided to remove jaw joints mainly due to the desire to get rid of non-functional implants, which could in the future be the cause of artifacts that would hamper proper assessment of CT images, MRI, and the risk of foreign body reactions. Another reason for the removal of miniplates and screws was discomfort connected with palpably felt fixations (6 patients), increased cold sensitivity (4 patients) and planned implant treatment of lost teeth (4 patients). During removal of the fixations the presence of gray- pigmented periosteum covering the miniplates and screws (**Supplementary Image 1**) was observed. Our clinical observations and literature data on the impact of metallic products on the surrounding tissues prompted us to evaluate the redox balance parameters in tissues surrounding titanic jaw fixtures.

It was documented that under a ROS increase, the level/activity of antioxidants may be increased, decreased or unchanged, which depends on the efficiency of a given tissue in fighting ROS and the level of ROS generation. Thus, both situations in which the increase in ROS generation (↑ TOS- 99% mandible, 80% maxilla) is accompanied by: a significant reduction in TAC concentrations, with unchanged concentrations/activity of other antioxidants (except for reduced activity of SOD 1) in the periosteum of the mandible as well as a lack of observed changes (except for reduced activity of SOD 1) of the antioxidative barrier of the periosteum of the maxilla exposed to titanium ions are equivalent to a shifted oxidative/antioxidative balance in the gray- pigmented periosteum of both the mandible and maxilla toward the oxidative status. However the antioxidant system of the mandible seems to be more affected.

A significant reduction of SOD1 activity in periosteum of Max1 and Man1 vs. appropriate controls, is in agreement with the results of others (Case et al., 1994; Hallab et al., 2000; Urban et al., 2000; Tsaryk et al., 2007; Saquib et al., 2012; Lee et al., 2013) and could have serious consequences for the process of bone healing. The non-neutralized superoxide anion could bind to the nitric oxide (NO) (Reynolds et al., 2017), harming its action on endothelial cells, which could have a negative effect on the neoangiogenesis process, and thus differentiation of osteopregenitor cells in osteoblasts at the fixation site.

The weakening of antioxidant response of the cell/tissue leads to oxidative modification of cellular components and it is evidenced by enhanced level of oxidatively modified cellular constituents or increased OSI (Sen et al., 2014; Knaś et al., 2016; Kołodziej et al., 2017; Maciejczyk et al., 2017).

Our study showed that both gray-pigmented periosteum of the maxilla and mandible exhibit greater susceptibility of their cellular elements to oxidative damage when exposed to titanium oxide *vs*. appropriate controls, regardless of the time elapsed between the insertion of the implant and its removal. It should be noticed, however, that greater diversity and intensity of oxidative modifications were observed in the periosteum of the mandible than in the periosteum of the maxilla. Our results showed a significant increase only in AOPP (↑25%) concentrations in the gray-pigmented periosteum of the maxilla as compared to the control, whereas in gray- pigmented periosteum of the mandible, we observed a significant increase in AOPP (38%) and in MDA (29%) and 4-HNE protein adduct (114%) as compared to the periosteum of the mandible collected before insertion of titanium implants. The intensity of oxidative stress determined by the OSI also confirmed that the periosteum covering the implants in the mandible (↑OSI 250%) is more exposed than in the maxilla (↑OSI 98%) to an oxidant attack generated if exposed to the Ti6Al4V titanium alloy. The observed inability to respond effectively to ROS input could have a negative effect on bone healing. One important factor of bone healing process is the differentiation of osteoprogenitor cells into osteoblasts and synthesis of organic substance, i.e., mostly collagen and the components of ground substance of the organic stroma of the bone (Borys et al., 2004). ROS reactions with proteins lead to irreversible changes in the structure of oxidized proteins as well as to a loss of their biological function. In the aspect of bone healing, this may result in the formation of an incorrectly built organic part of the bone tissue with poor mechanical resistance. What is more, Sheikhi et al. (2001) provide evidence that oxidized fatty acids stimulate adipogenesis and suppress osteoblastogenesis and directly stimulate formation as well as activity of osteoclasts (Sheikhi et al., 2001). It is very likely that intensive oxidative protein modification, expressed by elevated AOPP and also a greater number of lipid peroxidation products may interfere with the metabolism of the organic part of bone tissue and cause apertures of the mandible fracture that we observed in x-ray images of some patients (data not published) 14–18 months after the implantation.

This experiment does not explain the difference in the antioxidative response and susceptibility to oxidative damage. In our research, we have confirmed that the gray color of the periosteum was caused by titanium ion incrustation, what is more, we have observed that titanium ion concentrations were significantly higher in the mandible than in the maxilla (work in review). The explanation of these observations exceeds the scope of current work and is not explained in the available literature. We can only assume that the latter result is the result of different "motor function" of both analyzed bones. Among bones of the facial part of the skull, the mandible is the only moveable bone whose movement is controlled by strong muscles. Micromovements of the fixed osteotomy

fragments caused by mandible movement may increase the phenomenon of friction (fretting) between the screws and the miniplate (metal on metal corrosion), which increases the corrosion of titanium elements and the release of titanium ions into the periosteum surrounding the implant and contributes to higher ROS generation, which in the situation of observed inefficiency of antioxidative systems results in greater intensity of oxidative damage as compared to the maxilla. We anticipate that antioxidant supplementation would be helpful in this group of patients, which could compensate for oxidant/antioxidant disturbances at the implantation site, and thus prevent cell damage by oxidative processes.

Kynurenic acid and N-formylkynurenine belong to the primary biologically- active tryptophan metabolites that are formed during enzymatic reactions in the kynurenine pathway. The results of our research reveal that the kynurenine pathway is not activated in the process of bone healing in the case of titanium implant fixation, which is advantageous from the point of view of the bone healing process. Tryptophan degradation products in the kynurenine pathway inhibit osteoprogenitor cell differentiation, which results in inhibition proliferation and differentiation of osteoblasts and impairs bone healing (Sas et al., 2007). Apart from reduced concentrations of N-formylkynurenine in the mandibular periosteum, which probably results from oxidative modifications of 2,3-dioxygenase or indoleamine 2,3-dioxygenase, which convert tryptophan to N-formylkynurenine and reduced tryptophan concentrations in the maxillary periosteum, we did not observe any significant changes in the kynurenine pathway. The negative correlation between the tryptophan content and protein concentrations in homogenate of the gray- pigmented periosteum of the maxilla may suggest the use of tryptophan in the process of the protein part formation in the bone tissue remodeling process.

The absence of determinations of titanium concentrations in the blood does not allow us to conclude, whether it is released into the human blood system as it was observed in the experimental models (Olmedo et al., 2008; El-Shenawy et al., 2012). However, we could claim that chronic exposure to titanium implants in the jaw did not cause the general redox disturbances, which is obviously an advantageous phenomenon considering the general health aspect.

While analyzing the results obtained, one needs to consider the limited panel of analyses used in the experiment. Determination of other antioxidants or oxidative stress markers or the use of other analytical methods may result in obtaining different results and conclusions. It should be also underlined that MDA was measured via its reaction with tiobarbituric acid (TBA), which is not specific for lipids only and many amino acids, carbohydrates and aldehydes may react with TBA under the assay conditions. A weakness of this study is also the small size of the groups; however, it should be emphasized that that the experiment included all patients meeting the inclusion criteria reporting operative treatment for class III dentofacial deformities or to continue this treatment (removal of jaw fixations). The fact that our research qualified patients within the same age group—20–30 years can be regarded as a strength of the experiment. At this stage of life, a human organism reaches its peak bone mass, and the processes of build-up and resorption of bone tissue are balanced. It should be also underlined that in a clinical trial in a significant majority of the treated patients redox balance disorders in the periosteum surrounding titanium implants in the jaw are difficult to detect with currently available non-invasive diagnostic methods (clinical and biochemical blood tests, conventional radiological picture–**Supplementary Image 2**) due to their subclinical nature. However, our results indicate a persistence of oxidative stress in patients around the jaw bone fixation regardless of the time elapsed since the operation face skeletal defects. Aseptic chronic inflammation resulting primarily in response to the consumption of the products introduced into the body of metallic bodies, may cause osteolysis around the implant process and lead to a loss e.g., prosthetic joints or bone fractures.

CONCLUSIONS

1. Exposure to the Ti6Al4V titanium alloy leads to oxidant/antioxidant disturbances as well as oxidative damage to the periosteum surrounding the titanium implants of the maxilla and the mandible, although no clinical signs are observed.
2. Antioxidants supplementation (e.g., specific diet: green tea, aronia and blueberries juice, cocoa, curcumin, nuts, tomato, red vine etc. or spirulin, vitamin E, C supplementation) should be recommended to the patients undergoing treatment of skeletal defects with the use of titanium implants, as it could alleviate and/or prevent the damaging effects of oxidative stress in periosteum surrounding the implants.
3. The obtained results suggest sustained presence of oxidative stress among patients in the area of jaw bones fixations, independently of time, that has passed from the surgery of dentofacial deformities. It may indicate a need to improve the quality of used jaw bone fixations through increase of TiO_2 passivation layer thickness in miniplates and screws in the process of hard anodizing or a need to search for new materials to produce fixations, preferably biodegradable in tissues of human organism. However, this requires further research.

AUTHOR CONTRIBUTIONS

We declare that the paper "Antioxidant response and oxidative stress in patients with titanium fixation of the jaws JB, MM, AK, BA, WR, PZa, DW, JŁ, and AZ has not been published before. The paper is not under consideration for publication anywhere else and it was read and approved by all co- authors. All authors agree to the submission of the manuscript to the Frontiers in Physiology. JB: conceptualized, collection of the material, interpreted of data, wrote of the manuscript. MM: conceptualized, did laboratory determination, did performance of the graphic part of the manuscript. AK: conceptualized, interpreted of data, final approval of the version to be published. BA: conceptualized, did literature survey. WR: did statistical analysis, EJ: final approval of the version to be published. DW: did literature survey, final approval of the version to be published.

PZa: collection of the material. JŁ: did literature survey, final approval of the version to be published. PŻu: final approval of the version to be published, English correction. AZ: conceptualized, did laboratory determination, interpreted of data, wrote of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2017.00386/full#supplementary-material

Supplementary Image 1 | The gray- pigmented periosteum.

Supplementary Image 2 | X ray picture of the patients 23 months after jaw fixation (Siemens Aristos VB20D, 55kV).

REFERENCES

Aebi, H. (1984). Catalase *in vitro*. *Methods Enzymol.* 105, 121–126. doi: 10.1016/S0076-6879(84)05016-3

Borys, J., Grabowska, S. Z., Antonowicz, B., Dryl, D., Citko, A., and Rogowski, F. (2004). Collagen type I and III metabolism in assessment of mandible fractures healing. *Adv. Med. Sci.* 49, 237–245.

Buege, J. A., and Aust, S. D. (1978). Microsomal lipid peroxidation. *Methods Enzymol.* 52, 302–310. doi: 10.1016/S0076-6879(78)52032-6

Case, C. P., Langkamer, V. G., James, C., Palmer, M. R., Kemp, A. J., Heap, P. F., et al. (1994). Widespread dissemination of metal debris from implants. *J. Bone Joint Surg. Br.* 76, 701–712.

El-Shenawy, N. S., Mohsen, Q., and Fadl-Allah, S. A. (2012). Oxidative stress and antioxidant responses of liver and kidney tissue after implantation of titanium or titanium oxide coated plate in rat tibiae. *J. Mater. Sci. Mater. Med.* 23, 1763–1774. doi: 10.1007/s10856-012-4648-9

Erel, O. (2004). A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clin. Biochem.* 37, 227–285. doi: 10.1016/j.clinbiochem.2003.11.015

Erel, O. (2005). A new automated colorimetric method for measuring total oxidant status. *Clin. Biochem.* 38, 1103–1111. doi: 10.1016/j.clinbiochem.2005.08.008

Federico, A., Morgillo, F., Tuccillo, C., Ciardiello, F., and Loguercio, C. (2007). Chronic inflammation and oxidative stress in human carcinogenesis. *Int. J. Cancer* 121, 2381–2386. doi: 10.1002/ijc.23192

Haliwell, B., Clement, M. V., and Long, L. H. (2000). Hydrogen peroxide in the human body. *FEBS Lett.* 486, 10–13. doi: 10.1016/S0014-5793(00)02197-9

Hallab, N. J., Jacobs, J. J., Skipor, A., Black, J., Mikecz, K., and Galante, J. O. (2000). Systemic metal-protein binding associated with total joint replacement arthroplasty. *J. Biomed. Mater. Res.* 49, 353–361. doi: 10.1002/(SICI)1097-4636(20000305)49:3<353::AID-JBM8>3.0.CO;2-T

Kalousová, M., Skrha, J., and Zima, T. (2002). Advanced glycation end-products and advanced oxidation protein products in patients with diabetes mellitus. *Physiol. Res.* 51, 597–604.

Kinov, P., Leithner, A., Radl, R., Bodo, K., Khoschsorur, G. A., Schauenstein, K., et al. (2006). Role of free radicals in aseptic loosening of hip arthroplasty. *J. Orthop. Res.* 24, 55–62. doi: 10.1002/jor.20013

Knaś, M., Maciejczyk, M., Daniszewska, I., Klimiuk, A., Matczuk, J., Kołodziej, U., et al. (2016). Oxidative damage to the salivary glands of rats with streptozotocin-induced diabetes-temporal study: oxidative stress and diabetic salivary glands. *J. Diabetes Res.* 2016:13. doi: 10.1155/2016/4583742

Kołodziej, U., Maciejczyk, M., Miąsko, A., Matczuk, J., Knaś, M., Żukowski, P., et al. (2017). Oxidative modification in the salivary glands of high fat-diet induced insulin resistant rats. *Front. Physiol.* 8:20. doi: 10.3389/fphys.2017.00020

Lee, M. C., Yoshino, F., Shoji, H., Takahashi, S., Todoki, K., Shimada, S., 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. doi: 10.1177/154405910508400213

Lee, Y. H., Bhattarai, G., Park, I. S., Kim, G. R., Kim, G. E., Lee, M. H., et al. (2013). Bone regeneration around N-acetyl cysteine-loaded nanotube titanium dental implant in rat mandible. *Biomaterials* 34, 10199–10208. doi: 10.1016/j.biomaterials.2013.08.080

Lushchak, V. L. (2014). Classification of oxidative stress based on its intensity. *EXCLI J.* 13, 922–937.

Maciejczyk, M., Mikołuc, B., Pietrucha, B., Heropolitanska-Pliszka, E., Pac, M., Motkowski, R., et al. (2017). Oxidative stress, mitochondrial abnormalities and antioxidant defense in Ataxia-telangiectasia, bloom syndrome and Nijmegen breakage syndrome. *Redox Biol.* 11, 375–383. doi: 10.1016/j.redox.2016.12.030

Mentus, S. A. (2004). Oxygen reduction on anodically formed titanium dioxide. *Electrochim. Acta* 50, 27–32. doi: 10.1016/j.electacta.2004.07.009

Misra, H. P., and Fridovich, I. (1972). The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J. Biol. Chem.* 247, 3170–3175.

Olmedo, D. G., Tasat, D. R., Evelson, P., Guglielmotti, M. B., and Cabrini, R. L. (2008). Biological response of tissues with macrophagic activity to titanium dioxide. *J. Biomed. Mater. Res.* 84, 1087–1093. doi: 10.1002/jbm.a.31514

Paglia, D. E., and Valentine, W. N. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J. Lab. Clin. Med.* 70, 158–169.

Peters, K., Unger, R. E., Gatti, A. M., Sabbioni, E., Tsaryk, R., and Kirkpatrick, C. J. (2007). Metallic nanoparticles exhibit paradoxical effects on oxidative stress and proinflammatory response in endothelial cells *in vitro*. *Int. J. Immunopathol. Pharmacol.* 20, 685–695. doi: 10.1177/039463200702000404

Reynolds, L. J., Credeur, D. P., Manrique, C., Padilla, J., Fadel, P. J., and Thyfaut, J. P. (2017). Obesity, type 2 diabetes, and impaired insulin-stimulated blood flow: role of skeletal muscle NO synthase and endothelin-1. *J. Appl. Physiol.* 122, 38–47. doi: 10.1152/japplphysiol.00286.2016

Saquib, Q., Al-Khedhairy, A. A., Siddiqui, M. A., Faisal, M., Abou-Tarboush, F. M., Azam, A., et al. (2012). Titanium dioxide nanoparticles induced cytotoxicity, oxidative stress and DNA damage in human amnion epithelial (WISH) cells. *Toxicol. In vitro* 26, 351–361. doi: 10.1016/j.tiv.2011.12.011

Sas, K., Robotka, H., Toldi, J., and Vécsei, L. (2007). Mitochondria, metabolic disturbances, oxidative stress and the kynurenine system, with focus on neurodegenerative disorders. *J. Neurol. Sci.* 257, 221–239. doi: 10.1016/j.jns.2007.01.033

Sen, V., Uluca, Ü., Ece, A., Kaplan, I., Bozkurt, F., Aktar, F., et al. (2014). Serum prolidase activity and oxidant-antioxidant status in children with chronic hepatitis B virus infection. *Ital. J. Pediatr.* 40:95. doi: 10.1186/s13052-014-0095-1

Sheikhi, M., Bouhafs, R. K., Hammarström, K. J., and 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. doi: 10.1034/j.1601-0825.2001.70109.x

Sung, F. L., Zhu, T. Y., Au-Yeung, K. K., and Siow, Y. L. (2002). Enhanced MCP-1 expression during ischaemia/reperfusion injury is mediated by oxidative stress and NF-kappaB. *Kidney Int.* 62, 1160–1170. doi: 10.1111/j.1523-1755.2002.kid577.x

Tengvall, P., Elwing, H., Sjoqvist, L., Lundström, I., and Bjursten, L. (1989). Interaction between hydrogen peroxide and titanium: a possible

role in the biocompatibility of titanium. *Biomaterials* 10, 118–120. doi: 10.1016/0142-9612(89)90043-4

Tsaryk, R., Kalbacova, M., Hempel, U., Scharnweber, D., Unger, R. E., Dieter, P., et al. (2007). Response of human endothelial cells to oxidative stress on Ti6AL4V alloy. *Biomaterials* 28, 806–813. doi: 10.1016/j.biomaterials.2006.09.033

Urban, R. M., Jacobs, J. J., and Tomlinson, M. J., Gavrilovic, J., Black, J., Peoc'h, M. (2000). Dissemination of wear particles to the liver, spleen, and abdominal lymph nodes of patients with hip or knee replacement. *J. Bone Joint Surg. Am.* 82, 457–477. doi: 10.2106/00004623-200004000-00002

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Pathways that Regulate ROS Scavenging Enzymes, and Their Role in Defense Against Tissue Destruction in Periodontitis

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Periodontitis, an inflammatory disease that affects the tissues surrounding the teeth, is a common disease worldwide. It is caused by a dysregulation of the host inflammatory response to bacterial infection, which leads to soft and hard tissue destruction. In particular, it is the excessive inflammation in response to bacterial plaque that leads to the release of reactive oxygen species (ROS) from neutrophils, which, then play a critical role in the destruction of periodontal tissue. Generally, ROS produced from immune cells exhibit an anti-bacterial effect and play a role in host defense and immune regulation. Excessive ROS, however, can exert cytotoxic effects, cause oxidative damage to proteins, and DNA, can interfere with cell growth and cell cycle progression, and induce apoptosis of gingival fibroblasts. Collectively, these effects enable ROS to directly induce periodontal tissue damage. Some ROS also act as intracellular signaling molecules during osteoclastogenesis, and can thus also play an indirect role in bone destruction. Cells have several protective mechanisms to manage such oxidative stress, most of which involve production of cytoprotective enzymes that scavenge ROS. These enzymes are transcriptionally regulated via NRF2, Sirtuin, and FOXO. Some reports indicate an association between periodontitis and these cytoprotective enzymes' regulatory axes, with superoxide dismutase (SOD) the most extensively investigated. In this review article, we discuss the role of oxidative stress in the tissue destruction manifest in periodontitis, and the mechanisms that protect against this oxidative stress.

Keywords: oxidative stress, cytoprotective enzymes, ROS, osteoclast, periodontitis

INTRODUCTION

Periodontitis is a common disease worldwide of the tissues surrounding the teeth, and is caused by bacterial infection. It is characterized by a dysregulation of the host inflammatory response, which eventually results in soft and hard tissue destruction (Mercado et al., 2003; Bartold et al., 2005). Tissue destruction in periodontitis is considered to result from an excessive inflammatory response

to bacterial plaque. This leads to release of reactive oxygen species (ROS), such as hydrogen peroxide and superoxide, from neutrophils (Waddington et al., 2000; Canakci et al., 2005; Dahiya et al., 2013; Miricescu et al., 2014; Callaway and Jiang, 2015; White et al., 2016), which results in oxidative stress—an imbalance between the production of ROS and antioxidant defenses.

Generally, ROS produced from immune cells exhibit an anti-bacterial effect and play a role in host defense and immune regulation (Baehner et al., 1975; Canakci et al., 2005). Excessive ROS however, can exert cytotoxic effects (Esterbauer et al., 1991), cause oxidative damage to proteins and DNA (Wells et al., 2009), can interfere with cell growth and cell cycle progression (Chang et al., 2013), and induce apoptosis (Yu et al., 2012) of gingival fibroblasts. This collectively enables ROS to directly induce periodontal tissue damage. Some ROS are also indirectly involved in periodontal tissue destruction through their role as intracellular signaling molecules in the osteoclastogenic pathway (Ha et al., 2004), and indeed, excessive activation of osteoclasts is a typical pathology in severe periodontitis.

Cells normally have several protective mechanisms against these oxidative stressors (Furukawa-Hibi et al., 2005; Kensler et al., 2007; Hsu et al., 2008), most of which involve the induction of cytoprotective enzymes that scavenge ROS (Mates and Sanchez-Jimenez, 1999). One protective mechanism involves nuclear factor E2-related factor 2 (NRF2), a master regulatory transcription factor for the synthesis of cytoprotective enzymes. NRF2 has been reported to be a negative regulator of osteoclastogenesis (Kanzaki et al., 2013, 2014, 2015).

The relationship between periodontitis and ROS is illustrated in **Figure 1**. In this review article, we will discuss; (1) the role of oxidative stress in tissue destruction in periodontitis, and (2) mechanisms that protect against oxidative stress.

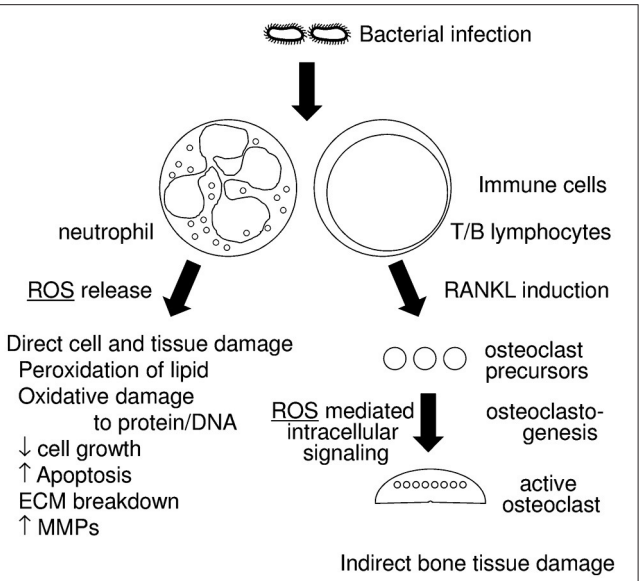

FIGURE 1 | Diagram of the relationship between periodontitis and reactive oxygen species (ROS). Infections with periodontopathogenic bacteria induce host immune responses, which includes neutrophil and T/B lymphocyte activation. Activated neutrophils generate ROS that have an anti-bacterial effect and are thus a form of host defense. Excessive ROS, however, result in direct cell and tissue damage, which includes (1) peroxidation of lipids, (2) oxidative damage to protein and DNA, (3) decrease of cell growth, (4) increased apoptosis, (5) breakdown of the extracellular matrix (ECM), (6) induction of matrix metalloproteases (MMPs). Activated T/B lymphocytes induce RANKL in periodontal tissue, which results in the augmentation of osteoclastogenesis. Some ROS work as intracellular signaling molecules in RANKL stimulation.

THE ROLE OF OXIDATIVE STRESS IN THE TISSUE DESTRUCTION IN PERIODONTITIS

Increased ROS are a hallmark of inflammation induced by neutrophils against invading bacteria, and are involved in tissue destruction (Sheikhi et al., 2000, 2001; Chapple et al., 2007; Matthews et al., 2007a). Periodontal tissue destruction is caused, in part, by neutrophils exhibiting an excessive inflammatory response to bacterial plaque (Canakci et al., 2005; Miricescu et al., 2014), with a high volume of ROS also generated by neutrophils in periodontal tissues with chronic periodontitis (Matthews et al., 2007b). ROS production in neutrophils is driven, in part, by nicotinamide adenine dinucleotide phosphate oxidase (NOX) and the purine degradation pathway, which is significantly accelerated in inflamed periodontal tissue (Giannopoulou et al., 2008; Barnes et al., 2009).

The release of ROS plays a critical role in periodontitis-associated tissue destruction, where ROS exert both direct and indirect effects on bone destruction. Directly, it can induce cytotoxic effects and oxidative damage to proteins and DNA. It can also interfere with cell growth and cell cycle progression (Chang et al., 2013), induce apoptosis (Yu et al., 2012) of gingival fibroblasts, and cause matrix degradation via the induction of matrix proteinases. Indirectly, ROS acts as an intracellular signaling molecule during osteoclastogenesis; an important process in hard tissue degeneration.

ROS exert cytotoxicity, such as peroxidation of lipids and phospholipids, against cells as well as the extracellular matrix (ECM). Additionally, protein aggregation through ROS-mediated oxidation (Squier and Bigelow, 2000; Squier, 2001; Friguet, 2002) leads to the breakdown of cell/tissue homeostasis (Hohn et al., 2014). ROS can also stimulate ECM degradation by inducing the breakdown of glycosaminoglycan (Fuchs and Schiller, 2014) and matrix proteinases (Dasgupta et al., 2009, 2010; Kar et al., 2010).

In addition to having a direct cytotoxic effect, ROS can indirectly promote hard tissue degeneration through their role in osteoclastogenesis. Intracellular signaling molecules that mediate osteoclastogenesis, a signaling cascade central to the destruction of alveolar bone, include ROS (Bax et al., 1992; Ha et al., 2004). In hard tissue destruction, alveolar bone resorption is driven by osteoclasts, the tissue-specific macrophage polykaryon created by the differentiation of monocyte/macrophage precursors cells on the bone surface (Teitelbaum, 2000). Generation of osteoclasts requires physical contact between osteoclast precursor cells and specific mesenchymal cells, such as marrow stromal cells or osteoblasts (Udagawa et al., 1990). The key

osteoclastogenic cytokine receptor activator of nuclear factor-kB ligand (RANKL) is a membrane-bound protein on osteoblasts and their precursors, which is recognized by the receptor, RANK, on marrow macrophages, thus prompting them to differentiate into osteoclasts (Lacey et al., 1998; Yasuda et al., 1998). In normal physiological circumstances, RANKL is principally expressed by mesenchymal cells of the osteoblast lineage, but in states of skeletal inflammation (Kong et al., 1999), as well as in periodontitis (Kawai et al., 2006), RANKL is produced in abundance by lymphocytes. Other key factors involved in osteoclastogenesis include TRAF6, RAC1, and NOX (Abo et al., 1991; Wong et al., 1998; Wang et al., 2008; Sasaki et al., 2009a). The NOX homologs, NOX1, and NOX2, complementarily generate ROS during osteoclastogenesis (Sasaki et al., 2009b).

Several reports have suggested that ROS signaling can lead to activation of mitogen-activated protein kinase (MAPK), phosphoinositide-3 kinase (PI3K), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) (Thannickal and Fanburg, 2000; Droge, 2002). NF-kB, which mediates IκBα phosphorylation and degradation, was the first transcription factor shown to respond to ROS (Schreck et al., 1991). Pretreatment of osteoclasts with antioxidants, to inhibit oxidation, was shown to reduce RANKL-induced AKT, NF-kB, and extracellular signal-regulated kinase (ERK) activation (Ha et al., 2004). Coincidentally, NF-kB also plays a pivotal role in cytokine-induced periodontal tissue damage (Chapple, 1997). Application of an antioxidant inhibited the responses of osteoclast precursors to RANKL; including activation of c-Jun N-terminal kinase, p38 MAPK and ERK, and inhibited osteoclast differentiation (Lee et al., 2005). RANKL-mediated ROS induces long lasting Ca^{2+} oscillations that activate the transcription factor, nuclear factor of activated T-cells, cytoplasmic 1 (NFATC1) (Kim et al., 2010). Taken together, ROS cause hard tissue destruction via osteoclastogenesis, as well as soft tissue destruction.

MECHANISMS THAT PROTECT AGAINST OXIDATIVE STRESS

Cells normally possess several regulatory pathways that protect against oxidative stress (Furukawa-Hibi et al., 2005; Kensler et al., 2007; Hsu et al., 2008), by producing cytoprotective enzymes that scavenge ROS (Mates and Sanchez-Jimenez, 1999). NRF2 (Thimmulappa et al., 2002), Sirtuin (Chen et al., 2011), and FOXO (Liu et al., 2005) are major regulatory pathways for cytoprotective enzymes (Kanzaki et al., 2016a). **Figure 2** summarizes the linking the periodontitis, the regulatory pathways of ROS scavenging enzymes, and the defense mechanism against tissue destruction.

NRF2 transcriptionally induces cytoprotective enzymes, such as heme oxygenase-1 (HO-1) (Alam et al., 1999), NAD(P)H: quinone reductase (NQO1) (Favreau and Pickett, 1991), gamma-glutamylcysteine synthetase (GCS) (Wild et al., 1998), and the auxiliary cellular NADPH regenerating enzyme, glucose 6-phosphate dehydrogenase (G6PD) (Thimmulappa et al., 2002). In addition, Sirtuin- and FOXO-induced superoxide dismutase

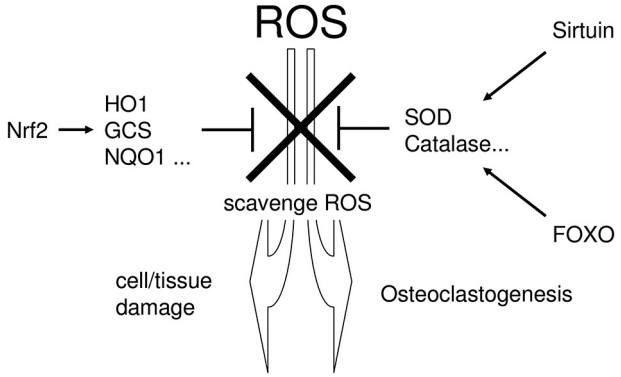

FIGURE 2 | The linking the periodontitis, the regulatory pathways of ROS scavenging enzymes, and the defense mechanism against tissue destruction. Excessive ROS exhibit cytotoxicity and induce tissue destruction in periodontal tissue. To protect such ROS-mediated cytotoxicity, cells possess several regulatory pathways which regulate the production of cytoprotective enzymes that scavenge ROS. NRF2, Sirtuin, and FOXO are situated in the major regulatory pathways for cytoprotective enzyme production.

(SOD) (Kops et al., 2002; Nemoto and Finkel, 2002) convert superoxide to hydrogen peroxide (Baehner et al., 1975), which is subsequently detoxified by catalase (CAT) (Essers et al., 2004). These cytoprotective enzymes play a critical role in the scavenging and detoxification of ROS.

Maintaining a balance between ROS and antioxidants is essential for periodontal health. In patients with severe chronic periodontitis, down-regulation of the NRF2 pathway was observed in polymorphonuclear leucocytes (Sima et al., 2016). In addition, neutrophils from patients with severe chronic periodontitis exhibited hyper-reactivity to bacterial stimuli (Dias et al., 2013). Conversely, activation of NRF2 prevented alveolar bone loss in an experimental animal study (Bhattarai et al., 2016). In this context, downregulation of NRF2 pathway might lead to the increase in ROS, which causes tissue destruction.

Cytoprotective enzymes are also thought to interfere with osteoclastogenesis, where ROS functions as an intracellular signaling molecule (Ha et al., 2004). Indeed, RANKL stimulation decreases expression of NRF2-dependent cytoprotective enzymes, thereby facilitating intracellular ROS signaling (Kanzaki et al., 2013, 2016b). Induction of cytoprotective enzymes by NRF2 activation subsequently inhibits osteoclastogenesis (Kanzaki et al., 2013, 2014, 2015; Sakai et al., 2013; Gambari et al., 2014; Lee et al., 2014; Lu et al., 2015; Bhattarai et al., 2016). Inversely, augmented osteoclastogenesis and bone destruction was observed with NRF2 deficiency (Rana et al., 2012; Hyeon et al., 2013; Ibanez et al., 2014; Lippross et al., 2014; Sun et al., 2015). Taken together, this shows that NRF2-dependent cytoprotective enzymes play a critical role in the regulation of bone destruction.

Enlargement of periapical lesions of the teeth in experimental animals has been associated with decreased expression of Sirtuin (SIRT6), causing increased apoptosis of osteoblasts (Kok et al., 2015). Activation of Sirtuin, by resveratrol (Tamaki et al., 2014) or overexpression of SIRT6 in osteoblasts by lentiviral

gene transfer (Kok et al., 2015; Hou et al., 2016), inhibited bone destruction. Therefore, not only NRF2- but also Sirtuin-mediated cytoprotective mechanisms control periodontal tissue homeostasis.

Activated FOXOs enhance antioxidant defense by augmenting cytoprotective enzymes, which successfully prevent inflammation and bone destruction (Chung et al., 2011; Kousteni, 2011). FOXO activation directly inhibits osteoclastogenesis (Bartell et al., 2014; Tan et al., 2015). However, FOXO binds to β-catenin, which exhibits inhibitory effects on osteoblastic differentiation. Thus, continuous stimulation of the FOXO/β-catenin pathway may reduce bone formation (Galli et al., 2011). In addition, *porphyromonas gingivalis*-induced ROS activate FOXO transcription factors through JNK signaling, which resulted in the FOXO1-controlled oxidative stress responses, such as inflammatory cytokine production and cell survival (Wang et al., 2015).

A relationship between cytoprotective enzymes themselves and periodontitis has also been reported. SOD is one of the most extensively investigated enzymes, and is closely associated with periodontitis. A positive relationship was demonstrated between the progression of periodontitis and serum SOD concentrations in experimental animals (Sobaniec and Sobaniec-Lotowska, 2000). This has been confirmed in human gingival crevicular fluid (GCF) (Akalin et al., 2005; Wei et al., 2010), serum (Wei et al., 2010), and saliva SOD (Canakci et al., 2009; Guentsch et al., 2012; Karim et al., 2012). Clinical studies in humans indicate that periodontal therapy returns elevated SOD levels to normal (Novakovic et al., 2013, 2014; Sukhtankar et al., 2013; Singh et al., 2014). Induction of SOD inhibited experimental periodontitis in animals (Petelin et al., 2000) and augmentation of anti-oxidant capacity by nutrient supplementation positively affected periodontal therapy in clinical trials (Biju et al., 2014; Daiya et al., 2014; Muniz et al., 2015). Furthermore, genetic mutation of *SOD* is considered to be a risk factor for periodontitis (Kazemi et al., 2015). These data support a role for SOD as a potential diagnostic marker for periodontitis. Therapeutically targeting SOD for the treatment of periodontitis may however produce substantive side effects, due to its beneficial effect on periodontopathogenic bacteria. SOD exhibited protective effects on the periodontopathogenic anaerobes *Porphyromonas gingivalis* (Lynch and Kuramitsu, 1999) and *Aggregatibacter actinomycetemcomitans* (Balashova et al., 2007).

HO-1 is another cytoprotective enzyme involved in the pathology of periodontitis. HO-1 inhibited RANKL upregulation in human cultured periodontal ligament cells (Lee et al., 2010) and lipopolysaccharide-induced production of proinflammatory mediators in cultured macrophages (Choi et al., 2014). Immunohistochemistry demonstrated that HO-1 was broadly expressed in periodontal tissue with chronic periodontitis (Gayathri et al., 2014). G6PD expression was also increased in the gingiva of patients with gingivitis (Di Paola et al., 2005; Yu et al., 2015). These data support the concept that the protective mechanisms provided by cytoprotective enzymes against ROS also play an inhibitory role in the progression of tissue destruction.

Clinically, it has been reported that markers of oxidative stress in saliva could serve as diagnostic markers for periodontitis (Sawamoto et al., 2005; Almerich-Silla et al., 2015; Banasova et al., 2015; Tothova et al., 2015) and therapeutic intervention against experimental periodontitis in rats improved total serum antioxidant levels (Saglam et al., 2015). A better understanding of the mechanisms that protect against oxidative stress will be useful for gaining a precise understanding of periodontitis pathology, which in turn, will contribute to the development of therapies for periodontitis treatment.

SUMMARY AND PERSPECTIVE

In this review, we have summarized recent evidence of the relationship between oxidative stress and periodontitis, a disease in which oxidative stress is both directly and indirectly involved with tissue destruction. Many reports describe a relationship between oxidative stress and periodontitis, with the balance between oxidative stress and defense mechanisms characterizing the pathological condition of the periodontitis. As such, some enzymes that are cytoprotective against oxidative stress could serve as diagnostic markers for periodontitis. Furthermore, a strong potential therapeutic approach for periodontitis would be to augment enzymes that protect against oxidative stress.

Although oxidative stress in periodontitis has been extensively investigated, there remains little information about the relationship between tissue damage and the immunological reactions exerted via ROS production in immune cells. Further investigation in this area is urgently needed for a comprehensive understanding of periodontitis.

AUTHOR CONTRIBUTIONS

Conception and design: HK and YN. Search references: SW, TN, YY, YN, YK, KI, SF, and YM. Drafted manuscript: HK, SW, YY, and YN. Critically revised the manuscript: HK, SW, YY, YK, KI, SF, YM, and YN.

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REFERENCES

Abo, A., Pick, E., Hall, A., Totty, N., Teahan, C. G., and Segal, A. W. (1991). Activation of the NADPH oxidase involves the small GTP-binding protein p21rac1. *Nature* 353, 668–670. doi: 10.1038/353668a0

Akalin, F. A., Toklu, E., and 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. doi: 10.1111/j.1600-051X.2005.00669.x

Alam, J., Stewart, D., Touchard, C., Boinapally, S., Choi, A. M., and Cook, J. L. (1999). Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. *J. Biol. Chem.* 274, 26071–26078. doi: 10.1074/jbc.274.37.26071

Almerich-Silla, J. M., Montiel-Company, J. M., Pastor, S., Serrano, F., Puig-Silla, M., and Dasi, F. (2015). Oxidative stress parameters in saliva and its association with periodontal disease and types of bacteria. *Dis. Markers* 2015:653537. doi: 10.1155/2015/653537

Baehner, R. L., Murrmann, S. K., Davis, J., and Johnston, R. B. Jr. (1975). The role of superoxide anion and hydrogen peroxide in phagocytosis-associated oxidative metabolic reactions. *J. Clin. Invest.* 56, 571–576. doi: 10.1172/JCI108126

Balashova, N. V., Park, D. H., Patel, J. K., Figurski, D. H., and Kachlany, S. C. (2007). Interaction between leukotoxin and Cu,Zn superoxide dismutase in *Aggregatibacter actinomycetemcomitans*. *Infect. Immun.* 75, 4490–4497. doi: 10.1128/IAI.00288-07

Banasova, L., Kamodyova, N., Jansakova, K., Tothova, L., Stanko, P., Turna, J., et al. (2015). Salivary DNA and markers of oxidative stress in patients with chronic periodontitis. *Clin. Oral Investig.* 19, 201–207. doi: 10.1007/s00784-014-1236-z

Barnes, V. M., Teles, R., Trivedi, H. M., Devizio, W., Xu, T., Mitchell, M. W., et al. (2009). Acceleration of purine degradation by periodontal diseases. *J. Dent. Res.* 88, 851–855. doi: 10.1177/0022034509341967

Bartell, S. M., Kim, H. N., Ambrogini, E., Han, L., Iyer, S., Serra Ucer, S., et al. (2014). FoxO proteins restrain osteoclastogenesis and bone resorption by attenuating H2O2 accumulation. *Nat. Commun.* 5:3773. doi: 10.1038/ncomms4773

Bartold, P. M., Marshall, R. I., and Haynes, D. R. (2005). Periodontitis and rheumatoid arthritis: a review. *J. Periodontol.* 76, 2066–2074. doi: 10.1902/jop.2005.76.11-S.2066

Bax, B. E., Alam, A. S., Banerji, B., Bax, C. M., Bevis, P. J., Stevens, C. R., et al. (1992). Stimulation of osteoclastic bone resorption by hydrogen peroxide. *Biochem. Biophys. Res. Commun.* 183, 1153–1158. doi: 10.1016/S0006-291X(05)80311-0

Bhattarai, G., Poudel, S. B., Kook, S. H., and Lee, J. C. (2016). Resveratrol prevents alveolar bone loss in an experimental rat model of periodontitis. *Acta Biomater.* 29, 398–408. doi: 10.1016/j.actbio.2015.10.031

Biju, T., Shabeer, M. M., Amitha, R., Rajendra, B. P., and Suchetha, K. (2014). Comparative evaluation of serum superoxide dismutase and glutathione levels in periodontally diseased patients: an interventional study. *Indian J. Dent. Res.* 25, 613–616. doi: 10.4103/0970-9290.147105

Callaway, D. A., and Jiang, J. X. (2015). Reactive oxygen species and oxidative stress in osteoclastogenesis, skeletal aging and bone diseases. *J. Bone Miner. Metab.* 33, 359–370. doi: 10.1007/s00774-015-0656-4

Canakci, C. F., Cicek, Y., and Canakci, V. (2005). Reactive oxygen species and human inflammatory periodontal diseases. *Biochemistry (Mosc).* 70, 619–628. doi: 10.1007/s10541-005-0161-9

Canakci, C. F., Cicek, Y., Yildirim, A., Sezer, U., and 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.

Chang, M. C., Tsai, Y. L., Chen, Y. W., Chan, C. P., Huang, C. F., Lan, W. C., et al. (2013). Butyrate induces reactive oxygen species production and affects cell cycle progression in human gingival fibroblasts. *J. Periodont. Res.* 48, 66–73. doi: 10.1111/j.1600-0765.2012.01504.x

Chapple, L. (1997). Reactive oxygen species and antioxidants in inflammatory diseases. *J. Clin. Periodontol.* 24, 287–296. doi: 10.1111/j.1600-051X.1997.tb00760.x

Chapple, L., Brock, G. R., Milward, M. R., Ling, N., and Matthews, J. B. (2007). Compromised GCF total antioxidant capacity in periodontitis: cause or effect? *J. Clin. Periodontol.* 34, 103–110. doi: 10.1111/j.1600-051X.2006.01029.x

Chen, Y., Zhang, J., Lin, Y., Lei, Q., Guan, K. L., Zhao, S., et al. (2011). Tumour suppressor SIRT3 deacetylates and activates manganese superoxide dismutase to scavenge ROS. *EMBO Rep.* 12, 534–541. doi: 10.1038/embor.2011.65

Choi, E. Y., Jin, J. Y., Choi, J. I., Choi, I. S., and Kim, S. J. (2014). DHA suppresses Prevotella intermedia lipopolysaccharide-induced production of proinflammatory mediators in murine macrophages. *Br. J. Nutr.* 111, 1221–1230. doi: 10.1017/S0007114513003681

Chung, H. Y., Lee, E. K., Choi, Y. J., Kim, J. M., Kim, D. H., Zou, Y., et al. (2011). Molecular inflammation as an underlying mechanism of the aging process and age-related diseases. *J. Dent. Res.* 90, 830–840. doi: 10.1177/0022034510387794

Dahiya, P., Kamal, R., Gupta, R., Bhardwaj, R., Chaudhary, K., and Kaur, S. (2013). Reactive oxygen species in periodontitis. *J. Indian Soc. Periodontol.* 17, 411–416. doi: 10.4103/0972-124X.118306

Daiya, S., Sharma, R. K., Tewari, S., Narula, S. C., and Kumar Sehgal, P. (2014). Micronutrients and superoxide dismutase in postmenopausal women with chronic periodontitis: a pilot interventional study. *J. Periodontal Implant Sci.* 44, 207–213. doi: 10.5051/jpis.2014.44.4.207

Dasgupta, J., Kar, S., Liu, R., Joseph, J., Kalyanaraman, B., Remington, S. J., et al. (2010). Reactive oxygen species control senescence-associated matrix metalloproteinase-1 through c-Jun-N-terminal kinase. *J. Cell. Physiol.* 225, 52–62. doi: 10.1002/jcp.22193

Dasgupta, J., Kar, S., Van Remmen, H., and Melendez, J. A. (2009). Age-dependent increases in interstitial collagenase and MAP Kinase levels are exacerbated by superoxide dismutase deficiencies. *Exp. Gerontol.* 44, 503–510. doi: 10.1016/j.exger.2009.04.005

Dias, H., Chapple, I. L., Milward, M., Grant, M. M., Hill, E., Brown, J., et al. (2013). Sulforaphane restores cellular glutathione levels and reduces chronic periodontitis neutrophil hyperactivity *in vitro*. *PLoS ONE* 8:e66407. doi: 10.1371/journal.pone.0066407

Di Paola, R., Mazzon, E., Rotondo, F., Dattola, F., Britti, D., De Majo, M., et al. (2005). Reduced development of experimental periodontitis by treatment with M40403, a superoxide dismutase mimetic. *Eur. J. Pharmacol.* 516, 151–157. doi: 10.1016/j.ejphar.2005.04.039

Droge, W. (2002). Free radicals in the physiological control of cell function. *Physiol. Rev.* 82, 47–95. doi: 10.1152/physrev.00018.2001

Essers, M. A., Weijzen, S., de Vries-Smits, A. M., Saarloos, I., de Ruiter, N. D., Bos, J. L., et al. (2004). FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. *EMBO J.* 23, 4802–4812. doi: 10.1038/sj.emboj.7600476

Esterbauer, H., Schaur, R. J., and Zollner, H. (1991). Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic. Biol. Med.* 11, 81–128. doi: 10.1016/0891-5849(91)90192-6

Favreau, L. V., and Pickett, C. B. (1991). Transcriptional regulation of the rat NAD(P)H:quinone reductase gene. Identification of regulatory elements controlling basal level expression and inducible expression by planar aromatic compounds and phenolic antioxidants. *J. Biol. Chem.* 266, 4556–4561.

Friguet, B. (2002). Protein repair and degradation during aging. *Sci. World J.* 2, 248–254. doi: 10.1100/tsw.2002.98

Fuchs, B., and Schiller, J. (2014). Glycosaminoglycan degradation by selected reactive oxygen species. *Antioxid. Redox Signal.* 21, 1044–1062. doi: 10.1089/ars.2013.5634

Furukawa-Hibi, Y., Kobayashi, Y., Chen, C., and Motoyama, N. (2005). FOXO transcription factors in cell-cycle regulation and the response to oxidative stress. *Antioxid. Redox Signal.* 7, 752–760. doi: 10.1089/ars.2005.7.752

Galli, C., Passeri, G., and Macaluso, G. M. (2011). FoxOs, Wnts and oxidative stress-induced bone loss: new players in the periodontitis arena? *J. Periodont. Res.* 46, 397–406. doi: 10.1111/j.1600-0765.2011.01354.x

Gambari, L., Lisignoli, G., Cattini, L., Manferdini, C., Facchini, A., and Grassi, F. (2014). Sodium hydrosulfide inhibits the differentiation of osteoclast progenitor cells via NRF2-dependent mechanism. *Pharmacol. Res.* 87, 99–112. doi: 10.1016/j.phrs.2014.06.014

Gayathri, G., Muthukumar, S., Joseph, L. D., and Suresh, R. (2014). Immunolocalization of heme oxygenase-1 in periodontal diseases. *Indian J. Dent. Res.* 25, 567–571. doi: 10.4103/0970-9290.147080

Giannopoulou, C., Krause, K. H., and Muller, F. (2008). The NADPH oxidase NOX2 plays a role in periodontal pathologies. *Semin. Immunopathol.* 30, 273–278. doi: 10.1007/s00281-008-0128-1

Guentsch, A., Hiese, I., Puklo, M., Kiehntopf, M., Pfister, W., and Eick, S. (2012). Variables of host response in saliva of patients with periodontitis: a pilot study. *Quintessence Int.* 43, e104–e114.

Ha, H., Kwak, H. B., Lee, S. W., Jin, H. M., Kim, H. M., Kim, H. H., et al. (2004). Reactive oxygen species mediate RANK signaling in osteoclasts. *Exp. Cell Res.* 301, 119–127. doi: 10.1016/j.yexcr.2004.07.035

Hohn, T., Jung, T., and Grune, T. (2014). Pathophysiological importance of aggregated damaged proteins. *Free Radic. Biol. Med.* 71, 70–89. doi: 10.1016/j.freeradbiomed.2014.02.028

Hou, K. L., Lin, S. K., Chao, L. H., Hsiang-Hua Lai, E., Chang, C. C., Shun, C. T., et al. (2016). Sirtuin 6 suppresses hypoxia-induced inflammatory response in human osteoblasts via inhibition of reactive oxygen species production and glycolysis-A therapeutic implication in inflammatory bone resorption. *Biofactors* 43, 170–180. doi: 10.1002/biof.1320

Hsu, C. P., Odewale, I., Alcendor, R. R., and Sadoshima, J. (2008). Sirt1 protects the heart from aging and stress. *Biol. Chem.* 389, 221–231. doi: 10.1515/BC.2008.032

Hyeon, S., Lee, H., Yang, Y., and Jeong, W. (2013). Nrf2 deficiency induces oxidative stress and promotes RANKL-induced osteoclast differentiation. *Free Radic. Biol. Med.* 65, 789–799. doi: 10.1016/j.freeradbiomed.2013.08.005

Ibanez, L., Ferrandiz, M. L., Brines, R., Guede, D., Cuadrado, A., and Alcaraz, M. J. (2014). Effects of Nrf2 deficiency on bone microarchitecture in an experimental model of osteoporosis. *Oxid. Med. Cell. Longev.* 2014:726590. doi: 10.1155/2014/726590

Kanzaki, H., Shinohara, F., Itohiya-Kasuya, K., Ishikawa, M., and Nakamura, Y. (2015). Nrf2 activation attenuates both orthodontic tooth movement and relapse. *J. Dent. Res.* 94, 787–794. doi: 10.1177/0022034515577814

Kanzaki, H., Shinohara, F., Itohiya, K., Yamaguchi, Y., Katsumata, Y., Matsuzawa, M., et al. (2016b). RANKL induces Bach1 nuclear import and attenuates Nrf2-mediated antioxidant enzymes, thereby augmenting intracellular reactive oxygen species signaling and osteoclastogenesis in mice. *FASEB J.* 31, 781–792. doi: 10.1096/fj.201600826R

Kanzaki, H., Shinohara, F., Kajiya, M., Fukaya, S., Miyamoto, Y., and Nakamura, Y. (2014). Nuclear nrf2 induction by protein transduction attenuates osteoclastogenesis. *Free Radic. Biol. Med.* 77, 239–248. doi: 10.1016/j.freeradbiomed.2014.09.006

Kanzaki, H., Shinohara, F., Kajiya, M., and Kodama, T. (2013). The Keap1/Nrf2 protein axis plays a role in osteoclast differentiation by regulating intracellular reactive oxygen species signaling. *J. Biol. Chem.* 288, 23009–23020. doi: 10.1074/jbc.M113.478545

Kanzaki, H., Shinohara, F., Kanako, I., Yamaguchi, Y., Fukaya, S., Miyamoto, Y., et al. (2016a). Molecular regulatory mechanisms of osteoclastogenesis through cytoprotective enzymes. *Redox Biol.* 8, 186–191. doi: 10.1016/j.redox.2016.01.006

Kar, S., Subbaram, S., Carrico, P. M., and Melendez, J. A. (2010). Redox-control of matrix metalloproteinase-1: a critical link between free radicals, matrix remodeling and degenerative disease. *Respir. Physiol. Neurobiol.* 174, 299–306. doi: 10.1016/j.resp.2010.08.019

Karim, S., Pratibha, P. K., Kamath, S., Bhat, G. S., Kamath, U., Dutta, B., et al. (2012). Superoxide dismutase enzyme and thiol antioxidants in gingival crevicular fluid and saliva. *Dent. Res. J. (Isfahan).* 9, 266–272.

Kawai, T., Matsuyama, T., Hosokawa, Y., Makihira, S., Seki, M., Karimbux, N. Y., et al. (2006). B and T lymphocytes are the primary sources of RANKL in the bone resorptive lesion of periodontal disease. *Am. J. Pathol.* 169, 987–998. doi: 10.2353/ajpath.2006.060180

Kazemi, E., Moradi, M. T., Yari, K., Mousavi, S. A., and Kahrizi, D. (2015). Association between Manganese Superoxide Dismutase (MnSOD Val-9Ala) genotypes with the risk of generalized aggressive periodontitis disease. *Cell. Mol. Biol. (Noisy-le-grand).* 61, 49–52.

Kensler, T. W., Wakabayashi, N., and Biswal, S. (2007). Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu. Rev. Pharmacol. Toxicol.* 47, 89–116. doi: 10.1146/annurev.pharmtox.46.120604.141046

Kim, M. S., Yang, Y. M., Son, A., Tian, Y. S., Lee, S. I., Kang, S. W., et al. (2010). RANKL-mediated reactive oxygen species pathway that induces long lasting Ca^{2+} oscillations essential for osteoclastogenesis. *J. Biol. Chem.* 285, 6913–6921. doi: 10.1074/jbc.M109.051557

Kok, S. H., Hou, K. L., Hong, C. Y., Chao, L. H., Hsiang-Hua Lai, E., Wang, H. W., et al. (2015). Sirtuin 6 modulates hypoxia-induced apoptosis in osteoblasts via inhibition of glycolysis: implication for pathogenesis of periapical lesions. *J. Endod.* 41, 1631–1637. doi: 10.1016/j.joen.2015.05.008

Kong, Y. Y., Feige, U., Sarosi, I., Bolon, B., Tafuri, A., Morony, S., et al. (1999). Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature* 402, 304–309. doi: 10.1038/46303

Kops, G. J., Dansen, T. B., Polderman, P. E., Saarloos, I., Wirtz, K. W., Coffer, P. J., et al. (2002). Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. *Nature* 419, 316–321. doi: 10.1038/nature01036

Kousteni, S. (2011). FoxOs: unifying links between oxidative stress and skeletal homeostasis. *Curr. Osteoporos. Rep.* 9, 60–66. doi: 10.1007/s11914-011-0054-3

Lacey, D. L., Timms, E., Tan, H. L., Kelley, M. J., Dunstan, C. R., Burgess, T., et al. (1998). Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 93, 165–176. doi: 10.1016/S0092-8674(00)81569-X

Lee, H. J., Jeong, G. S., Pi, S. H., Lee, S. I., Bae, W. J., Kim, S. J., et al. (2010). Heme oxygenase-1 protects human periodontal ligament cells against substance P-induced RANKL expression. *J. Periodont. Res.* 45, 367–374. doi: 10.1111/j.1600-0765.2009.01247.x

Lee, N. K., Choi, Y. G., Baik, J. Y., Han, S. Y., Jeong, D. W., Bae, Y. S., et al. (2005). A crucial role for reactive oxygen species in RANKL-induced osteoclast differentiation. *Blood* 106, 852–859. doi: 10.1182/blood-2004-09-3662

Lee, S. H., Kim, J. K., and Jang, H. D. (2014). Genistein inhibits osteoclastic differentiation of RAW 264.7 cells via regulation of ROS production and scavenging. *Int. J. Mol. Sci.* 15, 10605–10621. doi: 10.3390/ijms150610605

Lippross, S., Beckmann, R., Streubesand, N., Ayub, F., Tohidnezhad, M., Campbell, G., et al. (2014). Nrf2 deficiency impairs fracture healing in mice. *Calcif. Tissue Int.* 95, 349–361. doi: 10.1007/s00223-014-9900-5

Liu, J. W., Chandra, D., Rudd, M. D., Butler, A. P., Pallotta, V., Brown, D., et al. (2005). Induction of prosurvival molecules by apoptotic stimuli: involvement of FOXO3a and ROS. *Oncogene* 24, 2020–2031. doi: 10.1038/sj.onc.1208385

Lu, S. H., Chen, T. H., and Chou, T. C. (2015). Magnolol Inhibits RANKL-induced osteoclast differentiation of raw 264.7 macrophages through heme oxygenase-1-dependent inhibition of NFATc1 expression. *J. Nat. Prod.* 78, 61–68. doi: 10.1021/np500663y

Lynch, M. C., and Kuramitsu, H. K. (1999). Role of superoxide dismutase activity in the physiology of *Porphyromonas gingivalis*. *Infect. Immun.* 67, 3367–3375.

Mates, J. M., and Sanchez-Jimenez, F. (1999). Antioxidant enzymes and their implications in pathophysiologic processes. *Front. Biosci.* 4:D339–D345. doi: 10.2741/A432

Matthews, J. B., Wright, H. J., Roberts, A., Cooper, P. R., and Chapple, I. L. (2007b). Hyperactivity and reactivity of peripheral blood neutrophils in chronic periodontitis. *Clin. Exp. Immunol.* 147, 255–264. doi: 10.1111/j.1365-2249.2006.03276.x

Matthews, J. B., Wright, H. J., Roberts, A., Ling-Mountford, N., Cooper, P. R., and Chapple, I. L. (2007a). Neutrophil hyper-responsiveness in periodontitis. *J. Dent. Res.* 86, 718–722. doi: 10.1177/154405910708600806

Mercado, F. B., Marshall, R. I., and Bartold, P. M. (2003). Inter-relationships between rheumatoid arthritis and periodontal disease. A review. *J. Clin. Periodontol.* 30, 761–772. doi: 10.1034/j.1600-051X.2003.00371.x

Miricescu, D., Totan, A., Calenic, B., Mocanu, B., Didilescu, A., Mohora, M., et al. (2014). Salivary biomarkers: relationship between oxidative stress and alveolar bone loss in chronic periodontitis. *Acta Odontol. Scand.* 72, 42–47. doi: 10.3109/00016357.2013.795659

Muniz, F. W., Nogueira, S. B., Mendes, F. L., Rosing, C. K., Moreira, M. M., de Andrade, G. M., et al. (2015). The impact of antioxidant agents complimentary to periodontal therapy on oxidative stress and

periodontal outcomes: a systematic review. *Arch. Oral Biol.* 60, 1203–1214. doi: 10.1016/j.archoralbio.2015.05.007

Nemoto, S., and Finkel, T. (2002). Redox regulation of forkhead proteins through a p66shc-dependent signaling pathway. *Science* 295, 2450–2452. doi: 10.1126/science.1069004

Novakovic, N., Cakic, S., Todorovic, T., Raicevic, B. A., Dozic, I., Petrovic, V., et al. (2013). Antioxidative status of saliva before and after non-surgical periodontal treatment. *Srp. Arh. Celok. Lek.* 141, 163–168. doi: 10.2298/SARH1304163N

Novakovic, N., Todorovic, T., Rakic, M., Milinkovic, I., Dozic, I., Jankovic, S., et al. (2014). Salivary antioxidants as periodontal biomarkers in evaluation of tissue status and treatment outcome. *J. Periodont. Res.* 49, 129–136. doi: 10.1111/jre.12088

Petelin, M., Pavlica, Z., Ivanusa, T., Sentjurc, M., and Skaleric, U. (2000). Local delivery of liposome-encapsulated superoxide dismutase and catalase suppress periodontal inflammation in beagles. *J. Clin. Periodontol.* 27, 918–925. doi: 10.1034/j.1600-051x.2000.027012918.x

Rana, T., Schultz, M. A., Freeman, M. L., and Biswas, S. (2012). Loss of Nrf2 accelerates ionizing radiation-induced bone loss by upregulating RANKL. *Free Radic. Biol. Med.* 53, 2298–2307. doi: 10.1016/j.freeradbiomed.2012.10.536

Saglam, M., Koseoglu, S., Hatipoglu, M., Esen, H. H., and Koksal, E. (2015). Effect of sumac extract on serum oxidative status, RANKL/OPG system and alveolar bone loss in experimental periodontitis in rats. *J. Appl. Oral Sci.* 23, 33–41. doi: 10.1590/1678-775720140288

Sakai, E., Shimada-Sugawara, M., Yamaguchi, Y., Sakamoto, H., Fumimoto, R., Fukuma, Y., et al. (2013). Fisetin inhibits osteoclastogenesis through prevention of RANKL-induced ROS production by Nrf2-mediated up-regulation of phase II antioxidant enzymes. *J. Pharmacol. Sci.* 121, 288–298. doi: 10.1254/jphs.12243FP

Sasaki, H., Yamamoto, H., Tominaga, K., Masuda, K., Kawai, T., Teshima-Kondo, S., et al. (2009a). NADPH oxidase-derived reactive oxygen species are essential for differentiation of a mouse macrophage cell line (RAW264.7) into osteoclasts. *J. Med. Invest.* 56, 33–41. doi: 10.2152/jmi.56.33

Sasaki, H., Yamamoto, H., Tominaga, K., Masuda, K., Kawai, T., Teshima-Kondo, S., et al. (2009b). Receptor activator of nuclear factor- κB ligand-induced mouse osteoclast differentiation is associated with switching between NADPH oxidase homologues. *Free Radic. Biol. Med.* 47, 189–199. doi: 10.1016/j.freeradbiomed.2009.04.025

Sawamoto, Y., Sugano, N., Tanaka, H., and Ito, K. (2005). Detection of periodontopathic bacteria and an oxidative stress marker in saliva from periodontitis patients. *Oral Microbiol. Immunol.* 20, 216–220. doi: 10.1111/j.1399-302X.2005.00215.x

Schreck, R., Rieber, P., and Baeuerle, P. A. (1991). Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *EMBO J.* 10, 2247–2258.

Sheikhi, M., Bouhafs, R. K., Hammarstrom, K. J., and 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.

Sheikhi, M., Gustafsson, A., and Jarstrand, C. (2000). Cytokine, elastase and oxygen radical release by Fusobacterium nucleatum-activated leukocytes: a possible pathogenic factor in periodontitis. *J. Clin. Periodontol.* 27, 758–762. doi: 10.1034/j.1600-051x.2000.027010758.x

Sima, C., Aboodi, G. M., Lakschevitz, F. S., Sun, C., Goldberg, M. B., and Glogauer, M. (2016). Nuclear factor erythroid 2-related factor 2 down-regulation in oral neutrophils is associated with periodontal oxidative damage and severe chronic periodontitis. *Am. J. Pathol.* 186, 1417–1426. doi: 10.1016/j.ajpath.2016.01.013

Singh, N., Chander Narula, S., Kumar Sharma, R., Tewari, S., and Kumar Sehgal, P. (2014). Vitamin E supplementation, superoxide dismutase status, and outcome of scaling and root planing in patients with chronic periodontitis: a randomized clinical trial. *J Periodontol.* 85, 242–249. doi: 10.1902/jop.2013.120727

Sobaniec, H., and Sobaniec-Lotowska, M. E. (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.

Squier, T. C. (2001). Oxidative stress and protein aggregation during biological aging. *Exp. Gerontol.* 36, 1539–1550. doi: 10.1016/S0531-5565(01)00139-5

Squier, T. C., and Bigelow, D. J. (2000). Protein oxidation and age-dependent alterations in calcium homeostasis. *Front. Biosci.* 5:D504–D526. doi: 10.2741/A530

Sukhtankar, L., Kulloli, A., Kathariya, R., and Shetty, S. (2013). Effect of non-surgical periodontal therapy on superoxide dismutase levels in gingival tissues of chronic periodontitis patients: a clinical and spectophotometric analysis. *Dis. Markers* 34, 305–311. doi: 10.1155/2013/269027

Sun, Y. X., Li, L., Corry, K. A., Zhang, P., Yang, Y., Himes, E., et al. (2015). Deletion of Nrf2 reduces skeletal mechanical properties and decreases load-driven bone formation. *Bone* 74, 1–9. doi: 10.1016/j.bone.2014.12.066

Tamaki, N., R., Cristina Orihuela-Campos, Inagaki, Y., Fukui, M., Nagata, T., and Ito, H. O. (2014). Resveratrol improves oxidative stress and prevents the progression of periodontitis via the activation of the Sirt1/AMPK and the Nrf2/antioxidant defense pathways in a rat periodontitis model. *Free Radic. Biol. Med.* 75, 222–229. doi: 10.1016/j.freeradbiomed.2014.07.034

Tan, P., Guan, H., Xie, L., Mi, B., Fang, Z., Li, J., et al. (2015). FOXO1 inhibits osteoclastogenesis partially by antagnozing MYC. *Sci. Rep.* 5:16835. doi: 10.1038/srep16835

Teitelbaum, S. L. (2000). Bone resorption by osteoclasts. *Science* 289, 1504–1508. doi: 10.1126/science.289.5484.1504

Thannickal, V. J., and Fanburg, B. L. (2000). Reactive oxygen species in cell signaling. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, 279, L1005–L1028.

Thimmulappa, R. K., Mai, K. H., Srisuma, S., Kensler, T. W., Yamamoto, M., and Biswal, S. (2002). Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. *Cancer Res.* 62, 5196–5203.

Tothova, L., Kamodyova, N., Cervenka, T., and Celec, P. (2015). Salivary markers of oxidative stress in oral diseases. *Front. Cell. Infect. Microbiol.* 5:73. doi: 10.3389/fcimb.2015.00073

Udagawa, N., Takahashi, N., Akatsu, T., Tanaka, H., Sasaki, T., Nishihara, T., et al. (1990). Origin of osteoclasts: mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow-derived stromal cells. *Proc. Natl. Acad. Sci. U.S.A.* 87, 7260–7264. doi: 10.1073/pnas.87.18.7260

Waddington, R. J., Moseley, R., and Embery, G. (2000). Reactive oxygen species: a potential role in the pathogenesis of periodontal diseases. *Oral Dis.* 6, 138–151. doi: 10.1111/j.1601-0825.2000.tb00325.x

Wang, Q., Sztukowska, M., Ojo, A., Scott, D. A., Wang, H., and Lamont, R. J. (2015). FOXO responses to *Porphyromonas gingivalis* in epithelial cells. *Cell. Microbiol.* 17, 1605–1617. doi: 10.1111/cmi.12459

Wang, Y., Lebowitz, D., Sun, C., Thang, H., Grynpas, M. D., and Glogauer, M. (2008). Identifying the relative contributions of Rac1 and Rac2 to osteoclastogenesis. *J. Bone Miner. Res.* 23, 260–270. doi: 10.1359/jbmr.071013

Wei, D., Zhang, X. L., Wang, Y. Z., Yang, C. X., and 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. doi: 10.1111/j.1834-7819.2009.01123.x

Wells, P. G., McCallum, G. P., Chen, C. S., Henderson, J. T., Lee, C. J. J., Perstin, J., et al. (2009). Oxidative stress in developmental origins of disease: teratogenesis, neurodevelopmental deficits, and cancer. *Toxicol. Sci.* 108, 4–18. doi: 10.1093/toxsci/kfn263

White, P. C., Chicca, I. J., Cooper, P. R., Milward, M. R., and Chapple, I. L. (2016). Neutrophil extracellular traps in periodontitis: a web of intrigue. *J. Dent. Res.* 95, 26–34. doi: 10.1177/0022034515609097

Wild, C., Gipp, J. J., and Mulcahy, T. (1998). Overlapping antioxidant response element and PMA response element sequences mediate basal and β-naphthoflavone-induced expression of the human γ-glutamylcysteine synthetase catalytic subunit gene. *Biochem. J.* 332(Pt 2), 373–381. doi: 10.1042/bj3320373

Wong, B. R., Josien, R., Lee, S. Y., Vologodskaia, M., Steinman, R. M., and Choi, Y. (1998). The TRAF family of signal transducers mediates NF-κB activation by the TRANCE receptor. *J. Biol. Chem.* 273, 28355–28359. doi: 10.1074/jbc.273.43.28355

Yasuda, H., Shima, N., Nakagawa, N., Yamaguchi, K., Kinosaki, M., Mochizuki, S., et al. (1998). Osteoclast differentiation factor is a ligand

for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc. Natl. Acad. Sci. U.S.A.* 95, 3597–3602. doi: 10.1073/pnas.95.7.3597

Yu, J. Y., Lee, S. Y., Son, Y. O., Shi, X., Park, S. S., and Lee, J. C. (2012). Continuous presence of H(2)O(2) induces mitochondrial-mediated, MAPK- and caspase-independent growth inhibition and cytotoxicity in human gingival fibroblasts. *Toxicol. In vitro* 26, 561–570. doi: 10.1016/j.tiv.2012.01.022

Yu, N., Barros, S. P., Zhang, S., Moss, K. L., Phillips, S. T., and Offenbacher, S. (2015). Insulin response genes in different stages of periodontal disease. *J. Dent. Res.* 94(Suppl. 9), 194S–200S. doi: 10.1177/0022034515584384

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Evaluation of Salivary and Serum Antioxidant and Oxidative Stress Statuses in Patients with Chronic Periodontitis: A Case-Control Study

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Aim: Local bacteria stimulate polymorphonuclear neutrophils to release reactive oxygen species in periodontitis. Increased levels of oxidative stress play a significant role in the pathogenesis of periodontitis. Therefore, this study aimed to evaluate total salivary and serum antioxidant capacity and malondialdehyde in patients with chronic periodontitis.

Materials and methods: Fifty-five healthy subjects and 55 patients with chronic periodontitis, with an age range of 30–50 years, were evaluated. After clinical examination and case selection, unstimulated whole saliva was collected in the morning. Blood samples were taken from the antecubital vein. Total antioxidant capacity and malondialdehyde levels were evaluated by spectrophotometric assay. Data were analyzed with *t*-test, using Stata.11 software program.

Results: The periodontitis group exhibited lower salivary (0.16) and serum (0.36) total antioxidant capacity ($P = 0.11$) compared to the control group. Mean salivary malondialdehyde levels in the case and control groups were 0.80 ± 0.09 and 0.42 ± 0.08, respectively. The results showed significantly higher levels of salivary and serum malondialdehyde in the periodontitis group. Gender did not have any effect on antioxidant and oxidative stress levels.

Conclusion: This study indicated increased levels of salivary and serum oxidative stresses in patients with chronic periodontitis. Total antioxidant capacity was mildly lower in the saliva and serum of these patients. Higher malondialdehyde levels with no changes in antioxidant status can result in systemic and local complications in these patients.

Keywords: periodontitis, total antioxidant capacity, malondialdehyde, saliva, serum, oxidative stress, antioxidants, oral disease

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INTRODUCTION

Subgingival plaques in periodontitis have a plethora of microbial flora which cause inflammation and destruction of gingival tissues. Host cells release proinflammatory cytokines (interlukins 1, 6 and 8, and TNF-a) which stimulate infiltration of polymorphonuclear cells, the first line of cellular host defenses, against potential pathogens in the gingival sulcus. Polymorphonuclear cells produce

proteolytic enzymes and O^2 by oxidative burst. The interaction between bacteria increases oxygen consumption and produces reactive oxygen species (ROS) for killing of the bacteria which are capable of initiating periodontal tissue destruction (Sheikhi et al., 2001; Guentsch et al., 2008; Pendyala et al., 2008; Abou Sulaiman and Shehadeh, 2010; Žilinskas et al., 2011).

ROS is produced as a result of normal cellular metabolism processes (Akalin et al., 2007). It is important in cell signaling and metabolic processes (Brock et al., 2004; Gümüş et al., 2009).

Antioxidants (AOs) are substances that significantly delay or prevent oxidation of substrates that can be oxidized (Brock et al., 2004). AOs protect human body's cells from harmful oxidant effects. AOs eliminate oxidants and repair damage produced by ROS (Sheikhi et al., 2001). AOs are classified as chain- breaking AO, preventive AO and enzymes which control ROS (Baltacioglu et al., 2006; Akalin et al., 2007). AOs remove ROS which is necessary for safe aerobic life (Baltacioglu et al., 2006).

An imbalance between ROS production and antioxidant defense ability leads to oxidative stresses in tissues. ROS and oxidative stress have a main role in the pathogenesis of more than 100 inflammatory disorders (Brock et al., 2004; Gümüş et al., 2009; Tóthová et al., 2015) like periodontitis, diabetes, rheumatoid arthritis, stroke, and inflammatory lung diseases (Baltacioglu et al., 2006; Akalin et al., 2007; Chapple et al., 2007; Trivedi et al., 2015; Zhang et al., 2016).

ROS destroys DNA, lipid, protein, enzymes, and tissues (Sheikhi et al., 2001).

One of the most important reactions of free radicals is lipid peroxidation which changes the structural integrity and function of cell membranes. Malondialdehyde (MDA) is the end products of lipid peroxidation, as an oxidative stress marker, and results in tissue destruction by oxidative stress (Guentsch et al., 2008).

AO activities in saliva are strongly associated with periodontitis and various clinical variables (Tamaki et al., 2015). Several studies have shown relationships between periodontitis and oxidative stress and various clinical variables by measuring total antioxidant capacity (TAC) and MDA (Almerich-Silla et al., 2015; Baser et al., 2015; Tamaki et al., 2015; Trivedi et al., 2015). Despite contradictory results, some studies have shown a positive relationship between oxidative stress and periodontitis (Chapple et al., 2002, 2007; Brock et al., 2004; Baltacioglu et al., 2006; Akalin et al., 2007, 2009; Canakci et al., 2007, 2009; Guentsch et al., 2008; Pendyala et al., 2008; Žilinskas et al., 2011).

Recent data suggest increased oxidative stresses and MDA in periodontitis (Almerich-Silla et al., 2015; Trivedi et al., 2015; Zhang et al., 2016).

The hypothesis of this study was as follows: Periodontitis patients exhibit changes in local and systemic antioxidant and oxidative stresses. Saliva as a mirror of body health may reflect general health and may be used as a diagnostic material in future.

To the best of our knowledge, only one study to date has evaluated both salivary and serum antioxidant and oxidative stress statuses in periodontitis patients (Baltacioglu et al., 2014).

Abbreviations: ROS, Reactive oxygen species; AO, Antioxidants; MDA, Malondialdehyde; TAC, Total antioxidant capacity.

Given the controversies in the results of different studies and the importance of AO and oxidative stress levels in prevention, initiation, progression, and treatment of periodontal diseases, this study was undertaken to evaluate salivary and serum levels of TAC and MDA in chronic periodontitis in comparison to a healthy control group.

MATERIALS AND METHODS

In this case-control study 110 subjects were selected. The sample size was calculated according to previous study results (Guentsch et al., 2008). Fifty-five healthy subjects and 55 patients with chronic periodontitis with an age range of 30–50 years were included. Each group consisted of 27 females and 28 males. All the subjects were informed of the study procedures and written informed consent was obtained from all of them. The study protocol was approved by the Ethics Committee of Hamadan University of Medical Sciences. The research was conducted in full accordance with the ethical principles of the World Medical Association Declaration of Helsinki. Subjects with systemic diseases and those taking medications, pregnant women, smokers, alcohol users, and addicted individuals were excluded.

The subjects in the case group were selected from patients referred to the dental clinics in Hamadan and Tehran for treatment of periodontal diseases. The control group subjects were selected from those referring for routine dental examinations with eligible criteria in Hamadan Dental School during 2010–2013. We matched the case and control groups in relation to age and gender. All the participants were 30–50 years of age.

Periodontal disease and its severity were defined in terms of probing depth and clinical attachment loss. A full-mouth periodontal examination was performed for clinical attachment loss. The distance between the CEJ and the bottom of the gingival crevice was defined as the clinical attachment loss.

Patients with moderate periodontitis had 3–4 mm of attachment loss and patients with severe periodontitis had attachment loss >5 mm. Periodontitis was defined as the presence of proximal and mesial to distal clinical attachment loss of >4 mm in two or more teeth (Page and Eke, 2007). We selected patients with moderate and severe periodontitis.

Unstimulated whole salivary samples were collected using sterile Falcon tubes in 5 min in the morning (Navazesh, 1993). The salivary sample were immediately placed on to ice and stored at 4°C and transferred to the laboratory in a maximum of 20 min and kept at –80°C until the analysis.

Blood samples from antecubital vein were taken between 8:00 and 9:30 a.m. The blood samples were centrifuged for 10 min at 3,000 rpm within 30 min after venipuncture.

MDA levels were measures on the basis of reaction with thiobarbituric acid. In this method MDA was mixed thiobarbituric acid and colorful pigments were produced that extraction of colorful material in excellent phase, intensity of fluorescent was measured with stimulation wavelength of 520 nm and issuance wavelength of 550 nm with the unit of nmol/mL (Rai et al., 2006).

TAC was measured by FRAP method. In this method the revival power of ferrous ion was measured in the presence of tripyridyltriazine and by formation of colorful complex. Ferrous -tripyridyltriazine and examination of the variation of light absorption in 593 nm with mol/mL unit (Riviere and Papagiannoulis, 1987).

Student's *t*-test and chi-squared test were used for data analysis, using Stata 11. Statistical significance was set at $P < 0.05$.

RESULTS

Table 1 shows gender characteristics of the case and control groups.

Mean salivary TAC levels in the case and control groups were 0.16 ± 0.009 and 0.18 ± 0.1 (mol/mL), respectively.

Periodontitis group had lower salivary and serum TAC levels but the difference was not statistically significant. Mean and standard deviation of each data are presented in **Table 2**.

Mean salivary MDA levels in the case and control groups were 0.80 ± 0.09 and 0.42 ± 0.08, respectively.

The results showed significantly higher levels of salivary and serum MDA in the periodontitis group compared to the healthy control group.

Table 3 presents data in detail.

Gender did not have any effect on antioxidant and oxidative stress levels. Comparison of male and female subjects showed no statistically significant differences in their TAC and MDA levels (**Tables 1, 2**).

TABLE 1 | Characteristic of case and control group.

Sex	Number	Percent	Cum.
Male	56	50.45	50.45
Female	55	49.55	100
Total	111	100	
Sex	**Health**	**Perio**	**Total**
Male	28	28	56
Female	28	27	55
Total	56	55	111

TABLE 2 | Mean and standard deviation of salivary and serum total antioxidant capacity (TAC) in case and control group.

variables	Salivary TAC	*P*-value	Serum TAC	*P*-value
	Mean ± *SD*		Mean ± *SD*	
Male	0.17 ± 0.1	$p = 0.5$	0.37 ± 0.04	0.95
Female	0.17 ± 0.02		0.37 ± 0.02	
Periodontitis	0.16 ± 0.09	$p = 0.11$	0.36 ± 0.01	0.11
Control	0.18 ± 0.1		0.37 ± 0.05	

T-test.

TABLE 3 | Mean and standard deviation of salivary and serum malondialdehyde (MDA) in case and control group.

variables	Salivary MDA	*P*-value	Serum MDA	*P*-value
	Mean ± *SD*		**Mean ± *SD***	
Male	0.62 ± 0.2	$p = 0.44$	1.47 ± 0.31	0.66
Female	0.59 ± 0.2		1.49 ± 0.37	
Periodontitis	0.80 ± 0.09	$p = 0.0001$	1.76 ± 0.09	0.0001
Control	0.42 ± 0.08		1.15 ± 0.18	

DISCUSSION

Adult periodontitis is one of the most common chronic inflammatory diseases, in which microbial plaque causes periodontal ligament and bone destruction. Bacterial colonization, host immune response, and genetic predisposition are some of the main etiologic factors (Michalowicz et al., 2000; Kinane and Lappin, 2001; Sheikhi et al., 2001; Pihlstrom et al., 2005; Pussinen et al., 2007; Žilinskas et al., 2011).

High oxidative stress and low antioxidant capacity might have important roles in the etiopathogenesis of periodontitis (Tsai et al., 2005; Almerich-Silla et al., 2015; Baser et al., 2015; Tamaki et al., 2015; Trivedi et al., 2015).

We studied the salivary and serum levels of MDA and TAC in chronic periodontitis. The results showed significantly higher levels of salivary and serum MDA in the periodontitis group compared to the healthy control group. Salivary and serum TAC did not exhibit any statistically significant difference between the two groups although the case group had lower TAC levels compared to the healthy controls.

Based on the results of this study, periodontitis can also induce systemic oxidative stresses and alter serum MDA levels and vice versa.

Other studies have shown a reduction in both systemic and local antioxidant capacity and antioxidant concentration of gingival crevicular fluids in the periodontitis group as compared with the controls (Brock et al., 2004; Baltacioglu et al., 2006; Canakci et al., 2007; Guentsch et al., 2008).

Consistent with the results of this study, in a study by Panjamurthy MDA level was significantly higher in the periodontitis group (Panjamurthy et al., 2005). Synthesis of MDA might be due to a decrease in AO in destroyed in periodontal tissues.

Celec et al., too, showed high salivary MDA levels in periodontitis. No correlation was observed between salivary and serum MDA levels in their study. They concluded that local oxidative stress is a predisposing factor for MDA production in periodontitis (Celec et al., 2005).

Trivedi et al. showed significant MDA elevation and reductions in antioxidant enzymes in periodontitis patients. They reported a direct correlation between MDA levels and an inverse correlation of antioxidant enzymes with periodontitis (Trivedi et al., 2015).

Our results are consistent with studies demonstrating an increase in lipid peroxidation levels in serum, saliva, gingival crevicular fluid and gingiva in periodontitis (Sobaniec and

Sobaniec-Lotowska, 2000; Sheikhi et al., 2001; Mashayekhi et al., 2005; Panjamurthy et al., 2005; Tsai et al., 2005).

A number of recent studies measured different salivary oxidative stress markers in periodontitis and demonstrated that MDA was a more specific biomarker of lipid peroxidation in periodontitis patients. We assessed MDA which is a specific marker than others (Takane et al., 2002; Sculley and Langley-Evans, 2003; Sugano et al., 2003; Halliwell and Whiteman, 2004; Panjamurthy et al., 2005).

Similar to our results, in other studies salivary and serum TAC levels were lower in periodontal diseases compared to the control group (Baser et al., 2015; Tamaki et al., 2015).

One study suggested lower plasma antioxidant capacity in severe periodontitis, especially in the aggressive form and concluded that antioxidants might predict tissue destruction (Baser et al., 2015).

Based on our findings TAC was slightly lower in the case group. Zhang et al. also showed lower salivary TAC levels in periodontal patients (Zhang et al., 2016). We evaluated TAC because assays of TAC are biological interactions between individual antioxidants while specific antioxidant analysis might provide misleading information, and some of them might be undiscovered or difficult to assay, expensive, and time-consuming than TAC assays (Maxwell et al., 2006).

In our study we collected whole unstimulated saliva because it has major salivary gland composition and contains some elements of gingival crevicular fluids, immune cells, and tissue metabolites. It also most closely reflects the intraoral condition (Sculley and Langley-Evans, 2003; Tsai et al., 2005; Gümüş et al., 2009).

Oxidative stress and AO also change in smoking, diabetes mellitus, pregnancy, and systemic diseases. Therefore, we excluded all the subjects with systemic diseases and smokers from our study (Nagler et al., 2000; Canakci et al., 2007; Chapple et al., 2007; Gümüş et al., 2009).

In the present study gender had no effect on TAC and MDA levels, although other studies have shown gender differences in their levels (Brock et al., 2004; Maxwell et al., 2006). One study similar to the present study demonstrated that sex did not have any effect on gingival crevicular fluid TAC levels (Brock et al., 2004).

Based on other studies successful periodontal therapy increased gingival crevicular fluid TAC levels (Chapple et al., 2007) and decreased MDA levels (Guentsch et al., 2008); therefore, periodontal therapy can be very useful for the patient. TAC was significantly higher after scaling in a study by Yang et al. (2014).

It is still unclear whether periodontal disease leads to oxidative stresses or they happen as a result of it (Panjamurthy et al., 2005; Baltacioglu et al., 2006). Local and systemic antioxidant prescription might be helpful in periodontitis patients in lowering oxidative stress levels and might have beneficial effects on patients' general health.

In this study we only compared periodontitis patients with a healthy group and did not evaluate the effect of periodontitis severity on TAC and MDA levels.

Our results confirmed our primary hypothesis that local and systemic oxidative stresses and antioxidants play a role in periodontitis. Oxidative stress is produced in periodontitis, aggravating periodontal tissue destruction. MDA levels increased significantly and TAC levels decreased in periodontitis patients. Higher TAC levels can reduce MDA levels.

It seems our results had internal and external validity due to standard sample selection and examination. Therefore, the results of this study can be generalized and probably show the status of periodontal patients in the entire world.

The limitations of the present study were as follows: In this study we did not evaluate AO and oxidative stresses in terms of the disease severity and therapy. Studies on the measurement of MDA levels in terms of disease severity and therapy might be helpful in early diagnosis and prevention of periodontitis. In this study one trained postgraduate oral medicine student examined all the patients. It was advisable that the patients could be examined by two observers and they findings could be calibrated and the concordance between the two periodontal examinations calculated. In this study only TAC was evaluated as it can show all the antioxidant activity together. Evaluation of the subjects' antioxidants is recommended in future studies because it is very helpful.

Recently there has been more interest is prevention of disease by specific nutrient antioxidants (Panjamurthy et al., 2005; Canakci et al., 2007); therefore, further longitudinal investigations on large study populations, with age- and sex-matched groups, different periodontal status, and salivary, serum, plasma, and gingival crevicular fluid TAC and MDA levels and individual antioxidants are recommended to understand the mechanisms involved and whether it is the cause or effect of the disease.

The data presented in the current study indicated that periodontitis group had increased oxidative stress levels than the healthy control group. TAC mildly compromises in the saliva and serum of these patients. This high salivary and serum MDA levels with no change in antioxidant status can cause systemic and local complications in these patients.

CONCLUSION

This study indicated increased levels of salivary and serum oxidative stresses in patients with chronic periodontitis than a healthy control group. TAC was mildly lower in the saliva and serum of these patients. These high salivary and serum MDA levels, with no change in antioxidant status can cause systemic and local complications in these patients.

AUTHOR CONTRIBUTIONS

FA Idea, conception, and design of the work; Data collection; Data interpretation; Drafting of article; Final approval of article. MG Sialochemichal Analysis; Data collection; Final

approval of article. ZJ Data acquisition; Data analysis; Final approval of article. RK Data collection; Final approval of article. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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REFERENCES

Abou Sulaiman, A. E., and Shehadeh, R. M. (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

Akalin, F. A., Baltacioglu, E., Alver, A., and 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. doi: 10.1111/j.1600-051X.2007.01091.x

Akalin, F. A., Baltacioglu, E., Alver, A., and 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, 457–467. doi: 10.1902/jop.2009.080218

Almerich-Silla, J., Montiel-Company, J., Pastor, S., Serrano, F., Puig-Silla, M., and Dasí, F. (2015). Oxidative stress parameters in saliva and its association with periodontal disease and types of bacteria. *Dis. Markers* 2015:653537. doi: 10.1155/2015/653537

Baltacioglu, E., Akalin, F. A., Alver, A., Balaban, F., Unsal, M., and 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, 385–392. doi: 10.1111/j.1600-051X.2006.00923.x

Baltacioglu, E., Yuva, P., Aydin, G., Alver, A., Kahraman, C., Karabulut, E., et al. (2014). Lipid peroxidation levels and total oxidant/antioxidant status in serum and saliva from patients with chronic and aggressive periodontitis. Oxidative stress index: a new biomarker for periodontal disease? *J. Periodontol.* 85, 1432–1441. doi: 10.1902/jop.2014.130654

Baser, U., Gamsiz-Isik, H., Cifcibasi, E., Ademoglu, E., and Yalcin, F. (2015). Plasma and salivary total antioxidant capacity in healthy controls compared with aggressive and chronic periodontitis patients. *Saudi Med. J.* 36, 856–861. doi: 10.15537/smj.2015.7.11954

Brock, G. R., Butterworth, C. J., Matthews, J. B., and Chapple, I. L. (2004). Local and systemic total antioxidant capacity in periodontitis and health. *J. Clin. Periodontol.* 31, 515–521. doi: 10.1111/j.1600-051X.2004.00509.x

Canakci, C. F., Cicek, Y., Yildirim, A., Sezer, U., and 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.

Canakci, V., Yildirim, A., Canakci, C. F., Eltas, A., Cicek, Y., and Canakci, H. (2007). Total antioxidant capacity and antioxidant enzymes in serum, saliva, and gingival crevicular fluid of preeclamptic women with and without periodontal disease. *J. Periodontol.* 78, 1602–1611. doi: 10.1902/jop.2007.060469

Celec, P., Hodosy, J., Celecová, V., Vodrázka, J., Cervenka, T., Halcák, L., et al. (2005). Salivary thiobarbituric acid reacting substances and malondialdehyde–their relationship to reported smoking and to parodontal status described by the papillary bleeding index. *Dis. Markers* 21, 133–137. doi: 10.1155/2005/693437

Chapple, I. L., Brock, G. R., Milward, M. R., Ling, N., and Matthews, J. B. (2007). Compromised GCF total antioxidant capacity in periodontitis: cause or effect? *J. Clin. Periodontol.* 34, 103–110. doi: 10.1111/j.1600-051X.2006.01029.x

Chapple, I. L., Brock, G., Eftimiadi, C., and Matthews, J. B. (2002). Glutathione in gingival crevicular fluid and its relation to local antioxidant capacity in periodontal health and disease. *Mol. Pathol.* 55, 367–373. doi: 10.1136/mp.55.6.367

Guentsch, A., Preshaw, P. M., Bremer-Streck, S., Klinger, G., Glockmann, E., and Sigusch, B. W. (2008). Lipid peroxidation and antioxidant activity in saliva of periodontitis patients: effect of smoking and periodontal treatment. *Clin. Oral Investig.* 12, 345–352. doi: 10.1007/s00784-008-0202-z

Gümüş, P., Buduneli, N., Cetinkalp, S., Hawkins, S. I., Renaud, D., Kinane, D. F., et al. (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

Halliwell, B., and 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. doi: 10.1038/sj.bjp.0705776

Kinane, D. F., and Lappin, D. F. (2001). Clinical, pathological and immunological aspects of periodontal disease. *Acta Odontol. Scand.* 59, 154–160. doi: 10.1080/000163501750266747

Mashayekhi, F., Aghahoseini, F., Rezaie, A., Zamani, M. J., Khorasani, R., and Abdollahi, M. (2005). Alteration of cyclic nucleotides levels and oxidative stress in saliva of human subjects with periodontitis. *J. Contemp. Dent. Pract.* 6, 46–53.

Maxwell, S. R., Dietrich, T., and Chapple, I. L. (2006). Prediction of serum total antioxidant activity from the concentration of individual serum antioxidants. *Clin. Chim. Acta* 372, 188–194. doi: 10.1016/j.cca.2006.04.015

Michalowicz, B. S., Diehl, S. R., Gunsolley, J. C., Sparks, B. S., Brooks, C. N., Koertge, T. E., et al. (2000). Evidence of a substantial genetic basis for risk of adult periodontitis. *J. Periodontol.* 71, 1699–1707. doi: 10.1902/jop.2000.71.11.1699

Nagler, R., Lischinsky, S., Diamond, E., Drigues, N., Klein, I., and Reznick, A. Z. (2000). Effect of cigarette smoke on salivary proteins and enzyme activities. *Arch. Biochem. Biophys.* 379, 229–236. doi: 10.1006/abbi.2000.1877

Navazesh, M. (1993). Methods for collecting saliva. *Ann. N.Y. Acad. Sci.* 694, 72–77. doi: 10.1111/j.1749-6632.1993.tb18343.x

Page, R., and Eke, P. (2007). Case definitions for use in population-based surveillance of periodontitis. *J. Periodontol.* 78, 1387–1399. doi: 10.1902/jop.2007.060264

Panjamurthy, K., Manoharan, S., and Ramachandran, C. R. (2005). Lipid peroxidation and antioxidant status in patients with periodontitis. *Cell. Mol. Biol. Lett.* 10, 255–264.

Pendyala, G., Thomas, B., and Kumari, S. (2008). The challenge of antioxidants to free radicals in periodontitis. *J. Indian Soc. Periodontol.* 12, 79–83. doi: 10.4103/0972-124X.44100

Pihlstrom, B. L., Michalowicz, B. S., and Johnson, N. W. (2005). Periodontal diseases. *Lancet* 366, 1809–1820. doi: 10.1016/S0140-6736(05)67728-8

Pussinen, P. J., Paju, S., Mäntylä, P., and Sorsa, T. (2007). Serum microbial- and host-derived markers of periodontal diseases: a review. *Curr. Med. Chem.* 14, 2402–2412. doi: 10.2174/092986707781745604

Rai, B., Kharb, S., Jain, R., and Anand, S. (2006). Salivary lipid peroxidation product malonaldehyde in various dental diseases. *World J. Med. Sci.* 1, 100–101. Available online at: https://www.researchgate.net/profile/Simmi_Kharb2/publication/237662281_Salivary_Lipid_Peroxidation_Product_Malonaldehyde_in_Various_Dental_Diseases/links/53d71a240cf228d363ead345.pdf

Riviere, G. R., and Papagiannoulis, L. (1987). Antibodies to indigenous and laboratory strains of Streptococcus mutans in saliva from children with dental caries and from caries-free children. *Pediatr. Dent.* 9, 216–220.

Sculley, D. V., and Langley-Evans, S. C. (2003). Periodontal disease is associated with lower antioxidant capacity in whole saliva and evidence of increased protein oxidation. *Clin. Sci.* 105, 167–172. doi: 10.1042/CS20030031

Sheikhi, M., Bouhafs, R. K., Hammarström, K. J., and 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. doi: 10.1034/j.1601-0825.2001.0070109.x

Sobaniec, H., and Sobaniec-Lotowska, M. E. (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.

Sugano, N., Yokoyama, K., Oshikawa, M., Kumagai, K., Takane, M., Tanaka, H., et al. (2003). Detection of *Streptococcus anginosus* and 8-hydroxydeoxyguanosine in saliva. *J. Oral Sci.* 45, 181–184. doi: 10.2334/josnusd.45.181

Takane, M., Sugano, N., Iwasaki, H., Iwano, Y., Shimizu, N., and 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. doi: 10.1902/jop.2002.73.5.551

Tamaki, N., Yoshino, F., Fukui, M., Hayashida, H., Yoshida, A., Kitamura, M., et al. (2015). Relationship among salivary antioxidant activity, cytokines, and periodontitis: the Nagasaki Island study. *J. Clin. Periodontol.* doi: 10.1111/jcpe.12438. [Epub ahead of print].

Tóthováá, L., Kamodyová, N., Cervenka, T., and Celec, P. (2015). Salivary markers of oxidative stress in oral diseases. *Front. Cell. Infect. Microbiol.* 5:73. doi: 10.3389/fcimb.2015.00073

Trivedi, S., Lal, N., Mahdi, A. A., Singh, B., and Pandey, S. (2015). Association of salivary lipid peroxidation levels, antioxidant enzymes, and chronic periodontitis. *Int. J. Periodontics Restorative Dent.* 35, e14–e19. doi: 10.11607/prd.2079

Tsai, C. C., Chen, H. S., Chen, S. L., Ho, Y. P., Ho, K. Y., Wu, Y. M., et al. (2005). Lipid peroxidation: a possible role in the induction and progression of chronic periodontitis. *J. Periodont. Res.* 40, 378–384. doi: 10.1111/j.1600-0765.2005.00818.x

Yang, P. S., Huang, W. C., Chen, S. Y., Chen, C. H., Lee, C. Y., Lin, C. T., et al. (2014). Scaling-stimulated salivary antioxidant changes and oral-health behavior in an evaluation of periodontal treatment outcomes. *ScientificWorldJournal* 2014:814671. doi: 10.1155/2014/814671

Zhang, T., Andrukhov, O., Haririan, H., Müller-Kern, M., Liu, S., Liu, Z., et al. (2016). Total antioxidant capacity and total oxidant status in saliva of periodontitis patients in relation to bacterial load. *Front. Cell. Infect. Microbiol.* 5:97. doi: 10.3389/fcimb.2015.00097

Žilinskas, J., Kubilius, R., Žekonis, G., and 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.

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frontiers in Physiology

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Oxidative Modification in the Salivary Glands of High Fat-Diet Induced Insulin Resistant Rats

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Still little is known about the role of oxidative stress (OS) in the pathogenesis of the salivary gland dysfunction in the course of insulin resistance (IR). To induce IR rats was fed with a high fat diet (HFD) during 8 weeks. Stimulated and non-stimulated salivary flow rate, total protein, as well as oxidative damage markers: 4-HNE protein adduct, 8-isoprostanes (8-isoP), 8-hydroxy-D-guanosine (8-OHdG), advanced oxidation protein product (AOPP), and protein carbonyls (PC) were determined in the plasma and submandibular and parotid glands of IR and control rats. We have shown a significant decrease (45%) of the stimulated salivary flow rate, and in the total protein concentration in the parotid (35%) and submandibular (10%) glands of HFD IR as compared to the control rats. The level of 4-HNE protein adduct (15%) and 8-isoP (20%) in the submandibular glands of IR rats as well as total level of 4-HNE protein adduct (39%), 8-isoP (27%), AOPP (25%), PC (32%), and 8-OHdG (18%) in the parotid glands of IR rats were significantly higher as compared to the control group. We showed no correlation between the assessed OS parameters in the plasma and salivary glands. However, the redox balance in both glands shifted toward the oxidative status, parotid glands of IR rats are exposed to greater intensity OS. Stimulated secretory ability and mechanisms involved in the synthesis/secretion of proteins in the salivary glands are depressed in the course of IR. Oxidative damage in the salivary glands arises independently from the general OS in the course of insulin resistance induced by a high fat diet.

Keywords: insulin resistance, salivary glands, oxidative stress, oxidative damage, salivary dysfunction

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INTRODUCTION

The fact that global population is becoming more obese due to the growing sedentary lifestyle and higher caloric intake results in an increase in insulin resistance (IR), which may be defined as reduced sensitivity to insulin in target tissues (Erejuwa, 2012). Due to the fact that insulin resistance is one of the earliest factor in the etiology of type 2 diabetes mellitus and often leads to the development of primary arterial hypertension and renal failure, it is considered an extremely

serious health problem (Gan et al., 2013). Therefore, in recent years there has been a lot of interest, in this pathological states, both on the part of scientists, and clinicians.

One of the pathogenic factors leading to the development of IR is the phenomenon of oxidative stress (Aguirre et al., 2000; Kamata et al., 2005; Tinahones et al., 2009). According to Lushchak (2014), oxidative stress (OS) is "a situation where the steady-state reactive oxygen species (ROS) concentration is transiently or chronically enhanced, disturbing cellular metabolism, and its regulation and damaging cellular constituens."

Excess production of ROS damages all important cellular components, such as DNA, lipids, and proteins, as well as disrupts cellular metabolism including altered gene expression, signal transduction, cell growth, and apoptosis. It has been shown that lipid peroxidation is the first, the most sensitive marker of oxidative damage which is related to the fact that lipids are very easily and immediately oxidized (Erel, 2005). The lipid peroxidation process changes the physical properties of the cell membranes—it reduces the hydrophobic interior, damages the spatial organization of the lipid membrane, as well as affects various cellular functions and pathways. Moreover, oxidation of lipids components leads to the lipid radical species that may damage other biomolecules. The result of the amino acids oxidation may be a cleavage of the polypeptide chain, as well as formation of crosslinks within one or more polypeptide chains (Stadtman and Levine, 2003). All of these result in a loss of activity and function of oxidatively modified proteins, with all biological consequences for the cell. It should be noted that ROS inhibit the thiol-containing (-SH) receptors, including muscarinic, adrenergic, serotonergic, and histaminergic receptors. Oxidative damage to DNA primarily affect the nitrogenous bases, sugar moieties, DNA strand breaks, as well as formation of the cross-links between DNA and proteins (Lovell et al., 2011). OS and its cytopathological consequences have been implicated in the onset and pathology of periodontitis (Pendyala et al., 2013; Öngöz Dede et al., 2016), oral precancer (Agha-Hosseini et al., 2012), and cancer (Bahar et al., 2007; Agha-Hosseini et al., 2012) as well as in the alteration of the salivary glands function in the course of general diseases (Su et al., 2010; Zalewska et al., 2014a, 2015; Knaś et al., 2016a,b).

They are many reports on the increased oxidative damage products in human or experimental animals plasma, liver, skeletal muscles, subcutanues, and viscelar adipose tissue in the course of the IR, type 2 diabetes and obesity (Keaney et al., 2003; Furukawa et al., 2004; Milagro et al., 2006; Grimsrud et al., 2007; Frohnert et al., 2011). Evidence implicates OS in the alteration of salivary glands function and composition in the course of type 2 diabetes (Al-Rawi, 2011; Aitken-Saavedra et al., 2015), or obesity (Narotzki et al., 2014; Giuseppe et al., 2015; Knaś et al., 2016b). However, there are hardly any studies analyzing OS in the salivary glands in the course of IR. Zalewska et al. (2014b) showed alterations in the antioxidant enzymatic system of the salivary glands of rats with high fat diet (HFD) induced insulin resistance but did not prove if OS is clearly present. It is commonly accepted that the most reliable evidence of induction of OS is increased concentrations of oxidatively changed biomolecules, however changes in the activity or concentrations of antioxidants are being challenged in this regard.

This study is aimed at examining the OS level in the salivary glands of HFD induced insulin resistance and control rats by assessing the concentration of essential markers of oxidative damage as well as association between oxidatively modified cellular components and the secretory ability in the parotid and submandibular glands of rats in the course of HFD induced insulin resistance.

MATERIALS AND METHODS

The experimental procedures concerning animal treatment and maintenance were approved by the institutional Committee for Ethics use of Animals in the Medical University in Bialystok, Poland (protocol number 89/2015, 2015/109).

After arrival at the experimental animal house at the Department of Physiology, Medical University in Bialystok, Poland, the animals underwent 1-week adaptation period to the new conditions. Next, 16 male Wistar rats were divided randomly into two groups: control (C, $n = 8$) and experimental (HFD-IR, $n = 8$). The rats were housed in standard cages and maintained at controlled temperatures (20–21°C), under standard condition of light from 6.00 a.m. to 6.00 p.m. and with free access to tap water.

Diet

During the adaptation period, all rats were fed a standard diet which comprised 10.3% fat, 24.2% protein, and 65.5% carbohydrates (kcal) (Agropol, Motycz Poland).

For eight consecutive weeks of the experiment, the control group received the same standard diet (Agropol, Motycz Poland) as in the adaptation period. To develop insulin resistance (Qu et al., 2008; Gan et al., 2013), the HFD-IR group was fed a HFD (Research Diets, INC cat no. D12492) which was composed of 59.8% fat, 20.1% protein, and 20.1% carbohydrates (kcal) and had unlimited access to water.

The rats were weighed for the first time on the day of their arrival at the animal house and immediately prior to sacrifice.

After overnight fasting, rats were anesthetized by intraperitoneal injection with phenobarbital (80 mg/kg of body weight). Animals were placed on the heated couch (37°C) in the supine position to evaluate the salivary secretory ability. Whole non-stimulated saliva was collected from the oral cavity with the pre-weighted cotton balls for 15 min by the one experienced person (Sabino-Silva et al., 2013). Whole stimulated saliva was collected under parasymphatetic stimulation. Animals were injected with pilocarpine nitrate (5 mg/kg BW, intraperitoneal, Sigma Chemical Co, St. Louis, MO, USA). Five minutes after the pilocarpine administration stimulated whole saliva was collected, for 5 min (Picco et al., 2012). The salivary secretory ability was determined from the difference in the initial and final weight of the cotton balls. We assumed that 1 mg is equal to 1 μL (Romero et al., 2012).

Subsequently, whole blood was collected from the abdominal aorta and the salivary glands were removed. The right salivary glands were weighed (laboratory weight KERN PLI 510-3 M), immediately freeze-clamped with aluminum tongs, frozen

in liquid nitrogen and stored at −80°C until biochemical determinations. The left salivary glands were fixed with 10% formalin solution.

Blood was harvested into glass tubes with heparin and centrifuged (5 min, 4°C, 3000 g, MPW 351, MPW Med. Instruments, Warsaw, Poland). The obtained plasma was frozen in liquid nitrogen and stored at −80°C. There was no haemolysis in any of the obtained plasma.

Directly before the determinations, the salivary glands and plasma were thawed (4°C), cut into small pieces and diluted (1:10) in ice cold PBS [to assess concentrations of the carbonyl groups, part of the salivary glands were diluted in 50 mM phosphate buffer (1:10)]. Then, the salivary glands were homogenized with the addition of the protease inhibitor (1 tablet/10 mL of the buffer; Complete Mini Roche, France) and the addition of antioxidant butyl-hydroxytoluene (10 μL 0.5 M BHT in acetonitryle per 1 mL of the buffer; BHT; Sigma-Aldrich, Germany), on ice using glass homogenizer (Omni TH, Omni International, Kennesaw, GA, USA), and sonificated (1800 J/sample, 20 s three times, on ice; ultrasonic cell disrupter, UP 400S, Hielscher, Teltow, Germany). For plasma samples, solution of BHT and protease inhibitor were also added. The resulting homogenates were spinned for 20 min, 4°C, 5000 g (MPW Med Instruments, Warsaw, Poland). Only supernatants were further analyzed the same day.

Assays

The plasma free fatty acids (FFA), insulin, glucose concentrations and plasma and salivary glands 4-HNE protein adduct, 8-isoprostanes (8-isoP), 8-hydroxy-D-guanosine (8-OHdG), advanced oxidation protein product (AOPP), protein carbonyls (PC), and total proteins concentrations were performed in duplicates. The final result is the arithmetic average of the two measurements. Results were converted to the grams of the total protein. Normalisation to total protein is used to observe the differences in the ratio of biochemical parameters present in the salivary glands or plasma.

FFA were determined by the method Bligh and Dyer (1959), the fasting glucose was analyzed by glucometer (Accu-check glucometer, Byer, Germany), the insulin level was assessed by the ELISA method (BioVendor, Brno, Czech Republic). Based on these results, the insulin sensitivity was calculated using the HOMA index of insulin resistance (HOMA-IR) = fasting insulin (U/mL)x fasting glucose (mM)/ 22.5 (Ebertz et al., 2014).

The lipids (8-isoP, 4-HNE protein adduct) and DNA (8-OHdG) oxidations products were determined using commercial ELISA kits (Cell Biolabs, Inc. San Diego, CA, USA; Cayman Chemicals, Ann Arbor, MI, USA; USCN Life Science, Wuhan, China, respectively) according to the manufacturer's instructions. The absorbance of the colored reaction product was measured at 405 nm using a microplate reader MINDRAY MR- 96A.

The PC was assessed as described previously (Reznick and Packer, 1994). Briefly the supernatant and plasma were incubated for 60 min, 25°C with 10 mM DNPH (2,4-dinitrophenylhydrazine; POCH. SA (Polskie Odczynniki Chemiczne. Spółka Akcyjna, Gliwice, Poland) dissolved in 2.5 M HCl. The concentration of PC was determined spectrophotometrically, in the presence of the blank (guanidine hydrochloride), by measuring the absorbance at 355 nm and using the molar absorption coefficient for DNPH $\varepsilon = 22{,}000\ M^{-1}\,cm^{-1}$.

AOPP were determined colorimetrically according to the method (Kalousová et al., 2002). Two hundred microliter of tissue homogenate and serum diluted 1:5 in PBS were incubated with 10 μL of 1.16 M potassium iodide (Sigma-Aldrich, Germany) and 20 μL of glacial acetic acid (POCH SA, Gliwice, Poland). The absorbance of the mixture was measured immediately at 340 nm. A standard curve was made for chloramine T (Sigma-Aldrich, Germany) and the results were expressed in chloramine units per mg of total proteins.

The bicinchioninic method was used to determine the protein concentration. The bovine serum albumin was used as a standard (Thermo Scientific PIERCES BCA Protein Assay Kit, Rockford, IL, USA).

Histological Examination

The left salivary glands were embedded in paraffin for making five micron sections. Next, the sections were stained with hematoxylin-eosin and examined under light microscope (OPLYMPUS BX 51, OLYMPUS) using a gratitude 40 and 60 x magnification. Histological examination was carried out by a histologist.

Statistical Analysis

The data were expressed as median, minimum and maximum. Statistical analysis was performed using Statistica 10.0 (Statsoft, Cracow, Poland). For statistical analyses, the groups were compared using the nonparametric test (U Mann-Whitney test). The Spearman Correlation Coefficient was used to study the associations between the OS markers, protein concentrations in the salivary glands and blood glucose, insulin and FFA concentrations as well as salivary flow rate. The statistical significance was defined as $p \leq 0.05$.

RESULTS

Effects of High Fat Feeding on the Body Weight, Plasma Glucose, Free Fatty Acids, and Insulin Concentration and Salivary Glands Weight

Despite the fact that average daily food intake was similar in both groups, HFD-IR rats presented with a higher body weight when compared to the control rats ($p = 0.0002$) (**Table 1**). However, when comparing the HFD-IR and the control salivary glands' weight there was a significant increase only in the case of the parotid glands of HFD-IR group as compared to the control parotid glands ($p = 0.041$) (**Table 1**). The 8-week high fat intake also affected glucose homeostasis. We observed an increase in the fasting glucose concentration ($p = 0.001$) as well as in the insulin concentration ($p = 0.0009$) in the HFD-IR rats in comparison with the control rats. Moreover, the insulin sensitivity calculated by HOMA-IR, allowed us to conclude that high fat feeding results in insulin resistance. The median value of the HOMA-IR index

TABLE 1 | Effect of 8-weeks high fat feeding on body weight, fasting plasma glucose, insulin, and FFA level, HOMA-IR, salivary: glands weights, flow, and total proteins.

	Body weight (g) at the beginning/ at the end of the study M (min-max)	Glycaemia (mg/dL) M (min-max)	Insulin (mU/mL) M (min-max)	FFA (μmol/L) M (min-max)	HOMA-IR M (min-max)	Salivary gland weight (mg) parotid/ submandibular M (min-max)	Salivary flow rate (μL/min) unstimulated/ stimulated M (min-max)	Total protein (mg/dL) parotid/submandibular M (min-max)
C	50(46–57)/	95.3	1.14(0.79–	83.4(62.3–94.1)*	4.87(3.2–7.6)*	0.087(0.076–0.09)*/	0.4(0.11–0.66)/	5037.1(4188.2–7553.6)*/
$n = 8$	314.7(273–331)*	(91–101.3)*	1.35)*			0.25(0.23–0.285)	118.49(86.88–193.3)*	4819(4679–5251.1)*
HFD-IR	53(46–61)/407.7(376–450)	175.6(125.1–	2.69(2.19–2.83)	185.9	21.0(12.2–26.5)	0.108(0.093–0.11)/	0.39(0.07–0.59)/	3274.9(3108.4–3998.9)/
$n = 8$		210.5)		(157.5–198.4)		0.27(0.22–0.34)	65.11(35.6–79.89)	4337(3606.2–4592.9)

C, control; HFD-IR, high fat diet insulin resistant rats; FFA, free fatty acids; HOMA-IR, HOMA index of insulin resistance;
**p < 0.05; M (min-max)- Median (minimum-maximum).*

was significantly higher in the group of HFD-IR rats as compared to the reference rats ($p = 0.00001$) (**Table 1**).

Finally, rats fed with HFD were characterized by an elevated plasma free fatty acids concentration as compared to the control rats ($p = 0.001$).

The Effect of High Fat Feeding on Plasma 4-HNE-Protein Adduct, 8-isoP, 8-OHdG, AOPP, and PC

Plasma 4-HNE-protein adduct, 8-isoP, 8-OHdG, AOPP and PC concentration of HFD-IR and control rats are given in **Figure 1**. The obtained results proved that 8-week high fat feeding increased the general oxidative stress. We have shown that comparison of plasma OS biomarkers within groups revealed that HFD-IR group presented an elevated plasma 4-HNE-protein adduct (51%, $p = 0.0008$), 8-isoP (37%, $p = 0.0007$), 8-OHdG (27%, $p = 0.003$), AOPP (169%, $p = 0.00043$), and PC (42%, $p = 0.0005$) as compared to the control rats.

The Effect of High Fat Feeding on Salivary Flow Rate and Protein Concentration in the Parotid and Submandibular Glands

Our results showed that 8-week high fat feeding had only a minor effect on the unstimulated secretory ability of the salivary glands HFD-IR rats as compared to the control group, but significantly reduced the stimulated flow rate compared with the control group ($p = 0.0003$) (**Table 1**).

On the contrary, we observed that, high fat feeding influenced protein concentrations in both salivary glands. As shown in **Table 1** protein concentration in the parotid (35%) and submandibular (10%) glands' tissue of HFD-IR rats decreased significantly when compared with that of the control glands ($p = 0.031$, $p = 0.0009$, respectively; **Table 1**).

Submandibular Glands

Submandibular glands 4-HNE-protein adduct, 8-isoP, 8-OHdG, AOPP and PC concentration of HFD-IR and control rats are presented on **Figure 2**. Submandibular glands: 4-HNE-protein adduct (15%) and 8-isoP (20%) concentrations of HFD-IR rats increased significantly as compared to the control rats ($p = 0.02$, $p = 0.03$, respectively). On the other hand, the submandibular glands of the HFD-IR and control rats were comparable in terms of 8-OHdG, AOPP and PC concentration.

Parotid Glands

Figure 3 summarizes the parotid gland markers of OS in the HFD-IR and control groups. On the contrary to submandibular glands, parotid glands of HFD-IR rats showed the severity of oxidative modifications of all types of cellular elements, which we observed in the form of significant increases in concentrations of 4-HNE-protein adduct (39%), 8-isoP (27%), 8-OHdG (18%), AOPP (25%), and PC (32%) when compared to the control group ($p = 0.003$, $p = 0.03$, $p = 0.035$, $p = 0.023$ $p = 0.002$, respectively).

Parotid vs. Submandibular

Comparison of parotid and submandibular glands OS biomarkers revealed quite different results within control and

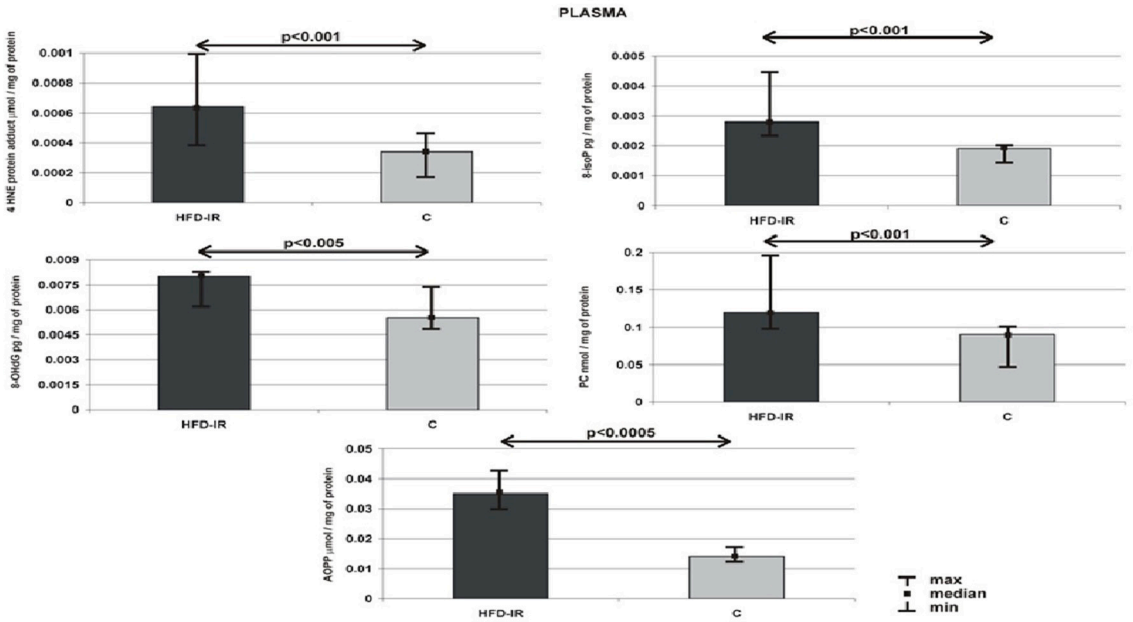

FIGURE 1 | Plasma 4-HNE protein adduct, 8-isoP, 8-OHdG, PC and AOPP of the HFD-IR and control rats. M, median; min, minimum; max, maximum; HFD-IR, high fat diet- insulin resistant rats; C, control group; 8-isoP, 8-isoprostanes; 8-OHdG, 8-D-hydroxyguanosine; PC, protein carbonyl groups; AOPP, advanced oxidation protein products.

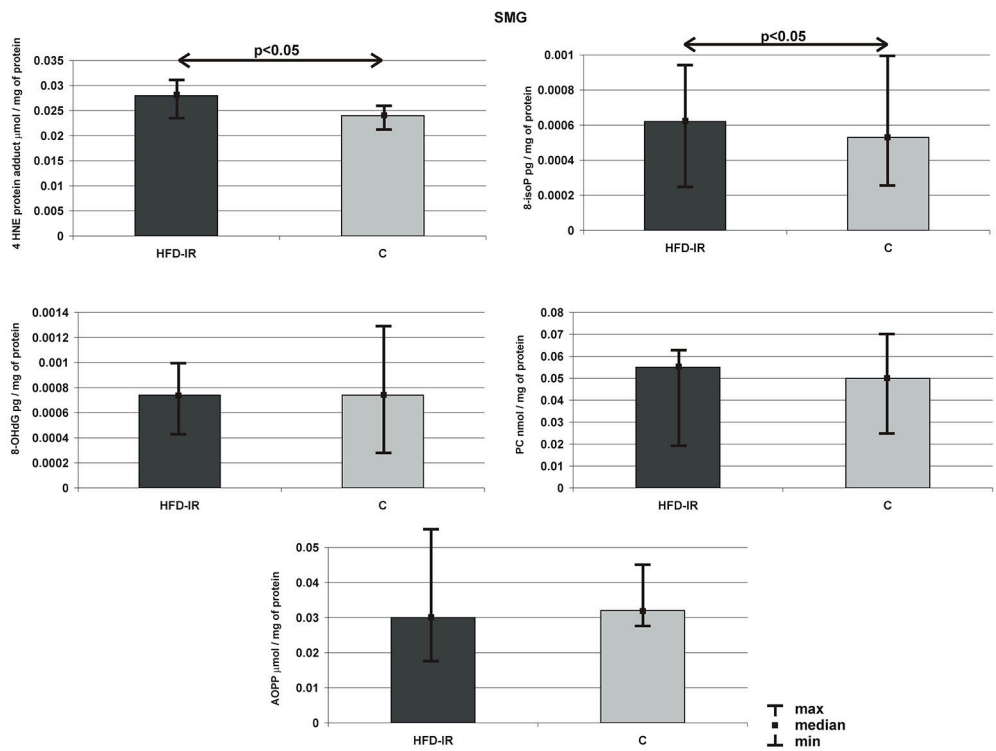

FIGURE 2 | Submandibular 4-HNE protein adduct, 8-isoP, 8-OHdG, PC, and AOPP of the HFD-IR and control rats. SMG, submandibular glands; M, median; min, minimum; max, maximum; HFD-IR, high fat diet insulin resistant rats; C, control group; 8-isoP, 8-isoprostanes; 8-OHdG, 8-D-hydroxyguanosine; PC, protein carbonyl groups; AOPP, advanced oxidation protein products.

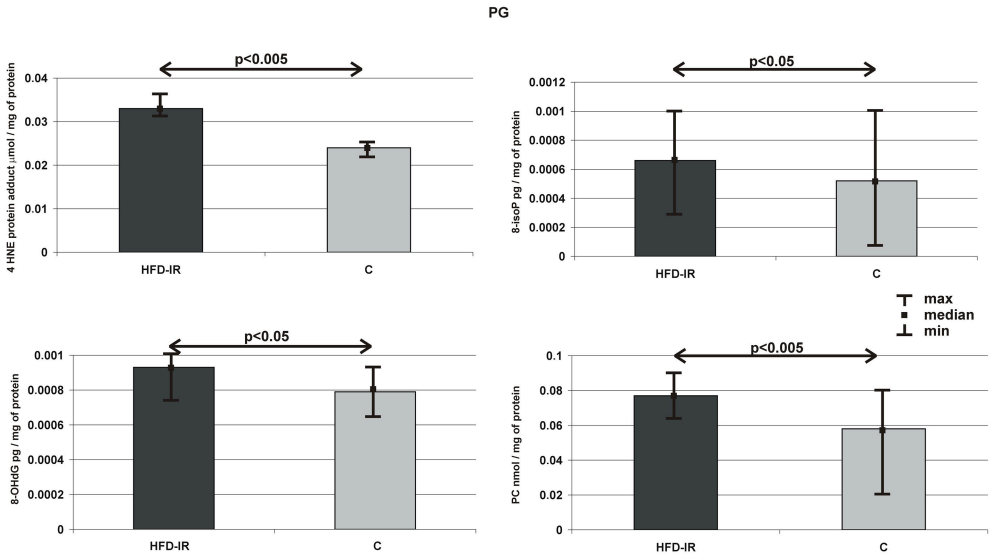

FIGURE 3 | Parotid 4-HNE protein adduct, 8-isoP, 8-OHdG, PC, and AOPP of the HFD-IR and control rats. PG, parotid glands; M, median; min, minimum; max, maximum; HFD-IR, high fat diet insulin resistant rats; C, control group; 8-isoP, 8-isoprostanes; 8-OHdG, 8-D-hydroxyguanosine; PC, protein carbonyl groups; AOPP, advanced oxidation protein products.

HFD-IR groups (**Table 2**). Our results showed that the control parotid and submandibular glands were comparable concerning all examined parameters of the oxidative stress. On the contrary, the cellular elements of the parotid glands of HFD-IR rats underwent more intensive oxidative modification as compared to the submandibular ones. The median concentrations of the parotid glands: 4-HNE-protein adduct, 8-isoP, 8-OHdG, AOPP and PC increased significantly as compared to the submandibular glands ($p = 0.02$, $p = 0.04$, $p = 0.043$, $p = 0.03$, $p = 0.001$, respectively).

The Effect of High Fat Feeding on Histological Observation of Salivary Glands

In the high fat feeding group, acinar cells of both the submandibular and parotid glands demonstrated a degenerative changes in the form of vacuolation (**Figure 4**), wherein the significantly more vacuoles were observed in the parotid gland in comparison to the submandibular gland (**Table 3**).

Correlation

The Spearman correlation analysis showed that 4-HNE protein adduct ($p = 0.001$, $r = 0.63$) and PC ($p = 0.03$, $r = 0.43$) in parotid glands of HFD-IR rats were positively correlated with plasma insulin concentration. In parotid glands of HFD-IR rats positive correlations were also noted between plasma HOMA-IR and 4-HNE protein adduct ($p = 0.02$, $r = 0.57$) and 8-isoP ($p = 0.003$, $r = 0.71$). We also observed a positive correlation between plasma insulin concentration and 4-HNE protein adduct ($p = 0.01$, $r = 0.48$) as well as negative correlation between plasma FFA and protein concentration ($p = 0.02$, $r = -0.43$) in submandibular glands of HFD-IR rats.

DISCUSSION

Salivary glands are responsible for maintaining oral cavity homeostasis. Their dysfunction results in a change of the composition and amount of saliva provided to the oral cavity. If the pathology persists for a longer period of time, these changes may impair oral health. Convincing evidence has established the role of ROS in the pathogenesis and development of salivary glands pathology in diabetes, obesity and insulin resistance. It is well documented that diabetes mellitus, obesity and IR alter the antioxidants function of salivary glands (Nogueira et al., 2005; Deconte et al., 2011; Zalewska et al., 2013, 2014b, 2015; Narotzki et al., 2014). Oxidative damage to lipids, proteins and DNA included in salivary glands cellular organelles was reported both in experimental diabetes in animals (Deconte et al., 2011; Knaś et al., 2016a) and in diabetic (Al-Rawi, 2011) and obese patients (Narotzki et al., 2014; Knaś et al., 2016b). However, in conjunction with a limited number of the research articles, the phenomenon of OS in the salivary glands in the course of IR still has not been sufficiently explored.

Evidence showed that chronic high fat feeding induces obesity, IR (Ebertz et al., 2014; Matczuk et al., 2016), as well as increases general body OS and oxidative damage (Furukawa et al., 2004). It is not surprising that the HFD in our study resulted in a significant increase in the body weight of rats, a significant increase in plasma glucose, insulin, and fatty acids concentrations as well as all the examined parameters of oxidative stress. We have also shown significantly higher medians of the HOMA-IR index in the group of HFD-IR rats ($p < 0.00001$) as compared to rats fed a standard diet, which confirmed that chronic high fat feeding decreased whole body insulin sensitivity. Based on the available literature and guidelines on diagnosing insulin-resistance (Qu et al., 2008; Gan et al., 2013; Ebertz et al., 2014)

TABLE 2 | Differences between submandibular and parotid salivary glands.

Group		SMG M (min-max)	PG M (min-max)	P SMG: PG
HFD-IR	4-HNE-protein adduct (μg/mg of protein)	0.027 (0.024–0.031)	0.034 (0.037–0.029)	0.02
	8-isoP (pg/mg of protein)	0.00062 (0.00026–0.00094)	0.00066 (0.00031–0.001)	0.04
	8-OHdG (pg/mg of protein)	0.00074 (0.00042–0.00096)	0.00093 (0.00073–0.001)	0.03
	PC (nmol/mg of protein)	0.055 (0.019–0.062)	0.077 (0.065–0.091)	0.001
	AOPP (μmol/mg of protein)	0.03 (0.018–0.056)	0.035 (0.029–0.037)	0.03
C	4-HNE-protein adduct (μg/mg of protein)	0.023 (0.021–0.026)	0.024 (0.022–0.025)	ns
	8-isoP (pg/mg of protein)	0.00053 (0.00027–0.00099)	0.00052 (0.00014–0.0011)	ns
	8-OHdG (pg/mg of protein)	0.00074 (0.00028–0.0013)	0.00079 (0.00065–0.00088)	ns
	PC (nmol/mg of protein)	0.05 (0.035–0.07)	0.058 (0.02–0.08)	ns
	AOPP (μmol/mg of protein)	0.032 (0.028–0.045)	0.028 (0.016–0.042)	ns

SMG, submandibular gland; PG, parotid gland; M, median; min, minimum; max, maximum; ns, not significant; HFD-IR, high fat diet insulin resistant rats; C, control group; 8-isoP, 8-isoprostanes; 8-OHdG, 8-D-hydroxyguanosine; PC, protein carbonyl groups; AOPP, advanced oxidation protein products.

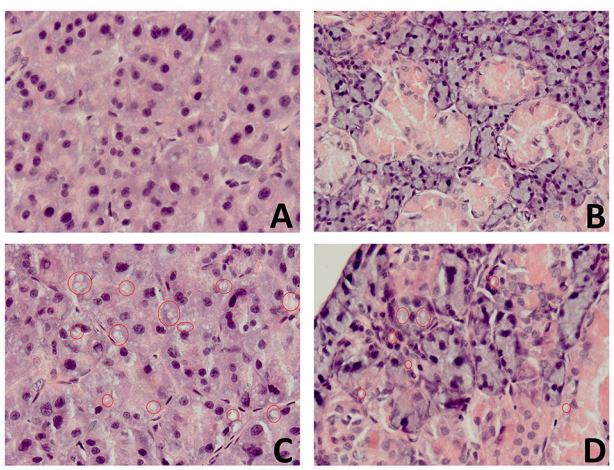

FIGURE 4 | The effect of high fat feeding on histological observation of salivary glands. (A) —parotid gland of the control rats **(C)**, **(B)**—submandibular gland of the control rats **(C)**, **(C)**—parotid gland of the high fat diet insulin resistant rats (HFD-IR), **(D)**— submandibular gland of the high fat diet insulin resistant rats (HFD-IR); red circles indicate vacuoles.

TABLE 3 | The effect of high fat feeding on histological observation of salivary glands.

	Parotid glands *N* = 8	Submandibular glands *N* = 8
C	8 (+)*	8 (+)*
HFD-IR	8 (+ + ++)°	7 (++), 1 (+ + +)

+ single vacuoles in the cytoplasm of acinar cells.
++5–15% of the each section occupied by pathological changes.
+ + + 16–30% of the each section occupied by pathological changes.
+ + ++ > 30% of the each section occupied by pathological changes.
C, control rats; HFD-IR, high fat diet insulin resistant rats.
**p< 0.05 control parotid/submandibular glands vs. HFD-IR parotid/submandibular glands.*
°p< 0.05 HFD-IR parotid vs. HFD-IR submandibular glands.

our results confirmed IR in the group of rats fed a high fat diet.

The control of ROS stationary level is the result of a balance between their production and their elimination. Disequilibrium in this balance causes OS, which is usually accompanied by oxidative damage defined as "biomolecular damage caused-by attack of ROS upon the constituents of living organisms." As was mentioned in the introduction, ROS can damage all classes of molecular cell components. Therefore, any single redox biomarker in isolation may be of limited value in diagnosis, staging, and prognosis of the oxidative stress-related human diseases. Many approaches for the assessment of oxidatively changed cellular components were selected. We used the most common assessment to evaluate oxidative damage: oxidized lipids (8-isoP and 4-HNE protein adduct), proteins (AOPP, PC), and DNA (8-OHdG).

Our study showed that both parotid and submandibular glands of HFD-IR rats had impaired ability to maintain normal redox balance compared to the data obtained in the salivary glands of the insulin-sensitive control rats, resulting in a

significant increase in the level of oxidized biomolecules in comparison to the salivary glands from control group.

However, a greater percentage increase in the concentration of lipid oxidation products (parotid gland (4-HNE protein adduct ↑39%, 8-isoP ↑27%) vs. submandibular gland (4-HNE protein adduct ↑15%, 8-isoP ↑20%), as well as new types of oxidatively modified components (AOPP ↑25, PC ↑32, and 8-OHdG ↑18%) in the parotid glands of HFD-IR rats as compared to the control proved that parotid glands are subjected to more intense OS, or they are more susceptible than submandibular glands to oxidant attack generated in the course of HFD induced IR. In addition, selective, from all oxidation products, a significant increase in lipoperoxidation products levels in the submandibular glands of rats feed with HFD vs. control prove once again that these glands are subject to lower intensity OS than parotid glands. It has been shown that the earliest sign of developing OS are the oxidative lipid modification products, due to the fact that the cell membrane and its lipids are the first to be exposed to the harmful effects of the free radicals. As the concentration of ROS further increases, the concentration of lipid peroxidation products grow and also proteins undergo oxidation and later DNA (Ayala et al., 2014).

The observed a significant increase in 8-OHdG level in the parotid glands of high fat feeding insulin resistant rats may have serious consequences for oral and also general health. The formation of 8-OHdG is the best known DNA damage occurring via OS and it is considered a "bio indicator" of carcinogenesis (Birben et al., 2012). It was shown that IR is a risk factor for the development of not only cardiovascular complications but also salivary glands tumors (Suba et al., 2005). However, the authors did not specify which salivary glands are more pronounced but a claim that a better control of IR seems to be necessary not only to reduce the cardiovascular risk but also prevent tumor promotion.

The present experiment does not explain the causes of a different course of OS in both salivary glands, however, it may be partly related to the observations of Zalewska et al. (2014b). Their report revealed that the antioxidant system of the parotid glands of rats feed with a HFD is more deficient to combine ROS as compared to submandibular one (Zalewska et al., 2014b). On the other hand, more intense oxidative damage to the parotid glands of HFD-induced insulin resistance rats may also be related to their physiological morphology, namely the presence of adipocytes in the parenchyma of the parotid glands (Amano et al., 2012). It not without significance is also the fact that only parotid glands displayed weight alteration from chronic high fat feeding, which was also observed in STZ diabetes (Mori et al., 1990) and obese (Bozzato et al., 2008) as well as type 2 diabetic (Carda et al., 2006) patients. The present study did not explain the nature of severe intracytoplasmic vacuolization in the parotid glands (with minimal changes in submandibular glands); however these vacuoles appeared to be a lipid nature since they were removed during fixation and processing of the samples. It was also shown that obese and type 2 diabetic parotid glands exhibited a significant enlargement, which is a result of enhanced storage of lipid droplets/adipocytes in the parotid parenchyma. Exposure to HFD leads to activation of inflammatory signaling in adipocytes and macrophages (↑ secretion of pro-inflammatory cytokines (TNFα, IL-6, IL-1β), activation of NADPH oxidase, and ↑ ROS production). Moreover, high fat feeding causes adipocyte to release monocyte chemoattractant protein-1 (MCP-1) that attracts monocytes in adipose tissue and transforms them in tissue resident inflammatory M1 phenotype macrophage (Solinas and Karin, 2010). The deepening inflammation, resulting in a further increase in production of the free radicals, which in the weakening of the antioxidant barrier leads to the oxidative damage to the organ and its dysfunction.

There was no obvious difference in the secretory response of the salivary glands under no stimulation, phenomena observed in obese patients (Knaś et al., 2016b). On the other hand, pilocarpine-stimulated whole saliva flow rate was 45% decreased in HFD-IR rats, compared to the control group. These results could suggest that salivary glands of HFD-IR rats have lowered susceptibility to muscarinic receptor stimulation, as was presented in STZ rats (Watanabe et al., 2001). On the other hand, if we assume that after stimulation only parotid glands increase their secretion (Zalewska et al., 2014a), reduced secretion of the stimulated saliva may be a result of severe acinar cell vacuolation in the parotid glands, and thus leads to reduction in the active secretory surface of the salivary glands. Affected stimulated saliva flow may be also due to the existence of disturbances at the level of neurotransmission. It can be assumed that extracellular matrix reconstruction caused by the parenchyma vacuolation of the salivary glands, chronic low grade inflammation (observed in parotid glands of obese and type 2 diabetes mellitus), ROS and acinar degeneration prevent function and/or communication of residual of the neural and the residual secretory units; however, to confirm or exclude this mechanism requires further studies.

In contrast to the saliva secretion, the mechanism involved in the synthesis/secretion of protein seems to be more sensitive to the effects induced by a high-fat diet. Protein concentration was significantly depressed in both glands, wherein in parotid glands was more disrupted than in submandibular one. There may be many reasons for reduced synthesis, secretion and total protein concentration. One of them is the reduced tissue sensitivity to insulin, which can be confirmed by a negative correlation between the plasma FFA level and the concentration of protein in the submandibular glands. It has been demonstrated that an increased plasma FFA level seriously affects insulin signaling pathway and contributes to the development of intracellular insulin resistance. Not without significance can be a morphologic changes as well as described above disturbances of neurotransmission.

Noteworthy is the absence of any correlation between the parameters of oxidative damage to both salivary glands and parameters of oxidative damage recorded in the serum. This may prove that oxidatively modified cellular components arise directly in the salivary glands, and they are not a result of diffusion of oxidation products from the blood vessels. However, we have shown a positive correlation between serum levels of insulin and 4-HNE protein adduct and PC in the parotid glands as well as concentrations of insulin and 4-HNE protein adduct in submandibular glands, which is in agreement with the data that insulin deficiency via enhancement of fatty acyl coenzyme A oxidase results in an increase in the oxidative environment and the same oxidative damage (Schönfeld et al., 2009). We also observed the positive correlation between 4-HNE

protein adduct and 8-isoP in parotid glands of HFD-IR rats and plasma HOMA-IR, which may suggest that only parotid lipid peroxidation products concentration increase as a function of insulin resistance, whereas parotid protein carbonyls, 8-OHdG and submandibular lipid peroxidation products are independent of insulin sensitivity.

They are a few limitation to our study. Most importantly, salivary glands: oxidative damage, dysfunction and morphological changes could be a consequences of high fat feeding or insulin resistance *per se* as well. Only kinetic studies could address this question. Secondly, they are a lot of other markers of ROS induced modification, and OS markers used by us are only the most frequently used. Using other markers of OS may partially or completely change our observations and conclusions. The applied animal model, very helpful in explaining occurring pathology, cannot be postponed directly to the humans.

CONCLUSION

In this study we have shown that high fat feeding results in the salivary gland's OS and oxidative damage as well as in the salivary glands dysfunction. We observed that mechanisms involved in the synthesis/secretion of proteins are affected in the parotid and submandibular glands of IR rats, however only stimulated secretory ability in the salivary glands is depressed in the course of HFD-induced insulin resistance. Moreover, we noted that the oxidative/antioxidative balance in both glands of IR rats shifted toward the oxidative status; however the parotid glands are exposed to a greater intensity oxidative stress. We also demonstrated that insulin resistance results in oxidative damage to DNA, proteins, and lipids, however oxidative damage in the salivary glands arises independently from the general OS as well as only the parotid lipid peroxidation products concentration seems to increase as a function of insulin resistance. IR is a prediabetic state, so one can see that salivary glands dysfunction manifests early in diabetes progression and it is detectable in prediabetic state. So IR should be taken seriously by the dentists, because at this stage diabetes can adversely affect the health of the oral cavity. Summarizing, OS may be a major phenotypic hallmark in the salivary insulin resistance. We believe that antioxidants supplementation could alleviate and/or prevent the damaging effects of OS and oxidative damage in patients with insulin resistance, improving function of the salivary glands as well as homeostasis of the oral cavity.

AUTHOR CONTRIBUTIONS

UK conceptualized, interpreted of data, wrote of the manuscript. MM conceptualized, did laboratory determination, did performance of the graphic part of the manuscript. AM did histological examination and interpreted these data. JM conceptualized, interpreted of data. MK- did statisctical analysis. PŻ conceptualized, did literature survey. MŻ and JB did literature survey, final approval of the version to be published. AZ conceptualized, did laboratory determination, interpreted of data, wrote of the manuscript.

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REFERENCES

Agha-Hosseini, F., Mirzaii-Dizgah, I., Farmanbar, N., and Abdollahi, M. (2012). Oxidative stress status and DNA damage in saliva of human subjects with oral lichen planus and oral squamous cell carcinoma. *J. Oral Pathol. Med.* 41, 736–740. doi: 10.1111/j.1600-0714.2012.01172.x

Aguirre, V., Uchida, T., Yenush, L., Davis, R., and White, M. F. (2000). The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). *J. Biol. Chem.* 275, 9047–9054. doi: 10.1074/jbc.275.12.9047

Aitken-Saavedra, J., Rojas-Alcayaga, G., Maturana-Ramírez, A., Escobar-Álvarez, A., Cortes-Coloma, A., Reyes-Rojas, M., et al. (2015). Salivary gland dysfunction markers in type 2 diabetes mellitus patients. *J. Clin. Exp. Dent.* 7, e501–e505. doi: 10.4317/jced.52329

Al-Rawi, N. H. (2011). Oxidative stress, antioxidant status and lipid profile in the saliva of type 2 diabetics. *Diab. Vasc. Dis. Res.* 8, 22–28. doi: 10.1177/1479164110390243

Amano, O., Mizobe, K., Bando, Y., and Sakiyama, K. (2012). Anatomy and histology of rodent and human major salivary glands-overview of the Japan salivary gland society-sponsored workshop. *Acta. Histochem. Cytochem.* 45, 241–250. doi: 10.1267/ahc.12013

Ayala, A., Mu-oz, M. F., and Arguelles, S. (2014). Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxide Med. Cell. Longev*. 2014:360438. doi: 10.1155/2014/360438

Bahar, G., Feinmesser, R., Shpitzer, T., Popovtzer, A., and Nagler, R. M. (2007). Salivary analysis in oral cancer patients: DNA and protein oxidation, reactive nitrogen species, and antioxidant profile. *Cancer* 109, 54–59. doi: 10.1002/cncr.22386

Birben, E., Sahiner, U. M., Sackesen, C., Erzurum, S., and Kalayci, O. (2012). Oxidative stress and antioxidant defense. *World Allergy Organ. J.* 5, 9–19. doi: 10.1097/WOX.0b013e3182439613

Bligh, E. G., and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37, 911–917. doi: 10.1139/o59-099

Bozzato, A., Burger, P., Zenk, J., Ulter, W., and Iro, H. (2008). Salivary gland biometry in female patients with eating disorders. *Eur. Arch. Otorhinolaryngol.* 265, 1095–1102. doi: 10.1007/s00405-008-0598-8

Carda, C., Mosquera-Lloreda, N., Salom, L., Gomez De Ferraris, M. E., and Peydró, A. (2006). Structural and functional salivary disorders in type 2 diabetic patients. *Med. Oral Pathol. Oral Cir. Bucal.* 11, 309–313.

Deconte, S. R., Oliveira, R. J., Calabria, L. K., Oliveira, V. N., Gouveia, N. M., Moraes Ada, S., et al. (2011). Alterations of antioxidant biomarkers and type I collagen deposition in the parotid gland of streptozotocin-induced diabetic rats. *Arch. Oral Biol.* 56, 744–751. doi: 10.1016/j.archoralbio.2011.01.005

Ebertz, C. E., Bonfleur, M. L., Bertasso, I. M., Mendes, M. C., Lubaczeuski, C., Araujo, A. C., et al. (2014). Duodenal jejunal bypass attenuates non-alcoholic fatty liver disease in western diet-obese rats. *Acta. Cir. Bras.* 29, 609–614. doi: 10.1590/S0102-8650201400150009

Erejuwa, O. O. (2012). "Oxidative stress in diabetes mellitus: is there a role for hypoglycemic drugs and/or antioxidants?," in *Oxidative Stress and Diseases,* ed V. Lushchak (InTech). Available online at: http://www.intechopen.com/books/oxidative-stress-and-diseases/oxidative-stress-in-diabetes-mellitus-is-there-a-role-for-hypoglycemic-drugs-and-or-antioxidants

Erel, O. (2005). A new automated colorimetric method for measuring total oxidant status. *Clin. Biochem.* 38, 1103–1111. doi: 10.1016/j.clinbiochem.2005.08.008

Frohnert, B. I., Sinaiko, A. R., Serrot, F. J., Foncea, R. E., and Moran, A. (2011). Increased adipose protein carbonylation in human obesity. *Obesity* 19, 1735–1741. doi: 10.1038/oby.2011.115

Furukawa, S., Fujita, T., Shimabukuro, M., Iwaki, M., and Yamada, Y. (2004). Increased oxidative stress in obesity and its impact on metabolic syndrome. *J. Clin. Invest.* 114, 1752–1761. doi: 10.1172/JCI21625

Gan, K. X., Wang, C., Chen, J. H., Zhu, C. J., and Song, G. Y. (2013). Mitofusin-2 ameliorates high-fat diet-induced insulin resistance in liver of rats. *World J. Gastroenterol.* 19, 1572–1581. doi: 10.3748/wjg.v19.i10.1572

Giuseppe, R. D., Cossellu, G., Vigna, L., Dicorato, F., De Vita, C., Venturelli, G., et al. (2015). Correlation between salivary and serum oxidized LDL levels: a pilot study on overweight/obese subjects. *J. Oral Pathol. Med.* 44, 884–887. doi: 10.1111/jop.12322

Grimsrud, P. A., Picklo, M. J., Griffin, T. J., and Bernlohr, D. A. (2007). Carbonylation of adipose proteins in obesity and insulin resistance. *Mol. Cell. Proteomics* 6, 624–637. doi: 10.1074/mcp.M600120-MCP200

Kalousová, M., Skrha, J., and Zima, T. (2002). Advanced glycation end-products and advanced oxidation protein products in patients with diabetes mellitus. *Physiol. Res.* 51, 597–604.

Kamata, H., Honda, S., Maeda, S., Chang, L., Hirata, H., and Karin, M. (2005). Reactive oxygen species promote TNFalpha- induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. *Cell* 120, 649–661. doi: 10.1016/j.cell.2004.12.041

Keaney, J. F. J., Larson, M. G., Vasan, R. S., Wilson, P. W., and Lipinska, I. (2003). Obesity and systemic oxidative stress: clinical correlates of oxidative stress in the Framingham Study. *Arterioscler. Thromb. Vasc. Biol.* 23, 434–439. doi: 10.1161/01.ATV.0000058402.34138.11

Knaś, M., Maciejczyk, M., Daniszewska, I., Klimiuk, A., Matczuk, J., Kołodziej, U., et al. (2016a). Oxidative damage to the salivary glands of rats with streptozotocin-induced diabetes-temporal study: oxidative stress and diabetic salivary glands. *J. Diabetes Res.* 2016:4583742. doi: 10.1155/2016/4583742

Knaś, M., Maciejczyk, M., Sawicka, K., Hady Razak, H., Niczyporuk, M., Ładny, J. R., et al. (2016b). Impact of morbid obesity and bariatric surgery on antioxidant/oxidant balance of the unstimulated and stimulated human saliva. *J. Oral Pathol. Med.* 45, 455–464. doi: 10.1111/jop.12383

Lovell, M. A., Soman, S., and Bradley, M. A. (2011). Oxidatively modified nucleic acids in preclinical Alzheimer's disease (PCAD) brain. *Mech. Ageing Dev.* 132, 443–448. doi: 10.1016/j.mad.2011.08.003

Lushchak, V. L. (2014). Classification of oxidative stress based on its intensity. *EXCLI J.* 13, 922–937.

Matczuk, J., Zalewska, A., Łukaszuk, B., Knaś, M., Maciejczyk, M., Grabowska, M., et al. (2016). Insulin resistance and obesity affect lipid profile in the salivary glands. *J. Diabetes Res.* 2016:8163474. doi: 10.1155/2016/8163474

Milagro, F. I., Campión, J., and Martínez, J. A. (2006). Weight gain induced by high-fat feeding involves increased liver oxidative stress. *Obesity* 14, 1188–1123. doi: 10.1038/oby.2006.128

Mori, Y., Muratsu, K., Nara, Y., and Morioka, T. (1990). The histophatological observation of the salivary glands in hamster with streptozotocin induced diabetes. *Fukuoka Igaku Zasshi* 81, 298–302.

Narotzki, B., Reznick, A. Z., Mitki, T., Aizenbud, D., and Levy, Y. (2014). Enhanced cardiovascular risk and altered oxidative status in elders with moderate excessive body fat. *Rejuvenation Res.* 17, 334–340. doi: 10.1089/rej.2013.1540

Nogueira, F. N., Cavalho, A. M., Yamaguti, P. M., and Nicolau, J. (2005). Antioxidant parameters and lipid peroxidation in salivary glands of streptozotocin-induced diabetic rats. *Clin. Chim. Acta* 353, 133–139. doi: 10.1016/j.cccn.2004.11.004

Öngöz Dede, F., Bozkurt Doğan, Ş., Balli, U., Avci, B., Durmuşlar, M. C., and Baratzade, T. (2016). Glutathione levels in plasma, saliva and gingival crevicular fluid after periodontal therapy in obese and normal weight individuals. *J. Periodontol. Res.* doi: 10.1111/jre.12349

Pendyala, G., Thomas, B., and Joshi, S. R. (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, 51–57. doi: 10.4103/1947-2714.106208

Picco, D. C. R., Costa, L. F., Delbem, A. C., Sassaki, K. T., Sumida, D. H., and Antoniali, C. (2012). Spontaneously hypertensive rat as experimental model of salivary hypofunction. *Arch. Oral Biol.* 57, 1320–1326. doi: 10.1016/j.archoralbio.2012.07.008

Qu, D. M., Song, G. Y., Gao, Y., Wang, J., Hu, S. G., and Han, M. (2008). Expression pattern of PGC-1 alpha and Mfn2 in insulin resistance state and after recovery in rat. *Jichu Yixue Yu Linchuang* 28, 133–137.

Reznick, A. Z., and Packer, L. (1994). Oxidative damage to proteins: spectrophotometric method for carbonyl assay. *Meth. Enzymol.* 233, 357–363. doi: 10.1016/S0076-6879(94)33041-7

Romero, A. C., Ibuki, F. K., and Noguiera, F. N. (2012). Sialic acid reduction in the saliva of streptozotocin induced diabetic rats. *Arch. Oral Biol.* 57, 1189–1193. doi: 10.1016/j.archoralbio.2012.02.016

Sabino-Silva, R., Okamoto, M. M., David-Silva, A., Mori, R. C., Freitas, H. S., and Machado, U. F. (2013). Increased SGLT1 expression in salivary gland ductal cells correlates with hyposalivation in diabetic and hypertensive rats. *Diabetol. Metab. Syndr.* 5:64. doi: 10.1186/1758-5996-5-64

Schönfeld, P., Dymkowska, D., and Wojtczak, L. (2009). Acyl-CoA-induced generation of reactive oxygen species in mitochondrial preparations is due to the presence of peroxisomes. *Free Radic. Biol. Med.* 47, 503–509. doi: 10.1016/j.freeradbiomed.2009.05.006

Solinas, G., and Karin, M. (2010). JNK1 and IKKbeta: molecular links between obesity and metabolic dysfunction. *FASEB J.* 24, 2596–2611. doi: 10.1096/fj.09-151340

Stadtman, E. R., and Levine, R. L. (2003). Free radical-mediated oxidation of free amino acids and amino acids residues in proteins. *Amino Acids* 25, 207–218. doi: 10.1007/s00726-003-0011-2

Su, H., Baron, M., Benarroch, M., Velly, A. M., Gravel, S., Schipper, H. M., et al. (2010). Altered salivary redox homeostasis in patients with systemic sclerosis. *J. Rheumatol.* 37, 1858–1863. doi: 10.3899/jrheum.091451

Suba, Z., Barabás, J., Takács, D., Szabó, G., and Ujpál, M. (2005). Epidemiological correlation of insulin resistance and salivary glands tumors. *Orv. Hetil.* 146, 1727–1732.

Tinahones, F. J., Murri-Pierri, M., Garrido-Sánchez, L., García-Almeida, J. M., García-Serrano, S., García-Arnés, J., et al. (2009). Oxidative stress in severely obese persons is greater in those with insulin resistance. *Obesity* 17, 240–246. doi: 10.1038/oby.2008.536

Watanabe, M., Yamagishi-Wang, H., and Kawaguchi, M. (2001). Lowered susceptibility of muscarinic receptor involved in salivary secretion of streptozotocin-induced diabetic rats. *Jpn. J. Pharmacol.* 87, 117–124. doi: 10.1254/jjp.87.117

Zalewska, A., Knaś, M., Kuźmiuk, A., Waszkiewicz, N., Niczyporuk, M., Waszkiel, D., et al. (2013). Salivary innate defense system in type 1 diabetes mellitus in children with mixed and permanent dentition. *Acta Odontol. Scand.* 71, 1493–1500. doi: 10.3109/00016357.2013.773071

Zalewska, A., Knaś, M., Maciejczyk, M., Waszkiewicz, N., Klimiuk, A., Choromańska, M., et al. (2015). Antioxidant profile, carbonyl and lipid oxidation markers in the parotid and submandibular glands of rats in different periods of streptozotocin induced diabetes. *Arch. Oral Biol.* 60, 1375–1386. doi: 10.1016/j.archoralbio.2015.06.012

Zalewska, A., Knaś, M., Żendzian-Piotrowska, M., Waszkiewicz, N., Szulimowska, J., Prokopiuk, S., et al. (2014b). Antioxidant profile of salivary glands in high fat diet- induced insulin resistance rats. *Oral Dis.* 20, 560–566. doi: 10.1111/odi.12173

Zalewska, A., Knaś, M., Waszkiewicz, N., Klimiuk, A., Litwin, K., Sierakowski, S., et al. (2014a). Salivary antioxidants in patients with systemic sclerosis. *J. Oral Pathol. Med.* 43, 61–68. doi: 10.1111/jop.12084

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frontiers in Physiology

REVIEW
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Does Oxidative Stress Induced by Alcohol Consumption Affect Orthodontic Treatment Outcome?

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HIGHLIGHTS

- Ethanol, Periodontal ligament, Extracellular matrix, Orthodontic movement.

Alcohol is a legal drug present in several drinks commonly used worldwide (chemically known as ethyl alcohol or ethanol). Alcohol consumption is associated with several disease conditions, ranging from mental disorders to organic alterations. One of the most deleterious effects of ethanol metabolism is related to oxidative stress. This promotes cellular alterations associated with inflammatory processes that eventually lead to cell death or cell cycle arrest, among others. Alcohol intake leads to bone destruction and modifies the expression of interleukins, metalloproteinases and other pro-inflammatory signals involving GSKβ, Rho, and ERK pathways. Orthodontic treatment implicates mechanical forces on teeth. Interestingly, the extra- and intra-cellular responses of periodontal cells to mechanical movement show a suggestive similarity with the effects induced by ethanol metabolism on bone and other cell types. Several clinical traits such as age, presence of systemic diseases or pharmacological treatments, are taken into account when planning orthodontic treatments. However, little is known about the potential role of the oxidative conditions induced by ethanol intake as a possible setback for orthodontic treatment in adults.

Keywords: ethanol, oxidative stress, orthodontic movement, periodontal ligament, orthodontic treatment

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INTRODUCTION

Since prehistoric ages alcohol (chemically known as ethyl alcohol or ethanol; EtOH) has been consumed by humans. In fact, EtOH is present in beer, wine, spirits and many other drinks. In fact, these products are usually consumed on a daily basis in several countries worldwide. EtOH dependence and abuse are the most abundant mental disorders worldwide. In America, approximately 14% of the population meets chronic alcoholic criteria during some period of their lives (Elkstrom and Ingelman-Sundberg, 1989; Caro and Cederbaum, 2004). EtOH is involved in almost 50% of traffic accidents, the majority of homicides, suicides and domestic violence cases (Graham et al., 1998; Ofori-Adjei et al., 2007). Additionally, EtOH is also implicated in several organic diseases as well as in diverse forms of cancer, including oral cancer (Nelson et al., 2013).

Many reports are available on EtOH-related oral health (Kranzler et al., 1990; Franceschi, 1993; Gelbier and Harris, 1996). There are some important studies on gingival margin recession and oral mucosae alterations (Harris et al., 1996, 2004; Khocht et al., 2003, 2013). Little evidence is reported about the direct effects of EtOH consumption on dental tissues, aside from cancer. One important reason to explain this lack of evidence is the existence of a diversity of factors in connection with alcoholism, e.g., vitamin/nutrients deficiency, other drugs of abuse, smoking, deficient oral care, caries, etc. All these elements impair the identification of concrete factors directly and exclusively related to EtOH in oral diseases (Gelbier and Harris, 1996; Marques et al., 2015).

According to the American Association of Orthodontists, around 4.8 million Americans wear braces. From 1994 to 2010, the percentage of adults getting braces rose from 680,000 to 1.1 million a year (58%). This fact suggests that a relevant number of adults getting braces or other tooth-related devices might consume EtOH along a great part of the orthodontic treatment.

There are several, and sometimes unknown, factors that determine orthodontic outcome. This review focuses on the potential role of EtOH consumption during orthodontic treatment as a plausible factor affecting orthodontic outcome. Surprisingly, EtOH exposure and orthodontic movement affect the same cellular and molecular signaling pathways, giving support to this hypothesis.

GENERAL AND LOCAL EtOH METABOLISM: OXIDATIVE STRESS AND EtOH-RELATED DISEASES

Because the liver is the main EtOH-detoxifying organ, EtOH-induced alterations have been mostly studied in hepatic tissue. However, nervous tissue, diverse connective-related tissues and others are also affected. EtOH exerts its deleterious effects in several tissues via oxidative and non-oxidative metabolic pathways (Bondy and Guo, 1995) involving free radical production and lipid peroxidation (Sun et al., 1997; Bosch-Morell et al., 1998; Ramachandran et al., 2003; Almansa et al., 2013; Flores-Bellver et al., 2014). One of the most important factor in this toxic process deals with the properties of EtOH to promote reactive oxygen species (ROS). These ROS ultimately react with macromolecules, among them membrane lipids, producing aldehydes such as 4-Hydroxynonenal (4-HNE) and Malondialdehyde (MDA). It is well known that aldehydes and ROS can directly affect both proteins or DNA, leading to transcription-repression of concrete genes. In fact, the role of ROS and aldehydes seems to be a key factor for these alterations, partially confirmed by the fact that administration of antioxidants prevents these EtOH-induced cellular alterations (Herrera et al., 2003; Bati et al., 2015; Han et al., 2015).

Cytochrome P450 and alcohol dehydrogenase (ADH) are the most relevant enzymes involved in EtOH metabolism. Both enzymes can be found not only in liver but also in other tissues (detailed below). The cytochrome P450 family proteins are involved in the oxidative metabolism of both endogenous and xenobiotic products (Tsutsumi et al., 1993; Miksys and Tyndale, 2002). It is known that CYP2E1 isoform is specifically involved in EtOH oxidation; furthermore, CYP2E1 has more affinity for EtOH than alcohol dehydrogenase (ADH) (Albano, 2008). In fact, CYP2E1 assumes an important role in ethanol metabolism, being considered as a major component of the microsomal ethanol-oxidizing system (MEOS) (Lieber and DeCarli, 1970; Koop et al., 1982). Despite EtOH being mostly catabolized in the liver by CYP2E1, the presence of CYP2E1 and ADH in other tissues indicates that EtOH could also be processed by a non-hepatic route (Martinez-Gil et al., 2015).

CYP2E1 is present in the digestive system, one of the most threatening environments because it is continuously exposed to different media containing chemicals, toxins, etc. In fact, CYP2E1 and ADH are strongly expressed not only in liver and the digestive tract, but also in other human oral cells as gingival fibroblasts, pulp, tongue and osteoblasts (Redetzki, 1960; Dong et al., 1996; Chen et al., 2006; Reichl et al., 2010; Plapp et al., 2015). Interestingly enough, it is well established that there is a good relation between CYP2E1 and EtOH in several digestive-related forms of cancer, e.g., mouth, pharynx, esophagus, colorectum and liver cancer (reviewed by Seitz and Wang, 2013). The presence of CYP2E1 and ADH in other cell types could explain a local and direct EtOH-detoxifying process (Flores-Bellver et al., 2014). In this sense, ethanol diffuses rapidly into saliva. Thirty minutes after alcohol intake, EtOH salivary and plasmatic levels are equilibrated. At the same time the levels of acetaldehyde in saliva exceed the systemic blood levels. Acetaldehyde and ethanol from saliva easily reach all the local tissues (Waszkiewicz et al., 2011, 2012; Zalewska et al., 2011). So it seems reasonable that EtOH and acetaldehyde can directly affect oral related structures.

Despite the fact that ADH has lower affinity for EtOH than CYP2E1, ADH is also relevant for EtOH detoxification. CYP2E1 and ADH are both present in the liver (Redetzki, 1960; Plapp et al., 2015) and also expressed in human attached gingiva and tongue (Dong et al., 1996). Surprisingly enough, whereas ADH is expressed in stromal osteoblasts, CYP2E1 seems to be unexpressed (Chen et al., 2006). Although these enzymes are not ubiquitously present in all tissues, their presence in liver and other tissues, clearly indicates the existence of extra-hepatic EtOH metabolism and that it might be related with some EtOH-related forms of cancer (Seitz and Wang, 2013).

PERIODONTUM, EXTRACELLULAR MATRIX, AND BONE DYNAMICS

The periodontum must be briefly presented as a complex histological area surrounding teeth relevant for root-tooth stability. This periodontal structure includes fibroblasts surrounded by the extracellular matrix (ECM) of hyaluronic acid (HA) and other extracellular proteins as collagen, mostly produced by periodontal fibroblasts. The most abundant collagen form is the type I collagen (Bornstein and Sage, 1980; Zhang et al., 1993).

Other important components of the periodontal ligament (PDL) are the matrix metalloproteinase enzyme family (MMP's) that degrade collagen, and its counterpart, tissue inhibitor metalloproteinases (TIMP) that do inhibit MMP's, being MMP-1 enzyme the most abundant in PDL (Birkedal-Hansen et al., 1993). Obviously, the balance between collagen production and MMP's activity determines the PDL quality and consequently dental stability. MMP's also degrade collagen under pathological conditions and therefore MMP-1, MMP-8, MMP-2, MMP-13 are locally and temporarily expressed during tooth movement phases (Apajalahti et al., 2003; Ingman et al., 2005; Cantarella et al., 2006; Leonardi et al., 2006; Huang et al., 2008; Meeran, 2012).

Root and bone resorption are both directly regulated by a group of tumor necrosis factor (TNF)-related proteins with paracrine-regulatory properties (Schoppet et al., 2002). Osteoprotegerin (OPG) is a soluble protein secreted by osteoblasts that acts as an inhibitor of both osteoclast differentiation and resorptive activity, promoting osteoclast apoptosis (Oshiro et al., 2002). Receptor activator of nuclear factor kappa-b ligand (RANKL) is expressed on the cell surface of osteoblast precursors (Schoppet et al., 2002), whereas its receptor (RANK) is expressed by osteoblastic cell lineages and activated T-cells (Katagiri and Takahashi, 2002). RANKL acts, together with macrophage colony stimulating-factor (M-CSF), promoting osteoclast formation, differentiation and activation, enhancing bone resorption activities (Kong et al., 1999; Liu and Zhang, 2015; Martin and Sims, 2015).

One important step for osteoclast fusion and activation is the coupling of RANK to RANKL. This union can be blocked by OPG, so the balance "resorption vs. reposition" depends on the prevalence of RANK vs. OPG, respectively.

ORTHODONTIC FORCES AFFECT PERIODONTAL STRUCTURES MODIFYING INTRA- AND EXTRA-CELLULAR PROTEINS

Orthodontic Forces Lead to Extracellular Modifications

During orthodontic movement, applied forces modulate both molecular and cellular configurations, e.g., those producing collagen (Bumann et al., 1997), modifying the periodontal structure and therefore dental position (Nakagawa et al., 1994; Krishnan and Davidovitch, 2006). Some evidence indicates that mechanical forces modulate the expression of integrins, MMP's or collagen (Bolcato-Bellemin et al., 2000; Von den Hoff, 2003; He et al., 2004). On the hypothetical model for periodontal remodeling summarized by Meikle (2006), tension and compressive sides present some similarities. In the tensile strain, periodontal fibroblasts release IL-1 and IL-6; these interleukins can stimulate MMP's and inhibit TIMP synthesis, so bone and matrix lose structure in order to facilitate bone and PDL regeneration. At the same time, mechanically activated fibroblasts can induce angiogenesis by vascular endothelial growth factor (VEGF) release, helping bone renewal. In the compression side, similarly to the tensile side, IL-1, IL-6, and MMP's are released. One of the differences between both complementary processes seems to be the prevalence of OPG vs. RANK, leading to bone reconstruction and bone destruction, respectively (Tyrovola et al., 2008).

This represents an interesting issue for orthodontics or periodontal management, since both conditions involve these type of cellular responses, e.g., during tooth movement or periodontal disease. In this regard, some reports have found different biological markers in the gingivo-crevicular fluid (GCF): elevated levels of Prostaglandin E, IL-1β, IL-6, TNF-α and epidermal growth factor (EGF) have been found in GCF during tooth movement or periodontal disease (Grieve et al., 1994; Uematsu et al., 1996). Hyaline material and sterile necrosis in local pressure zones have been found also after tooth movement (Kurol and Owman-Moll, 1998). Unfortunately, the significance of these changes is only partially known.

Extracellular matrix degradation facilitates cell proliferation and capillary growth leading to the synthesis of new PDL and bone structures. However, on the compression side, periodontal cells also release IL-1 and IL-6, up-regulating not only MMP's, but also RANKL, leading to osteoclast-mediated bone resorption (Nakano et al., 2011).

Cathepsins are lysosomal cysteine proteases that play an important role in bone resorption. Cathepsin B levels can be increased by orthodontic tooth movement, being involved in extracellular matrix degradation in response to mechanical stress (Maeda et al., 2007). Since Cathepsins K, B and L are over-expressed in the compression side, they may be related to bone resorption (Domon et al., 1999; Sugiyama et al., 2003).

Orthodontic Forces Lead to Intracellular Modifications

Mechanical strain generates diverse intracellular responses in cells during orthodontic movements that could be of clinical interest. Integrins are transmembrane proteins whose extracellular side connects to the ECM via fibronectin (Wang et al., 1993; Clarke and Brugge, 1995), and the intracellular one connects with actin of the cytoskeleton. In fact, this actin-cytoskeletal connection is mediated by proteins as paxillin, talin and vinculin leading to focal adhesions that are crucial for cell adhesion and migration (Sastry and Burridge, 2000; Meikle, 2006). Although little is known about the role of integrin receptors in ECM for cell adhesion, the intracellular side is associated to cAMP and inositol phosphate activation pathways both involved in downstream cell signaling (Wang et al., 1993; DeMali et al., 2003). Well known integrin-mediated extracellular signals are mitogen-activated protein kinases (MAPKs) and Rho pathways, both are activated by mechanic stimuli in periodontal fibroblasts and osteoblasts (Basdra et al., 1995; Peverali et al., 2001).

MAPKs regulate several cellular responses such as cell division, metabolic processes, survival-apoptosis and differentiation, among others. Five distinct groups of MAPKs have been characterized in mammals: extracellular signal-regulated kinases (ERKs) 1 and 2 (namely ERK1/2), c-Jun amino-terminal kinases (JNKs) 1, 2, and 3, p38 isoforms α,

β, γ, and δ, ERKs 3 and 4, and ERK5 (reviewed by Chen et al., 2001; Kyriakis and Avruch, 2001; Roux and Blenis, 2004). ERK 1 and 2 have been described to, when activated, phosphorylate membrane proteins (CD120a, Syk, and calnexin), nuclear substrates and cytoskeletal proteins (neurofilaments and paxillin) (reviewed by Chen et al., 2001; Roux and Blenis, 2004). Cyclic tensile forces up-regulate bone marrow protein-2 (BMP-2) expression via ERK1/2 and p38 MAP kinase pathways, with COX and PGE2 implication, in human periodontal ligament cells (Suzuki et al., 2014) and promote the migration of periodontal cells via ERK signaling pathway activation (Pan et al., 2010). Moreover, multiple data indicates that mechanical forces modulate ERK activities in periodontal fibroblasts increasing type I collagen, ostepontin and MMP-1 production (Liedert et al., 2006; Jeon et al., 2009; Kook et al., 2009, 2011).

Also related to extracellular stimuli-mediated signaling, Rho is a family of serine/threonine kinases (Wennerberg et al., 2005; Bustelo et al., 2007) involved in cell recruitment-migration, proliferation and apoptosis (Ridley, 2001; Etienne-Manneville and Hall, 2002). It has been recently described that Rho is involved in experimental orthodontic tooth movement by increasing Rho-kinase (ROCK) activity on the tension side (Meng et al., 2015). This fits with the finding that ROCK1 acts as a suppressor of inflammatory cell migration by regulating PTEN phosphorylation (Vemula et al., 2010). In line with this, it has been reported that Rho-ROCK enhances the formation of actin stress fibers and focal adhesion in fibroblasts (Amano et al., 1997). More concretely, during experimental orthodontic movement, the tension areas showed increased expression of actin stress fibers as well as increased number of myofibroblasts in the periodontal area (Meng et al., 2010, 2007). These facts are related to focal adhesion phenomena related to dental movement.

Not related to integrin-mediated signaling, Toll-like receptors (TLRs) are transmembrane proteins playing a critical role in innate immune system. TLRs are made up of an extracellular and of a cytoplasmic domain, homologous to the cytoplasmic domain of the human IL-1 receptor (Medzhitov, 2001). TLRs can recognize different patterns, known as pathogen-associated molecular patterns (PAMP). These PAMPs include lipids, proteins, lipoproteins, nucleic acids, and lipopolysaccarides (LPS) (Medzhitov and Janeway, 1997; Yang et al., 1998). TLR4 is particularly interesting in oral tissues because it is highly expressed by periodontal fibroblasts and specifically recognizes *Porphyromonas gingivalis* LPS (Takeuchi and Akira, 2001). The activation of TLR4 promotes pro-inflammatory signaling processes leading to periodontal alterations, osteoclast activation-recruitment and cytokine expression (Kikkert et al., 2007; Gelani et al., 2009; Nussbaum et al., 2009). TLR4 has been recently associated with mechanical forces on fibroblasts: its activation increased the expression of MMP-1, 3, and 10, increased phosphorylation of p38, JNK, and NF-κB, strongly suggesting that TLR4 may play an important role during orthodontic treatment (Lisboa et al., 2013). Hyaluronic acid (HA) is a classic and abundant component of connective tissue also present in the PDL. HA is an endogenous ligand for TLR4 that promotes protective responses in skin and lung injury models (Jiang et al., 2005; Taylor et al., 2007). Although the anti-inflammatory properties of HA and its mechanisms are partially unknown, direct interactions with inflammatory cells and the physical properties of the molecule, seem to be implicated. It is shown that HA reduces TNF-α and IFN-γ production and induces NF-κB activation in macrophages (Noble et al., 1996; Wang et al., 2006). As an example, this TLR4-HA interaction seems to be related to Cox-2 and PGE_2 production to protect the colon mucosa from injury (Chen et al., 2011). More research is needed to explain the concrete role and mechanism of the HA-TLR4 interaction that could make it be of interest for orthodontic and periodontal clinical care.

A graphic summary of the periodontal area with the extracellular processes is detailed in **Figures 1**, **2**.

EtOH MODULATES EXTRACELLULAR PROTEINS AND PROMOTES INTRACELLULAR CHANGES

EtOH Modifies Extracellular Protein Activities and Bone Dynamics

It is well documented how EtOH affects osteoclastic/osteoblastic dynamics producing osteopenia and osteoporosis (Manolagas, 2000; Turner, 2000). Although the mechanisms are not fully understood, EtOH may promote bone loss inhibiting osteoblastogenesis (Friday and Howard, 1991) by altering bone remodeling-related genes (Chakkalakal, 2005; Callaci et al., 2009). It has also been shown an inverse correlation between EtOH intake and bone mineral density in both pre- and post-menopausal women (Turner and Sibonga, 2001). IL-6 seems to be responsible, at least in part, for this EtOH-induced bone loss (Dai et al., 2000). Interestingly enough, IL-6 is also increased during orthodontic movement (Grieve et al., 1994; Uematsu et al., 1996) and therefore it seems plausible that EtOH consumption during orthodontic treatment would affect the outcome of the intervention via IL-6. More research is needed to analyze the IL-6 levels and bone remodeling under these circumstances (EtOH+ orthodontic forces).

Some reports indicate that EtOH exposure preferentially alters the periodontal area, developing periodontitis by increasing the loss of attachment through recession of gingival margins (Khocht et al., 2003) or by altering the oral mucosa (Harris et al., 1996, 2004). Regarding the influence of EtOH and other drugs on tooth decay, some studies focus the attention on the EtOH-induced oral micro-flora alterations due to EtOH-acetaldehyde metabolism, leading to the progression of dental caries (Dasanayake et al., 2010; Rooban et al., 2011), and little is known about the role of EtOH on orthodontic movement.

Estrogens can protect from bone resorption (Kousteni et al., 2001; Chen et al., 2005) and this inhibitory effect seems to be related to the RANKL-RANK-OPG system (Syed and Khosla, 2005). In fact, estrogens can suppress RANKL expression in osteoblasts (Bord et al., 2003). Chen et al. (2006, 2008) found that the protective effects of estradiol on EtOH-induced bone loss was related to the inhibition of ROS production in osteoblasts. Additionally, NADPH oxidase (NOx) and estradiol

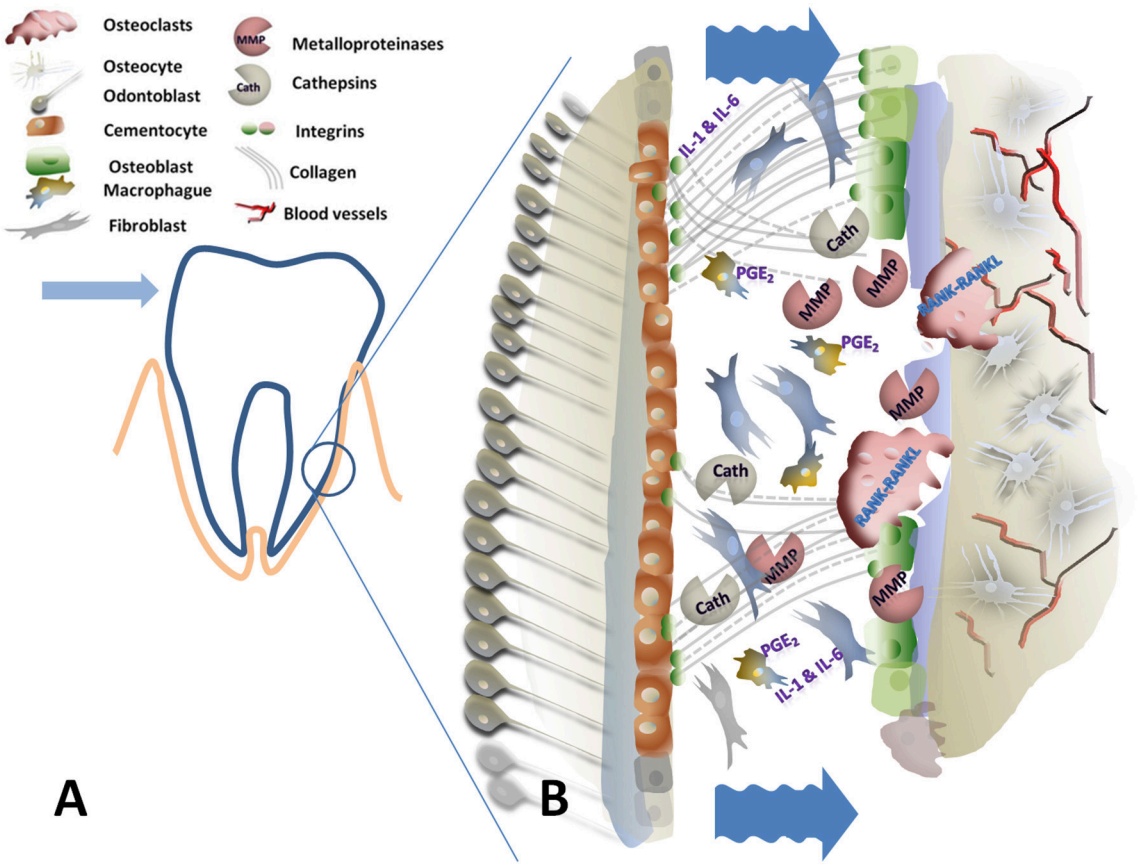

FIGURE 1 | Graphical scheme of the periodontal ligament and alveolar bone under compressive forces. (A) Is the schematic representation of a tooth in the socket where the arrowhead indicates the sense of the applied force and the encircled area represents the compressive side. **(B)** Detailed view of the compressive side: Wrinkled arrows indicate the reduction on the periodontal gap due to compression. This mechanical signal affects cells and extracellular matrix components promoting extracellular release of matrix degrading enzymes as Metalloproteinases (MMP) and Cathepsins (Cath), macrophage activation (IL1, 6, and PGE2) and RANK-RANKL osteoclast activation. This results on bone resorption with periodontal destruction-reconstruction in the new dental position.

would play a critical role on EtOH-induced bone loss via the ERK/STAT3/RANKL pathway.

It has been demonstrated that chronic EtOH consumption promotes bone loss, increases PGE2 expression and other inflammatory markers in rats (Dantas et al., 2012; Surkin et al., 2014). All these markers are related to periodontal disease, so the hypothesis of EtOH-induced oxidative burden as a modulator of the extracellular environment during tooth movement is supported.

Some reports indicate that MMP-1, Cathepsins K, B, and L are increased in the compression side during tooth movement (Domon et al., 1999; Sugiyama et al., 2003; Maeda et al., 2007). EtOH-induced osteoclastogenesis increases RANK and Cathepsin K activities (Domon et al., 1999). However, it has been shown that EtOH reduces proteolytic activity in hepatic Cathepsins B and L (Kharbanda et al., 1995, 1996). EtOH and tooth movement may act in the same way by increasing bone resorption and PDL remodeling in the compression side despite this tissue difference. Future studies should be addressed to know whether this fact is synergistic or accumulative leading to excessive bone resorption and eventually to root resorption.

EtOH Promotes Intracellular Responses

EtOH affects intestinal epithelial tight junction integrity via Ca^{++}-mediated Rho/ROCK activation (Elamin et al., 2014). It has been described that EtOH exposure disorganizes actin-cytoskeleton in astrocytes and this process is mediated by RhoA signaling pathway (Guasch et al., 2003). Although nothing is known about the effect of EtOH on the cytoskeletal periodontal fibroblasts and osteoblasts, some evidence indicates that both EtOH and periodontal movement act in the same Rho-ROCK pathway. It seems reasonable that EtOH exposure during orthodontic movement may alter the cytoskeletal organization affecting orthodontic outcome.

ROS is a relevant extracellular ERK-triggering stimulus that up-regulates ERK-dependent genes such as RANKL (Torres, 2003). Supporting this fact, it was found that the administration of antioxidants such as N-Acetyl cysteine, estradiol or vitamin C, suppress RANKL mRNA expression and induces PDL progenitor cell differentiation via ERK activation pathway (Chen et al., 2008; Yan et al., 2013). In this regard, it is well documented that EtOH metabolism results in ROS production and subsequently leads to

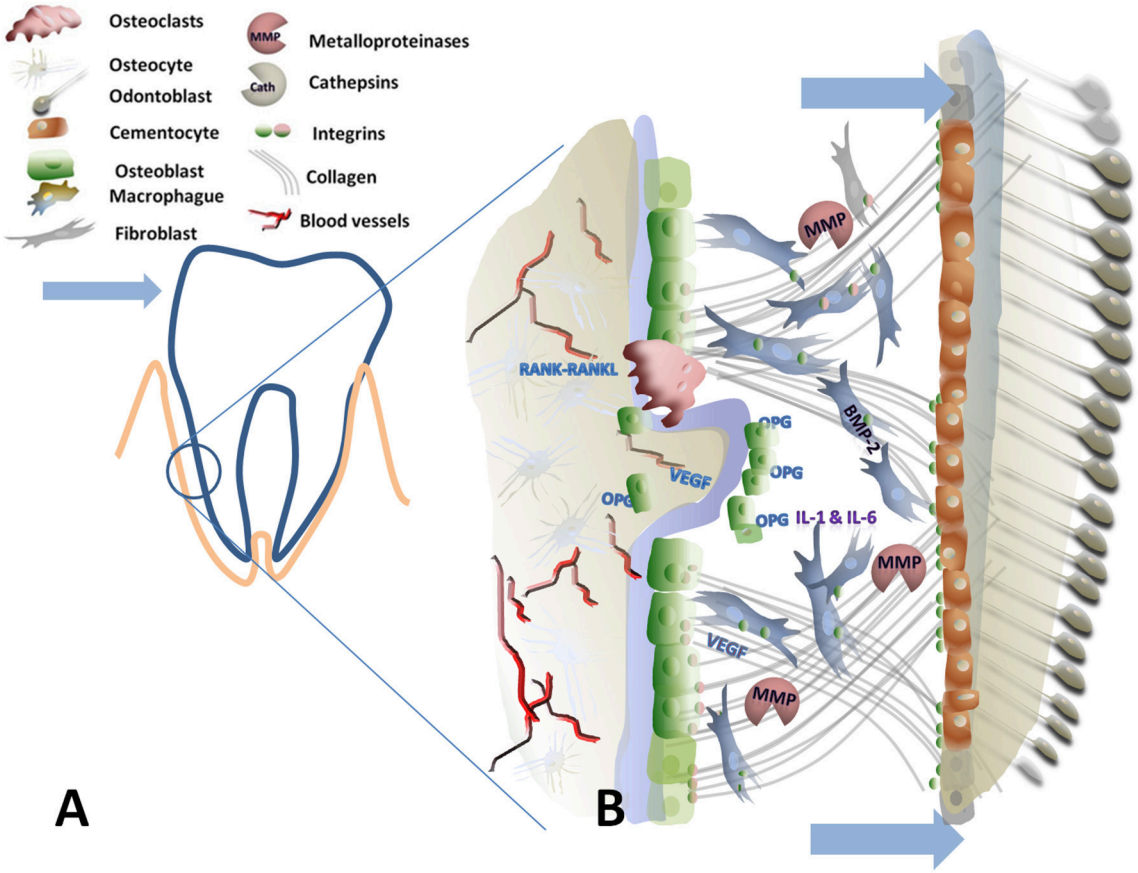

FIGURE 2 | Graphical scheme of the periodontal ligament and alveolar bone under tensile forces. (A) Is the schematic representation of a tooth in the socket where the arrowhead indicate the applied force. The encircled area represents the tensile side. **(B)** Detailed view of the tensile side: Arrows indicate the increase on the periodontal gap due to tensile force. Tensile forces are transmitted via collagen-coupled Integrins to different cell types promoting new bone generation. Bone marrow protein 2 (BMP-2), osteoprotegerin (OPG), vascular endothelial growth factor (VEGF) are necessary for new bone formation.

cell damage and eventually death (Johnsen-Soriano et al., 2007; Flores-Bellver et al., 2014). Even more, EtOH-induced oxidative stress seems to be crucial for these negative effects on cells, since the administration of antioxidants restores the oxidative misbalance and prevents the negative effects on cells (Herrera et al., 2003; Koch et al., 2004; Crews et al., 2007). ROS and EtOH activate MMP- 1,-2, and -9 via protein tyrosine kinase signaling, leading to basal membrane disruption (Haorah et al., 2007, 2008).

It is well documented that EtOH promotes inflammatory responses via TLR4 in different tissues, e.g., brain, lung and liver (Vaneker et al., 2008; Fernandez-Lizarbe et al., 2013; Zmijewski et al., 2014; Pascual et al., 2015). So, after considering the aforementioned data on TLR4, it seems plausible that EtOH exposure could be closely related to periodontal stability (see **Figure 3**) and therefore it becomes an important factor on clinical practice.

Glycogen synthase kinase 3 β (GSK3β) regulates the production of cytokines after TLR4 stimulation (Martin et al., 2005). TLR4-GSK3β route activation has been closely related to periodontal alterations induced by *P. gingivalis* and other pathogens (Wang et al., 2011). In this sense, and fitting with this, it has been shown how alcoholic fatty liver pathogenesis implicates GSK3β route activation (Zeng et al., 2014) and it has been demonstrated that GSK3β inhibition suppresses bacterial-induced periodontal bone loss (Adamowicz et al., 2012), supporting the idea that TLR4-GSK3β pathway could be particularly affected in alcohol-users. It is well documented that EtOH promotes inflammatory responses via TLR4 in different tissues, e.g., brain, lung and liver (Vaneker et al., 2008; Fernandez-Lizarbe et al., 2013; Zmijewski et al., 2014; Pascual et al., 2015). Considering the aforementioned data on TLR4, it seems plausible that EtOH exposure could be closely related to periodontal stability (see **Figure 3**) and therefore it becomes an important factor on clinical practice.

FINAL DISCUSSION

Despite the fact that several studies indicate the potential deleterious effect of EtOH on periodontum and alveolar

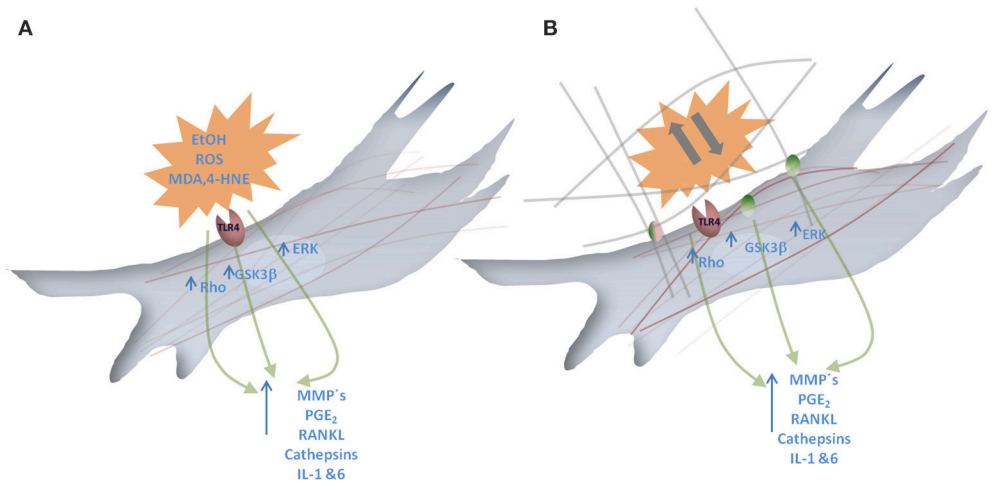

FIGURE 3 | Graphical scheme of the molecular responses promoted by EtOH exposure and mechanical forces. (A) Represents a fibroblast where EtOH and EtOH-derived reactive oxygen species (ROS), lipid peroxidation products such as 4-hydroxynonenal (4-HNE) and Malondialdehyde (MDA) interact with Toll like receptor4 (TLR4). This interaction results in Rho, GSKβ and ERK pathway activation that leads to MMP/Cath, PGE_2, RANKL and IL-1 and 6 release. **(B)** Representation of a fibroblast where mechanical forces affecting extracellular matrix can interact with integrins. This interaction results in Rho, GSKβ, and ERK pathway activation that leads to MMP/Cath, PGE_2, RANKL and IL-1 and 6 release.

bone, there is only one report on the effects of ethanol during orthodontic movement. In this work, Araujo and collaborators describe less bone resorption at the end of tooth movement suggesting a delay of tooth movement in a rat model of binge drinking (Araujo et al., 2014). Obviously, this model simulates a drinking pattern where high EtOH concentrations are acutely consumed, which is different from the chronic pattern, where high EtOH levels are daily maintained for several weeks. According to the typically long lasting orthodontic treatments, mild and chronic EtOH exposure could interfere with this chronic orthodontic treatment by modifying the aforementioned proteins and genes leading to orthodontic failure or undesired outcome.

Systemic diseases are of relevance in oral medicine and dentistry. *Diabetes mellitus* (DM) is considered a common systemic disease with oral manifestations and profuse literature deals with the considerations of orthodontic treatment on diabetic patients (Burden et al., 2001; Vernillo, 2001; Bensch et al., 2003; McKenna, 2006). Experimental data widely show that DM promotes molecular and structural changes in the periodontal area after orthodontic treatment including MMP's or bone alterations (Feng et al., 2007; Abbassy et al., 2010; Braga et al., 2011; Villarino et al., 2011; Zhang et al., 2011). There are some similarities between alcohol exposure and DM in terms of molecular signaling and gene expression (Barcia et al., 2015). Additionally, experimental and clinical studies strongly indicate a close relationship between alcohol intake and risk of diabetes development (Cullmann et al., 2012; Kim et al., 2013). In consonance with this issue, orthodontic movement promotes intra and extracellular alterations, finally affecting periodontum and alveolar bone. Since the influence of DM in orthodontic treatment outcome seems clear, it seems appropriate to further investigate the effects of chronic EtOH exposure on orthodontic treatment.

As a hypothetical model, EtOH exposure during orthodontic movement may interfere with osteogenesis at the tension side, accepting that EtOH produces osteoblastogenesis inhibition (Friday and Howard, 1991). IL-6/ROS and PGE2 mediated bone loss is induced by EtOH (Dai et al., 2000; Chen et al., 2006) and it also increases RANKL (Chen et al., 2008). Additionally, as mentioned above, TLR4 over-activation (EtOH+ tensile strain) may lead to GSKβ activation, negatively affecting the periodontum (Kikkert et al., 2007; Gelani et al., 2009; Nussbaum et al., 2009). On the compression side, where bone destruction and reorganization takes place, probably bone resorption would be increased during EtOH metabolism leading to a rapid but unstable teeth position.

In view of the close similarities found between EtOH- and mechanical strain-induced responses on periodontal tissues, the aim of this review is to spark attention on the potential effect of EtOH consumption during orthodontic or periodontal treatment as a factor that needs to be considered in clinical practice. Further research is necessary to fully and experimentally support the actual indications suggesting that alcoholic beverages consumption should be discouraged during orthodontic treatment in adults.

AUTHOR CONTRIBUTIONS

JB proposed the subject of the revision and distributed the tasks; supervised each of the topics that were revised.

SP, VA, and LP revised the existing literature on tooth movement regulation and effects of alcohol on oral diseases. AU and GP supervised references content and the different perspectives studied. VV and VA supervised manuscript writing. FR supervised the whole manuscript and its final version.

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REFERENCES

Abbassy, M. A., Watari., I., and Soma, K. (2010). The effect of *Diabetes mellitus* on rat mandibular bone formation and microarchitecture. *Eur. J. Oral Sci.* 118, 364–369. doi: 10.1111/j.1600-0722.2010.00739.x

Adamowicz, K., Wang, H., Jotwani, R., Zeller, I., Potempa, J., and Scott, D. A. (2012). Inhibition of GSK3 abolishes bacterial-induced periodontal bone loss in mice. *Mol. Med.* 18, 1190–1196. doi: 10.2119/molmed.2012.00180

Albano, E. (2008). Oxidative mechanisms in the pathogenesis of alcoholic liver disease. *Mol. Aspects Med.* 29, 9–16. doi: 10.1016/j.mam.2007.09.004

Almansa, I., Barcia, J. M., López-Pedrajas, R., Muriach, M., Miranda, M., and Romero, F. J. (2013). Naltrexone reverses ethanol-induced rat hippocampal and serum oxidative damage. *Oxid. Med. Cell. Longev.* 2013:296898. doi: 10.1155/2013/296898

Amano, M., Chihara, K., Kimura, K., Fukata, Y., Nakamura, N., Matsuura, Y., et al. (1997). Formation of actin stress fibers and focal adhesions enhanced by rho-kinase. *Science* 275, 1308–1311. doi: 10.1126/science.275.5304.1308

Apajalahti, S., Sorsa, T., Railavo, S., and Ingman, T. (2003). The *in vivo* levels of matrix metalloproteinase-1 and -8 in gingival crevicular fluid during initial orthodontic tooth movement. *J. Dent. Res.* 82, 1018–1022. doi: 10.1177/154405910308201216

Araujo, C. M., Johann, A. C., Camargo, E. S., and Tanaka, O. M. (2014). The effects of binge-pattern alcohol consumption on orthodontic tooth movement. *Dental Press J. Orthod.* 19, 93–98. doi: 10.1590/2176-9451.19.6.093-098.oar

Barcia, J. M., Flores-Bellver, M., Muriach, M., Sancho-Pelluz, J., Lopez-Malo, D., Urdaneta, A. C., et al. (2015). Matching diabetes and alcoholism: oxidative stress, inflammation, and neurogenesis are commonly involved. *Mediators Inflamm.* 2015:624287. doi: 10.1155/2015/624287

Basdra, E. K., Papavassiliou, A. G., and Huber, L. A. (1995). Rab and rho GTPases are involved in specific response of periodontal ligament fibroblasts to mechanical stretching. *Biochim. Biophys. Acta* 1268, 209–213. doi: 10.1016/0167-4889(95)00090-F

Bati, B., Celik, I., and Dogan, A. (2015). Determination of hepatoprotective and antioxidant role of walnuts against ethanol-induced oxidative stress in rats. *Cell. Biochem. Biophys.* 71, 1191–1198. doi: 10.1007/s12013-014-0328-3

Bensch, L., Braem, M., Van Acker, K., and Willems, G. (2003). Orthodontic treatment considerations in patients with *Diabetes mellitus*. *Am. J. Orthod. Dentofacial Orthop.* 123, 74–78. doi: 10.1067/mod.2003.53

Birkedal-Hansen, H., Moore, W. G., Bodden, M. K., Windsor, L. J., Birkedal-Hansen, B., DeCarlo, A., et al. (1993). Matrix metalloproteinases: a review. *Crit. Rev. Oral Biol. Med.* 4, 197–250. doi: 10.1177/10454411930040020401

Bolcato-Bellemin, A. L., Elkaim, R., Abehsera, A., Fausser, J. L., Haikel, Y., and Tenenbaum, H. (2000). Expression of mRNAs encoding for α and β integrin subunits, MMPs, and TIMPs in stretched human periodontal ligament and gingival fibroblasts. *J. Dent. Res.* 79, 1712–1716. doi: 10.1177/00220345000790091201

Bondy, S. C., and Guo, S. X. (1995). Regional selectivity in ethanol-induced pro-oxidant events within the brain. *Biochem. Pharmacol.* 6, 69–72. doi: 10.1016/0006-2952(94)00442-O

Bord, S., Ireland, D. C., Beavan, S. R., and Compston, J. E. (2003). The effects of estrogen on osteoprotegerin, RANKL, and estrogen receptor expression in human osteoblasts. *Bone* 32, 136–141. doi: 10.1016/S8756-3282(02)00953-5

Bornstein, P., and Sage, H. (1980). Structurally distinct collagen types. *Ann. Rev. Biochem.* 49, 957–1003. doi: 10.1146/annurev.bi.49.070180.004521

Bosch-Morell, F., Martínez-Soriano, F., Colell, A., Fernández-Checa, J. C., and Romero, F. J. (1998). Chronic ethanol feeding induces cellular antioxidants decrease and oxidative stress in rat peripheral nerves. Effect of S-adenosyl-L-methionine and N-acetyl-L-cysteine. *Free Radic. Biol. Med.* 25, 365–368. doi: 10.1016/S0891-5849(98)00036-7

Braga, S. M., Taddei, S. R., Andrade, I. Jr., Queiroz-Junior, C. M., Garlet, G. P., Repeke, C. E., et al. (2011). Effect of diabetes on orthodontic tooth movement in a mouse model. *Eur. J. Oral Sci.* 119, 7–14. doi: 10.1111/j.1600-0722.2010.00793.x

Bumann, A., Carvalho, R. S., Schwarzer, C. L., and Yen, E. H. (1997). Collagen synthesis from human PDL cells following orthodontic tooth movement. *Eur. J. Orthod.* 19, 29–37. doi: 10.1093/ejo/19.1.29

Burden, D., Mullally, B., and Sandler, J. (2001). Orthodontic treatment of patients with medical disorders. *Eur. J. Orthod.* 23, 363–372. doi: 10.1093/ejo/23.4.363

Bustelo, X. R., Sauzeau, V., and Berenjeno, I. M. (2007). GTP-binding proteins of the Rho/Rac family: regulation, effectors and functions *in vivo*. *Bioessays* 29, 356–370. doi: 10.1002/bies.20558

Callaci, J. J., Himes, R., Lauing, K., Wezeman, F. H., and Brownson, K. (2009). Binge alcohol-induced bone damage is accompanied by differential expression of bone remodeling-related genes in rat vertebral bone. *Calcif. Tissue Int.* 84, 474–484. doi: 10.1007/s00223-009-9240-z

Cantarella, G., Cantarella, R., Caltabiano, M., Risuglia, N., Bernardini, R., and Leonardi, R. (2006). Levels of matrix metalloproteinases 1 and 2 in human gingival crevicular fluid during initial tooth movement. *Am. J. Orthod. Dentofacial Orthop.* 130, 568.e11–568.e16. doi: 10.1016/j.ajodo.2006.04.020

Caro, A. A., and Cederbaum, A. I. (2004). Oxidative stress, toxicology, and pharmacology of CYP2E1. *Annu. Rev. Pharmacol. Toxicol.* 44, 27–42. doi: 10.1146/annurev.pharmtox.44.101802.121704

Chakkalakal, D. A. (2005). Alcohol-induced bone loss and deficient bone repair. *Alcohol Clin. Exp. Res.* 29, 2077–2090. doi: 10.1097/01.alc.0000192039.21305.55

Chen, H., Mahaseth, M., and Zhang, Y. (2011). Hyaluronic acid as a rescue therapy for trinitrobenzene sulfonic acid-induced colitis through Cox-2 and PGE_2 in a Toll-like receptor 4-dependent way. *J. Zhejiang Univ. Sci. B.* 12, 712–719. doi: 10.1631/jzus.B1000362

Chen, J. R., Haley, R. L., Hidestrand, M., Shankar, K., Liu, X., Lumpkin, C. K., et al. (2006). Estradiol protects against ethanol-induced bone loss by inhibiting up-regulation of receptor activator of nuclear factor-κB ligand in osteoblasts. *J. Pharmacol. Exp. Ther.* 319, 1182–1190. doi: 10.1124/jpet.106.109454

Chen, J. R., Plotkin, L. I., Aguirre, J. I., Han, L., Jilka, R. L., Kousteni, S., et al. (2005). Transient versus sustained phosphorylation and nuclear accumulation of ERKs underlie anti-versus pro-apoptotic effects of estrogens. *J. Biol. Chem.* 280, 4632–4638. doi: 10.1074/jbc.M411530200

Chen, J. R., Shankar, K., Nagarajan, S., Badger, T. M., and Ronis, M. J. (2008). Protective effects of estradiol on ethanol-induced bone loss involve inhibition of reactive oxygen species generation in osteoblasts and downstream activation of the extracellular signal-regulated kinase/signal transducer and activator of transcription 3/receptor activator of nuclear factor-κB ligand signaling cascade. *J. Pharmacol. Exp. Ther.* 324, 50–59. doi: 10.1124/jpet.107.130351

Chen, Z., Gibson, T. B., Robinson, F., Silvestro, L., Pearson, G., Xu, B., et al. (2001). MAP kinases. *Chem. Rev.* 101, 2449–2476. doi: 10.1021/cr000241p

Clarke, E. A., and Brugge, J. S. (1995). Integrins and signal transduction pathways: the road taken. *Science* 268, 233–239. doi: 10.1126/science.7716514

Crews, F., Nixon, K., Kim, D., Joseph, J., Shukitt-Hale, B., Qin, L., et al. (2007). BHT blocks NF-κB activation and ethanol-induced brain damage. *Alcohol Clin. Exp. Res.* 30, 1938–1949. doi: 10.1111/j.1530-0277.2006.00239.x

Cullmann, M., Hilding, A., and Östenson, C. G. (2012). Alcohol consumption and risk of pre-diabetes and type 2 diabetes development in a Swedish population. *Diabet. Med.* 29, 441–452. doi: 10.1111/j.1464-5491.2011.03450.x

Dai, J., Lin, D., Zhang, J., Habib, P., Smith, P., Murtha, J., et al. (2000). Chronic alcohol ingestion induces osteoclastogenesis and bone loss through IL-6 in mice. *J. Clin. Invest.* 106, 887–895. doi: 10.1172/JCI10483

Dantas, A. M., Mohn, C. E., Burdet, B., Zorrilla-Zubilete, M., Mandalunis, P. M., Elverdin, J. C., et al. (2012). Ethanol consumption enhances periodontal inflammatory markers in rats. *Arch. Oral Biol.* 57, 1211–1217. doi: 10.1016/j.archoralbio.2012.02.008

Dasanayake, A. P., Warnakulasuriya, S., Harris, C. K., Cooper, D. J., Peters, T. J., and Gelbier, S. (2010). Tooth decay in alcohol abusers compared to alcohol and drug abusers. *Int. J. Dent.* 2010:786503. doi: 10.1155/2010/786503

DeMali, K. A., Wennerberg, K., and Burridge, K. (2003). Integrin signaling to the actin cytoskeleton. *Curr. Opin. Cell Biol.* 15, 72–582. doi: 10.1016/S0955-0674(03)00109-1

Domon, S., Shimokawa, H., Matsumoto, Y., Yamaguchi, S., and Soma, K. (1999). *In situ* hybridization for matrix metalloproteinase-1 and cathepsin K in rat root-resorbing tissue induced by tooth movement. *Arch. Oral Biol.* 44, 907–915. doi: 10.1016/S0003-9969(99)00091-6

Dong, Y. J., Peng, T. K., and Yin, S. J. (1996). Expression and activities of class IV alcohol dehydrogenase and class III aldehyde dehydrogenase in human mouth. *Alcohol* 13, 257–262. doi: 10.1016/0741-8329(95)02052-7

Elamin, E., Masclee, A., Dekker, J., and Jonkers, D. (2014). Ethanol disrupts intestinal epithelial tight junction integrity through intracellular calcium-mediated Rho/ROCK activation. *Am. J. Physiol. Gastrointest. Liver Physiol.* 306, G677–G685. doi: 10.1152/ajpgi.00236.2013

Elkstrom, G., and Ingelman-Sundberg, M. (1989). Rat liver microsomal NADPH-supported oxidase activity and lipid peroxidation dependent on ethanol-inducible cytochrome P-450 (P-450IIEI). *Biochem. Pharmacol.* 38, 1313–1319. doi: 10.1016/0006-2952(89)90338-9

Etienne-Manneville, S., and Hall, A. (2002). Rho GTPases in cell biology. *Nature* 420, 629–635. doi: 10.1038/nature01148

Feng, Z. M., Wu, Y. S., and Yan, G. Y. (2007). Change of expression of matrix metalloproteinase-2 in the periodontal tissues of *Diabetes mellitus* rats during orthodontic toot movement. *Hua Xi Kou Qiang Yi Xue Za Zhi.* 25, 118–121.

Fernandez-Lizarbe, S., Montesinos, J., and Guerri, C. J. (2013). Ethanol induces TLR4/TLR2 association, triggering an inflammatory response in microglial cells. *J. Neurochem.* 126, 261–273. doi: 10.1111/jnc.12276

Flores-Bellver, M., Bonet-Ponce, L., Barcia, J. M., Garcia-Verdugo, J. M., Martinez-Gil, N., Saez-Atienzar, S., et al. (2014). Autophagy and mitochondrial alterations in human retinal pigment epithelial cells induced by ethanol: implications of 4-hydroxy-nonenal. *Cell Death Dis.* 5, e1328. doi: 10.1038/cddis.2014.288

Franceschi, S. (1993). Oesophageal and oral cancer: aetiological factors. *Eur. J. Cancer Prev.* 2, 2–3. doi: 10.1097/00008469-199301001-00007

Friday, K. E., and Howard, G. A. (1991). Ethanol inhibits human bone cell proliferation and function *in vitro*. *Metabolism* 40, 562–565. doi: 10.1016/0026-0495(91)90044-W

Gelani, V., Fernandes, A. P., Gasparoto, T. H., Garlet, T. P., Cestari, T. M., Lima, H. R., et al. (2009). The role of toll-like receptor 2 in the recognition of *Aggregatibacter actinomycetemcomitans*. *J. Periodontol.* 80, 2010–2019. doi: 10.1902/jop.2009.090198

Gelbier, S., and Harris, C. (1996). Oral and dental health in the alcohol misuser. *Addict. Biol.* 1, 165–169. doi: 10.1080/1355621961000124786

Graham, K., Leonard, K. E., Room, R., Wild, C., Pihl, R. O., Bois, C., et al. (1998). Alcohol and aggression: current directions in research on understanding and preventing intoxicated aggression. *Addiction* 93, 659–676. doi: 10.1046/j.1360-0443.1998.9356593.x

Grieve, W. G., Johnson, G. K., Moore, R. N., Reinhardt, R. A., and DuBois, L. M. (1994). Prostaglandin E (PGE) and interleukin-1β (IL-1β) levels in gingival crevicular fluid during human orthodontic tooth movement. *Am. J. Orthod. Dentofacial Orthop.* 105, 369–374. doi: 10.1016/S0889-5406(94)70131-8

Guasch, R. M., Tomas, M., Miñambres, R., Valles, S., Renau-Piqueras, J., and Guerri, C. (2003). RhoA and lysophosphatidic acid are involved in the actin cytoskeleton reorganization of astrocytes exposed to ethanol. *J. Neurosci. Res.* 15, 487–502. doi: 10.1002/jnr.10594

Han, J. H., Tian, H. Z., Lian, Y. Y., Yu, Y., Lu, C. B., Li, X. M., et al. (2015). Quetiapine mitigates the ethanol-induced oxidative stress in brain tissue, but not in the liver, of the rat. *Neuropsychiatr. Dis. Treat.* 15, 1473–1482. doi: 10.2147/NDT.S80505

Haorah, J., Schall, K., Ramirez, S. H., and Persidsky, Y. (2008). Activation of protein tyrosine kinases and matrix metalloproteinases causes blood-brain barrier injury: novel mechanism for neurodegeneration associated with alcohol abuse. *Glia* 56, 78–88. doi: 10.1002/glia.20596

Haorah, J., Ramirez S. H., Schall, K., Smith, S., Pandya, R., and Persidsky, Y. (2007). Oxidative stress activates protein tyrosine kinase and matrix metalloproteinases leading to blood-brain barrier dysfunction. *J. Neurochem.* 101, 566–576. doi: 10.1111/j.1471-4159.2006.04393.x

Harris, C. K., Warnakulasuriya, K. A., Cooper, D. J., Peters, T. J., and Gelbier, S. (2004). Prevalence of oral mucosal lesions in alcohol misusers in south London. *J. Oral Pathol. Med.* 33, 253–259. doi: 10.1111/j.0904-2512.2004.00142.x

Harris, C. K., Warnakulasuriya, K. A., Johnson, N. W., Gelbier, S., and Peters, T. J. (1996). Oral health in alcohol misusers. *Community Dent. Health.* 13, 199–203.

He, Y., Macarak, E. J., Korostoff, J. M., and Howard, P. S. (2004). Compression and tension: differential effects on matrix accumulation by periodontal ligament fibroblasts *in vitro*. *Connect. Tissue Res.* 45, 28–39. doi: 10.1080/03008200490278124

Herrera, D. G., Yague, A. G., Johnsen-Soriano, S., Bosch-Morell, F., Collado-Morente, L., Muriach, M., et al. (2003). Selective impairment of hippocampal neurogenesis by chronic alcoholism: protective effects of an antioxidant. *Proc. Natl. Acad. Sci. U.S.A.* 100, 7919–7924. doi: 10.1073/pnas.1230907100

Huang, S. F., Li, Y. H., Ren, Y. J., Cao, Z. G., and Long, X. (2008). The effect of a single nucleotide polymorphism in the matrix metalloproteinase-1 (MMP-1) promoter on force-induced MMP-1 expression in human periodontal ligament cells. *Eur. J. Oral Sci.* 116, 319–323. doi: 10.1111/j.1600-0722.2008.00552.x

Ingman, T., Apajalahti, S., Mäntylä, P., Savolainen, P., and Sorsa, T. (2005). Matrix metalloproteinase 1 and 8 in GCF during orthodontic tooth movement: a pilot study during 1 month follow- up after fixed appliance activation. *Eur. J. Orthod.* 27, 202–207. doi: 10.1093/ejo/cjh097

Jeon, Y. M., Kook, S. H., Son, Y. O., Kim, E. M., Park, S. S., Kim, J. G., et al. (2009). Role of MAPK in mechanical force-induced up-regulation of type I collagen and osteopontin in human gingival fibroblasts. *Mol. Cell. Biochem.* 320, 45–52. doi: 10.1007/s11010-008-9897-z

Jiang, D., Liang, J., Fan, J., Yu, S., Chen, S., Luo, Y., et al. (2005). Regulation of lung injury and repair by Toll-like receptors and hyaluronan. *Nat. Med.* 11, 1173–1179. doi: 10.1038/nm1315

Johnsen-Soriano, S., Bosch-Morell, F., Miranda, M., Asensio, S., Barcia, J. M., Romá, J., et al. (2007). Ebselen prevents chronic alcohol-induced rat hippocampal stress and functional impairment. *Alcohol. Clin. Exp. Res.* 31, 486–492. doi: 10.1111/j.1530-0277.2006.00329.x

Katagiri, T., and Takahashi, N. (2002). Regulatory mechanisms of osteoblast and osteoclast differentiation. *Oral Dis.* 8, 147–159. doi: 10.1034/j.1601-0825.2002.01829.x

Kharbanda, K. K., McVicker, D. L., Zetterman, R. K., and Donohue, T. M. (1995). Ethanol consumption reduces the proteolytic capacity and protease activities of hepatic lysosomes. *Biochim. Biophys. Acta* 1245, 421–429. doi: 10.1016/0304-4165(95)00121-2

Kharbanda, K. K., McVicker, D. L., Zetterman, R. K., and Donohue, T. M. (1996). Ethanol consumption alters trafficking of lysosomal enzymes and affects the processing of procathepsin L in rat liver. *Biochim. Biophys. Acta* 1291, 45–52. doi: 10.1016/0304-4165(96)00043-8

Khocht, A., Janal, M., Schleifer, S., and Keller, S. (2003). The influence of gingival margin recession on loss of clinical attachment in alcohol-dependent patients without medical disorders. *J. Periodontol.* 74, 485–493. doi: 10.1902/jop.2003.74.4.485

Khocht, A., Schleifer, S., Janal, M., and Keller, S. (2013). Neutrophil function and periodontitis in alcohol-dependent males without medical disorders. *J. Int. Acad. Periodontol.* 15, 68–74.

Kikkert, R., Laine, M. L., Aarden, L. A., and van Winkelhoff, A. J. (2007). Activation of toll-like receptors 2 and 4 by gram-negative periodontal bacteria. *Oral Microbiol. Immunol.* 22, 145–151. doi: 10.1111/j.1399-302X.2007.00335.x

Kim, S. J., Ju, A., Lim, S. G., and Kim, D. J. (2013). Chronic alcohol consumption, type 2 *Diabetes mellitus*, insulin-like growth factor-I (IGF-I), and growth hormone (GH) in ethanol-treated diabetic rats. *Life Sci.* 93, 778–782. doi: 10.1016/j.lfs.2013.09.018

Koch, O. R., Pani, G., Borrello, S., Colavitti, R., Cravero, A., Farrè, S., et al. (2004). Oxidative stress and antioxidant defenses in ethanol-induced cell injury. *Mol. Aspects Med.* 25, 191–198. doi: 10.1016/j.mam.2004.02.019

Kong, Y. Y., Feige, U., Sarosi, I., Bolon, B., Tafuri, A., Morony, S., et al. (1999). Activated T cells regulate bone loss and joint destruction in adjuvant

arthritis through osteoprotegerin ligand. *Nature* 402, 304–309. doi: 10.1038/46303

Kook, S. H., Hwang, J. M., Park, J. S., Kim, E. M., Heo, J. S., Jeon, Y. M., et al. (2009). Mechanical force induces type I collagen expression in human periodontal ligament fibroblasts through activation of ERK/JNK and AP-1. *J. Cell. Biochem.* 106, 1060–1067. doi: 10.1002/jcb.22085

Kook, S. H., Jang, Y. S., and Lee, J. C. (2011). Involvement of JNK-AP-1 and ERK-NF-κB signaling in tension-stimulated expression of Type I collagen and MMP-1 in human periodontal ligament fibroblasts. *J. Appl. Physiol.* 111, 1575–1583. doi: 10.1152/japplphysiol.00348.2011

Koop, D. R., Morgan, E. T., Tarr, G. E., and Coon, M. J. (1982). Purification and characterization of a unique isozyme of cytochrome P-450 from liver microsomes of ethanol-treated rabbits. *J. Biol. Chem.* 257, 8472–8480.

Kousteni, S., Bellido, T., Plotkin, L. I., O'Brien, C. A., Bodenner, D. L., Han, K., et al. (2001). Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. *Cell* 104, 719–730. doi: 10.1016/s0092-8674(02)08100-x

Kranzler, H., Babor, T., Goldstein, L., and Gold, J. (1990). Dental pathology and alcohol related indicators in an out patient clinic sample. *Community Dent. Oral Epidemiol.* 18, 204–207. doi: 10.1111/j.1600-0528.1990.tb00058.x

Krishnan, V., and Davidovitch, Z. (2006). Cellular, molecular, and tissue-level reactions to orthodontic force. *Am. J. Orthod. Dentofacial Orthop.* 129, 469.e1–32. doi: 10.1016/j.ajodo.2005.10.007

Kurol, J., and Owman-Moll, P. (1998). Hyalinization and root resorption during early orthodontic tooth movement in adolescents. *Angle Orthod.* 68, 161–165.

Kyriakis, J. M., and Avruch, J. (2001). Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol. Rev.* 81, 807–869.

Leonardi, R., Talic, N. F., and Loreto, C. (2006). MMP-13 (collagenase 3) immunolocalisation during initial orthodontic tooth movement in rats. *Acta Histochem.* 109, 215–220. doi: 10.1016/j.acthis.2007.01.002

Lieber, C. S., and DeCarli, L. M. (1970). Hepatic microsomal ethanol-oxidizing system. *In vitro* characteristics and adaptive properties *in vivo*. *J. Biol. Chem.* 245, 2505–2512.

Liedert, A., Kaspar, D., Blakytny, R., Claes, L., and Ignatius, A. (2006). Signal transduction pathways involved in mechanotransduction in bone cells. *Biochem. Biophys. Res. Commun.* 349, 1–5. doi: 10.1016/j.bbrc.2006.07.214

Lisboa, R. A., Andrade, M. V., and Cunha-Melo, J. R. (2013). Toll-like receptor activation and mechanical force stimulation promote the secretion of matrix metalloproteinases 1,3 and 10 of human periodontal fibroblasts via p38, JNK and NF-κB. *Arch. Oral Biol.* 58, 731–739. doi: 10.1016/j.archoralbio.2012.12.009

Liu, W., and Zhang, X. (2015). Receptor activator of nuclear factor-κB ligand (RANKL)/RANK/osteoprotegerin system in bone and other tissues. *Mol. Med. Rep.* 11, 3212–3218. doi: 10.3892/mmr.2015.3152

Maeda, A., Soejima, K., Bandow, K., Kuroe, K., Kakimoto, K., Miyawaki, S., et al. (2007). Force-induced IL-8 from periodontal ligament cells requires IL-1α. *J. Dent. Res.* 86, 629–634. doi: 10.1177/154405910708600709

Manolagas, S. C. (2000). Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. *Endocr. Rev.* 21, 115–137. doi: 10.1210/er.21.2.115

Marques, T. C., Sarracini, K. L., Cortellazzi, K. L., Mialhe, F. L., de Castro-Meneghim, M., Pereira, A. C., et al. (2015). The impact of oral health conditions, socioeconomic status and use of specific substances on quality of life of addicted persons. *BMC Oral Health* 15:38. doi: 10.1186/s12903-015-0016-8

Martin, M., Rehani, K., Jope, R. S., and Michalek, S. M. (2005). Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. *Nat. Immunol.* 6, 777–784. doi: 10.1038/ni1221

Martin, T. J., and Sims, N. A. (2015). RANKL/OPG; critical role in bone physiology. *Rev. Endocr. Metab. Disord.* 16, 131–139. doi: 10.1007/s11154-014-9308-6

Martinez-Gil, N., Flores-Bellver, M., Atienzar-Aroca, S., Lopez-Malo, D., Urdaneta-Alba, C., Sancho-Pelluz, F. J., et al. (2015). CYP2E1 in the human retinal pigment epithelium: expression, activity, and induction by ethanol. *Invest. Ophthalmol. Vis. Sci.* 11, 6855–6963. doi: 10.1167/iovs.14-16291

McKenna, S. (2006). Dental management of patients with diabetes. *J. Dent. Clin. North Am.* 50, 591–606. doi: 10.1016/j.cden.2006.06.008

Medzhitov, R. (2001). Toll-like receptors and innate immunity. *Nat. Rev. Immunol.* 1, 135–145. doi: 10.1038/35100529

Medzhitov, R., and Janeway, C. A. (1997). Innate immunity: impact on the adaptive immune response. *Curr. Opin. Immunol.* 9, 4–9. doi: 10.1016/S0952-7915(97)80152-5

Meeran, N. A. (2012). Biological response at the cellular level within the periodontal ligament on application of orthodontic force. An update. *J. Orthodont. Sci.* 1, 2–10. doi: 10.4103/2278-0203.94769

Meikle, M. C. (2006). The tissue, cellular, and molecular regulation of orthodontic tooth movement: 100 years after Carl Sandstedt. *Eur. J. Orthod.* 28, 221–240. doi: 10.1093/ejo/cjl001

Meng, R., Song, M., and Pan, J. (2015). Rho is involved in periodontal tissue remodelling with experimental tooth movement in rats. *Arch. Oral Biol.* 60, 923–931. doi: 10.1016/j.archoralbio.2015.01.017

Meng, Y., Bai, D., Han, X. L., Liu, M., Huang, L., and Bao, L. N. (2007). Proliferation of myofibroblast in periodental tension side during orthodontic tooth movement of beagle dogs. *Sichuan Da Xue Xue Bao Yi Xue Ban.* 38, 123–125.

Meng, Y., Han, X., Huang, L., Bai, D., Yu, H., He, Y., et al. (2010). Orthodontic mechanical tension effects on the myofibroblast expression of alpha-smooth muscle actin. *Angle Orthodont.* 80, 912–918. doi: 10.2319/101609-578.1

Miksys, S. L., and Tyndale, R. F. (2002). Drug-metabolizing cytochrome P450s in the brain. *J. Psychiatry Neurosci.* 27, 406–441.

Nakagawa, M., Kukita, T., Nakasima, A., and Kurisu, K. (1994). Expression of the type I collagen gene in rat periodontal ligament during tooth movement as revealed by *in situ* hybridization. *Arch. Oral Biol.* 39, 289–294. doi: 10.1016/0003-9969(94)90119-8

Nakano, Y., Yamaguchi, M., Fujita, S., Asano, M., Saito, K., and Kasai, K. (2011). Expressions of RANKL/RANK and M-CSF/c-fms in root resorption lacunae in rat molar by heavy orthodontic force. *Eur. J. Orthod.* 33, 335–343. doi: 10.1093/ejo/cjq068

Nelson, D. E., Jarman, D. W., Rehm, J., Greenfield, T. K., Rey, G., Kerr, W. C., et al. (2013). Alcohol-attributable cancer deaths and years of potential life lost in the United States. *Am. J. Public Health.* 103, 641–648. doi: 10.2105/AJPH.2012.301199

Noble, P. W., McKee, C. M., Cowman, M., and Shin, H. S. (1996). Hyaluronan fragments activate an NF-κB/I-κBα autoregulatory loop in murine macrophages. *J. Exp. Med.* 183, 2373–2378. doi: 10.1084/jem.183.5.2373

Nussbaum, G., Ben-Adi, S., Genzler, T., Sela, M., and Rosen, G. (2009). Involvement of Toll-like receptors 2 and 4 in the innate immune response to Treponema denticola and its outer sheath components. *Infect. Immun.* 77, 3939–3947. doi: 10.1128/IAI.00488-09

Ofori-Adjei, D., Casswell, S., Drummond, C., Wei, H., Medina-Mora, M. E., Ranganathan, S., et al. (2007). *World Health Organization Expert Committee on Problems Related to Alcohol Consumption, Second Report*. Geneva: WHO.

Oshiro, T., Shiotani, A., Shibasaki, Y., and Sasaki, T. (2002). Osteoclast induction in periodontal tissue during experimental movement of incisors in osteoprotegerin-deficient mice. *Anat. Rec.* 266, 218–225. doi: 10.1002/ar.10061

Pan, J. S., Han, Y., Chen, D. P., Xu, L., Qi, Y. X., and Yan, Z. Q. (2010). Cyclic strain promotes migration of human periodontal ligament cell via extracellular signal-regulated kinase (ERK) signaling pathway. *Zhonghua Kou Qiang Yi Xue Za Zhi.* 45, 80–84. doi: 10.3760/cma.j.issn.1002-0098.2010.02.005

Pascual, M., Baliño, P., Aragón, C. M. G., and Guerri, C. (2015). Cytokines and chemokines as biomarkers of ethanol-induced neuroinflammation and anxiety-related behavior: role of TLR4 and TLR2. *Neuropharmacol.* 89, 352–359. doi: 10.1016/j.neuropharm.2014.10.014

Peverali, F. A., Basdra, E. K., and Papavassiliou, A. G. (2001). Stretch-mediated activation of selective MAPK subtypes and potentiation of AP-1 binding in human osteoblastic cells. *Mol. Med.* 7, 68–78.

Plapp, B. V., Leidal, K. G., Murch, B. P., and Green, D. W. (2015). Contribution of liver alcohol dehydrogenase to metabolism of alcohols in rats. *Chem. Biol. Interact.* 178, 16–23. doi: 10.1016/j.cbi.2014.12.040

Ramachandran, V., Watts, L. T., Maffi, S. K., Chen, J., Schenker, S., and Henderson, G. (2003). Ethanol-induced oxidative stress precedes mitochondrially mediated apoptotic death of cultured fetal cortical neurons. *J. Neurosci. Res.* 74, 577–588. doi: 10.1002/jnr.10767

Redetzki, H. M. (1960). Alcohol-aldehyde transhydrogenation with liver alcohol dehydrogenase. *Tex. Rep. Biol. Med.* 18, 83–92.

Reichl, F. X., Seiss, M., Buters, J., Behrendt, H., Hickel, R., and Durner, J. (2010). Expression of CYP450-2E1 and formation of 2,3-epoxymethacrylic acid (2,3-EMA) in human oral cells exposed to dental materials. *Dent. Mater.* 26, 1151–1156. doi: 10.1016/j.dental.2010.08.005

Ridley, A. J. (2001). Rho proteins, PI 3-kinases, and monocyte/macrophage motility. *FEBS Lett.* 498, 168–171. doi: 10.1016/S0014-5793(01)02481-4

Rooban, T., Vidya, K., Joshua, E., Rao, A., Ranganathan, S., Rao, U. K., et al. (2011). Tooth decay in alcohol and tobacco abusers. *J. Oral. Maxillofac. Pathol.* 15, 14–21. doi: 10.4103/0973-029X.80032

Roux, P. P., and Blenis, J. (2004). ERK and p38 MAPK-activated protein kinases: a family protein kinases with diverse biological functions. *Microbiol. Mol. Biol. Rev.* 68, 320–344. doi: 10.1128/MMBR.68.2.320-344.2004

Sastry, S. K., and Burridge, K. (2000). Focal adhesions: a nexus for intracellular signaling and cytoskeletal dynamics. *Exptl. Cell. Res.* 261, 25–36. doi: 10.1006/excr.2000.5043

Schoppet, M., Preissner, K. T., and Hofbauer, L. C. (2002). RANK ligand and osteoprotegerin: paracrine regulators of bone metabolism and vascular function. *Arterioscler. Thromb. Vasc. Biol.* 22, 549–553. doi: 10.1161/01.ATV.0000012303.37971.DA

Seitz, H. K., and Wang, X. D. (2013). "The role of cytochrome P450 2E1 in ethanol-mediated carcinogenesis," in *Cytochrome P450 2E1: Its Role in Disease and Drug Metabolism*, ed A. Dey (Dordrecht: Springer), 131–143.

Sugiyama, Y., Yamaguchi, M., Kanekawa, M., Yoshii, M., Nozoe, T., Nogimura, A., et al. (2003). The level of cathepsin B in GCF during human orthodontic tooth movement. *Eur. J. Orthod.* 25, 71–76. doi: 10.1093/ejo/25.1.71

Sun, A. Y., Chen, Y. M., James-Kracke, M., Wixom, P., and Cheng, Y. (1997). Ethanol-induced cell death by lipid peroxidation in PC12 cells. *Neurochem. Res.* 22, 1187–1192. doi: 10.1023/A:1021968526696

Surkin, P. N., Ossola, C. A., Mohn, C. E., Elverdin, J. C., and Fernández-Solari, J. (2014). Chronic alcohol consumption alters periodontal health in rats. *Alcohol. Clin. Exp. Res.* 38, 2001–2007. doi: 10.1111/acer.12436

Suzuki, R., Nemoto, E., and Shimauchi, H. (2014). Cyclic tensile force up-regulates BMP-2 expression through MAP kinase and COX-2/PGE2 signaling pathways in human periodontal ligament cells. *Exp. Cell. Res.* 323, 232–241. doi: 10.1016/j.yexcr.2014.02.013

Syed, F., and Khosla, S. (2005). Mechanisms of sex steroid effects on bone. *Biochem. Biophys. Res. Commun.* 328, 688–696. doi: 10.1016/j.bbrc.2004.11.097

Takeuchi, O., and Akira, S. (2001). Toll-like receptors: their physiological role and signal transduction system. Int. *Immunopharmacol.* 1, 625–635. doi: 10.1016/S1567-5769(01)00010-8

Taylor, K. R., Yamasaki, K., Radek, K. A., di Nardo, A., Goodarzi, H., Golenbock, D., et al. (2007). Recognition of hayluronan released in sterile injury involves a unique receptor complex dependent on Toll-like receptor 4, CD44, and MD-2. *J. Biol. Chem.* 282, 18265–18275. doi: 10.1074/jbc.M606352200

Torres, M. (2003). Mitogen-activated protein kinase pathways in redox signaling. *Front. Biosci.* 8, d369–d391. doi: 10.2741/999

Tsutsumi, M., Lasker, J. M., Takahashi, T., and Lieber, C. S. (1993). *In vivo* induction of hepatic P4502E1 by ethanol: role of increased enzyme synthesis. *Arch. Biochem. Biophys.* 304, 209–218. doi: 10.1006/abbi.1993.1341

Turner, R. T. (2000). Skeletal response to alcohol. *Alcohol. Clin. Exp. Res.* 24, 1693–1701. doi: 10.1111/j.1530-0277.2000.tb01971.x

Turner, R. T., and Sibonga, J. D. (2001). Effects of alcohol use and estrogen on bone. *Alcohol. Res. Health* 25, 276–281.

Tyrovola, J. B., Spyropoulos, M. N., Makou, M., and Perrea, D. (2008). Root resorption and the OPG/RANKL/RANK system: a mini review. *J. Oral Sci.* 50, 367–376. doi: 10.2334/josnusd.50.367

Uematsu, S., Mogi, M., and Deguchi, T. (1996). Interleukin (IL)-1β, IL-6, tumor necrosis factor-α, epidermal growth factor, and β2-microglobulinlevels are elevated in gingival crevicular fluid during human orthodontic tooth movement. *J. Dent. Res.* 75, 562–567. doi: 10.1177/00220345960750010801

Vaneker, M., Joosten, L. A., Heunks, L. M., Snijdelaar, D. G., Halbertsma, F. J., van Egmond, J., et al. (2008). Low-tidal-volume mechanical ventilation induces a toll-like receptor 4-dependent inflammatory response in healthy mice. *Anesthesiology* 109, 465–472. doi: 10.1097/ALN.0b013e318182aef1

Vemula, S., Shi, J., Hanneman, P., Wei, L., and Kapur, R. (2010). ROCK1 functions as a suppressor of inflammatory cell migration by regulating PTEN phosphorylation and stability. *Blood* 115, 1785–1796. doi: 10.1182/blood-2009-08-237222

Vernillo, A. T. (2001). *Diabetes mellitus*: relevance to dental treatment. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol Endod.* 91, 263–270. doi: 10.1067/moe.2001.114002

Villarino, M. E., Lewicki, M., and Ubios, A. M. (2011). Bone response to orthodontic forces in diabetic Wistar rats. *Am. J. Orthod. Dentofacial Orthop.* 139(4 Suppl.), S76–S82. doi: 10.1016/j.ajodo.2010.06.021

Von den Hoff, J. W. (2003). Effects of mechanical tension on matrix degradation by human periodontal ligament cells cultured in collagen gels. *J. Periodontal Res.* 38, 449–457. doi: 10.1034/j.1600-0765.2003.00404.x

Wang, H., Brown, J., and Martin, M. (2011). Glycogen synthase kinase 3: a point of convergence for the host inflammatory response. *Cytokine* 53, 130–140. doi: 10.1016/j.cyto.2010.10.009

Wang, M. J., Kuo, J. S., Lee, W. W., Huang, H. Y., Chen, W. F., and Lin, S. Z. (2006). Translational event mediates differential production of tumor necrosis factor-α in hyaluronan-stimulated microglia and macrophages. *J. Neurochem.* 97, 857–871. doi: 10.1111/j.1471-4159.2006.03776.x

Wang, N., Butler, J. P., and Ingber, D. E. (1993). Mechanotransduction across the cell surface and through the cytoskeleton. *Science* 269, 1124–1127. doi: 10.1126/science.7684161

Waszkiewicz, N., Zalewska, A., Szajda, S. D., Szulc, A., Kępka, A., Minarowska, A., et al. (2012). The effect of chronic alcohol intoxication and smoking on the activity of oral peroxidase. *Folia Histochem. Cytobiol.* 50, 450–455. doi: 10.5603/FHC.2012.0062

Waszkiewicz, N., Zalewska, A., Szulc, A., Kepka, A., Konarzewska, B., Zalewska-Szajda, B., et al. (2011). The influence of alcohol on the oral cavity, salivary glands and saliva. *Pol. Merkur. Lekarski.* 30, 69–74.

Wennerberg, K., Rossman, K. L., and Der, C. J. (2005). The Ras superfamily at a glance. *J. Cell Sci.* 118, 843–846. doi: 10.1242/jcs.01660

Yan, Y., Zeng, W., Song, S., Zhang, F., He, W., Liang, W., et al. (2013). Vitamin C induces periodontal ligament progenitor cell differentiation via activation of ERK pathway mediated by PELP1. *Protein Cell.* 4, 620–627. doi: 10.1007/s13238-013-3030-0

Yang, R. B., Mark, M. R., Gray, A., Huang, A., Xie, M. H., Zhang, M., et al. (1998). Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signaling. *Nature* 395, 284–288. doi: 10.1038/26239

Zalewska, A., Waszkiewicz, N., Szajda, S. D., and Waszkiel, D. (2011). Impact of salivary flow and lysozyme content and output on the oral health of rheumatoid arthritis patients. *Postepy. Hig. Med. Dosw.* 65, 40–45. doi: 10.5604/17322693.932232

Zeng, T., Zhang, C. L., Song, F. Y., Zhao, X. L., and Xie, K. Q. (2014). CMZ reversed chronic ethanol-induced disturbance of PPAR-α possibly by suppressing oxidative stress and PGC-1α acetylation, and activating the MAPK and GSK3β pathway. *PLoS ONE* 9:e98658. doi: 10.1371/journal.pone.0098658

Zhang, L., Li, X., and Bi, L. J. (2011). Alterations of collagen-I, MMP-1 and TIMP-1 in the periodontal ligament of diabetic rats under mechanical stress. *J. Periodontal. Res.* 46, 448–455. doi: 10.1111/j.1600-0765.2011.01359.x

Zhang, X., Schuppan, D., Becker, J., Reichart, P., and Gelderblom, H. R. (1993). Distribution of undulin, tenascin, and fibronectin in the human periodontal ligament and cementum: comparative immunoelectron microscopy with ultra-thin cryosections. *J. Histochem. Cytochem.* 41, 245–251. doi: 10.1177/41.2.7678270

Zmijewski, E., Lu, S., and Harrison-Findik, D. D. (2014). TLR4 signaling and the inhibition of liver hepcidin expression by alcohol. *World J. Gastroenterol.* 20, 12161–12170. doi: 10.3748/wjg.v20.i34.12161

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