Increased Levels of 8-Hydroxydeoxyguanosine and Malondialdehyde and its Relationship with Antioxidant Enzymes in Saliva of Periodontitis Patients

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ABSTRACT

Objectives: The aim of this study was to evaluate 8-hydroxydeoxyguanosine (8-OHdG) and Malondialdehyde (MDA) levels, and superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities in whole saliva of patients with chronic periodontitis. Moreover, the relationship among the oxidative damage biomarkers, antioxidant enzymes activities and clinical periodontal status were investigated.

Methods: Whole saliva samples were collected from 30 patients with chronic periodontitis and 30 periodontally healthy control. To determine the clinical condition of each subject, the plaque index, gingival index, clinical attachment level, and probing depth were measured. The salivary 8-OHdG level was measured using the ELISA method. SOD and GPx activities and MDA levels were determined spectrophotometrically.

Results: Higher salivary 8-OHdG and MDA levels ($P<.001$), and lower salivary SOD and GPx activities ($P<.05$) were detected in periodontitis patients compared to the healthy controls. Additionally, there were significant negative correlations between salivary levels of 8-OHdG and both salivary SOD and GPx activities as well as between salivary levels of MDA and both salivary SOD and GPx activities ($P<.001$).

Conclusions: Higher salivary 8-OHdG and MDA levels and lower salivary antioxidant activities seem to reflect increased oxygen radical activity during periodontal inflammation. (Eur J Dent 2009;3:100-106)

Key words: 8-hydroxydeoxyguanosine; Malondialdehyde; Superoxide dismutase; Glutathione peroxidase; Chronic periodontitis; Saliva.

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INTRODUCTION

Oxidative stress, occurring as a consequence of imbalance between the formation of free oxygen radicals and inactivation of these species by antioxidant defense system, is capable of causing damage to various cellular and extracellular constituents.¹ The deleterious effects of increased oxidative stress are termed oxidative damage; generally appear after exposure to a relatively high concentration of reactive oxygen species (ROS) and/or a decrease in antioxidant (AO) defense system against ROS. Oxidative stress has been implicated as a major contributor in over 100 disorders and more recently periodontitis.²

Periodontitis is an oral inflammatory disorder that gives rise to tissue damage and loss, as a result of the complex interaction between pathogenic bacteria and the host's immune response. Pathogens such as Gram-negative species, mobile rods and spirochetes may have the ability to invade gingival tissues. The interaction between pathogenic bacteria and the host's immune response is accompanied by an increase in cytokine expression and immunological activity in gingival tissues. Several lines of evidence implicate polymorphonuclear leukocytes (PMN) as the primary mediators of host response against the pathogens.³ A study carried out by Moseley et al⁴ demonstrates that PMN have been produced a range of antimicrobial factors, which include ROS during phagocytosis of periodontopathic bacteria. Recently, ROS have been reviewed in the pathogenesis of periodontitis.² It has been suggested that result of stimulation by bacterial antigens, PMN produce and release a big quantity of ROS, culminating in heightened oxidative damage to gingival tissue, periodontal ligament and alveolar bone.⁵ ROS are active in depolymerization of extracellular matrix components, lipid peroxidation (LPO), oxidation of enzymes such as anti proteases, induction of pro inflammatory cytokines and DNA damage.^{2,6}

Free radicals and related species have well-defined roles in the inflammatory process. Superoxide (O_2^-) and hydrogen peroxide (H_2O_2) may react to form more highly reactive species, which may result in chain reactions of free radical formation.² The reduction of proteins with iron sulphur centers during inflammation leads to

the release of iron (Fe^{2+}) and this may react with H_2O_2 to form the highly reactive hydroxyl radical (OH). Also elevated peroxynitrite ($ONOO^-$) may be generated, where O_2^- is able to react with nitric oxide (NO) a product of NO synthase which has been shown to be increased during inflammation. These radical formations can extensive damage to proteins, lipids and DNA molecules.^{2,7} 8-hydroxydeoxyguanosine (8-OHdG) is an oxidized nucleoside that is excreted in the bodily fluids with DNA repair.⁷ Previous studies demonstrated that the 8-OHdG in body fluids can act as a biomarker of oxidative stress in disorders including chronic inflammatory diseases.^{8,9} Also, as a result of ROS interacts with polyunsaturated fatty acids in cell membranes or lipoproteins, the process of uncontrolled lipid peroxidation (LPO) occurs.¹⁰ Malondialdehyde (MDA) is an indicator of LPO and one of the final decomposition products of LPO, which has numerous deleterious effects on biological systems.^{11,12}

Antioxidants are present in all body fluids and tissues, and protect against endogenously-formed free radicals, usually produced by leakage of the electron transport system.¹³ Antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) provide protection within cells whilst low-molecular-weight scavenging antioxidants are present in extracellular fluid. Three SOD isoenzymes are known in humans, including Cu/Zn-SOD found in the cytoplasm and nucleus isoenzymes, mitochondrial Mn-SOD, and extracellular SOD (EC-SOD) (Cu,Zn-SOD).^{14,15} EC-SOD locates in the extracellular matrix of cells and protects tissue against damage from extracellularly produced ROS. Two forms of GPx are known: classical cellular GPx and extracellular GPx (eGPx) which serves an important antioxidant role in many extracellular surfaces and spaces.^{16,17}

In this study, we evaluated 8-OHdG and MDA levels, and SOD and GPx activities in whole saliva of patients with chronic periodontitis. The data obtained were compared with those from healthy control subjects. Moreover, the relationship among the oxidative damage biomarkers, antioxidant enzymes activities and clinical periodontal status were investigated.

MATERIALS AND METHODS

Patient selection

The study was carried out in 60 subjects: 30 patients with chronic periodontitis (15 males and 15 females, aged between 31 and 63 years, with a mean age of 45.3) and 30 periodontally healthy controls (15 males and 15 females, aged between 27 and 59 years, with a mean age of 42.7). The subjects were selected among the individuals who applied to Ataturk University, Faculty of Dentistry, Department of Periodontology, due to periodontal problems or for routine controls. Thirty chronic periodontitis patients (two sites with probing pocket depth of ≥ 4 mm, gingival inflammation, 30% bone loss) and 30 periodontally healthy subjects (no history of any periodontal disease, with no gingival inflammation and with good oral hygiene) were recruited.¹⁸ The subjects included in the study: had no history of systemic disease, had not received periodontal therapy and had not taken antibiotics, anti-inflammatory drugs or any other drugs for at least 6 months, were never-smokers, not alcohol or anti-oxidant consumers. This study was approved by the ethics committee of Ataturk University, and a written informed consent was obtained from each participant.

Periodontal status in all subjects was determined by measuring plaque index (PI),¹⁹ gingival index (GI),²⁰ probing depth (PD), and clinical attachment level (CAL). PD and CAL were measured on six sites of teeth. Full-mouth periapical radiographs were taken to determine the level of periodontal bone loss of the patients.

Saliva preparation

At clinical examination, paraffin wax-stimulated whole saliva was collected in a quiet room between 9 am and noon, at least 8 hours after food intake and was obtained by expectorating into disposable tubes before clinical measurements. About 2 ml of whole saliva was collected in tubes and centrifuged immediately to remove cell debris (1000 x g for 10 minutes at 4°C). The supernatant was removed and stored in small aliquots at -80°C until analysis.

Assay of GPx activities

GPx activity was measured according to Paglia and Valentine.²¹ A total of 2.65 ml of 50 mM potassium phosphate buffer (pH 7.0) including 5 mM EDTA, 100 µl of GSH (150 mM), 20 µl of glutathione

reductase (30 U/ml), 20 µl of NaN_3 (0.12 M), 100 µl of NADPH (8 mM) and 50 µl of saliva sample were mixed, and the tubes incubated for 30 min at 37°C. The reaction was started by the addition of 100 µl of H_2O_2 solution (2 mM), mixed rapidly by inversion, and the conversion of NADPH to NADP was measured spectrophotometrically for 5 min at 340 nm. The enzyme activity was expressed as U/l using an extinction coefficient for NADPH at 340 nm of $6.22 \times 10^{-6} \text{ M}^{-1} \cdot \text{cm}^{-1}$.

Assay of SOD activities

Cu, Zn-SOD activity was measured using the method described by Sun et al.²² A total of 2.45 ml of assay reagent [0.3 mM xanthine, 0.6 mM Na_2EDTA , 0.15 mM nitroblue tetrazolium (NBT), 0.4 M Na_2CO_3 , 1 g/l bovine serum albumin] was combined with 0.5 ml of saliva sample. Xanthine oxidase (50 µl, 167 U/L) was added to initiate the reaction and the reduction of NBT by superoxide anion radicals, which are produced by the xanthine-xanthine oxidase system, was determined by measuring the absorbance at 560 nm. Cu, Zn-superoxide dismutase activity was expressed as U/ml, where 1 U is defined as that amount of enzyme causing half-maximal inhibition of NBT reduction.

Assay of MDA levels

MDA levels were measured in the clinical samples by the method of Jain et al.²³ This method is based on the reaction of MDA with thiobarbituric acid to produce a complex that can be determined spectrophotometrically; 0.2 ml of sample were mixed thoroughly with 0.8 ml of phosphate buffered saline (pH 7.4) and 0.025 ml of butylated hydroxytoluene solution (0.88%). After addition of 0.5 mL of 30% trichloroacetic acid, the samples were placed on ice for 2 h and then centrifuged at 2000 x g at 25 °C for 15 min. One ml of supernatant was mixed with 0.075 ml of 0.1 M EDTA and 0.25 ml of 1% thiobarbituric acid in 0.05 N NaOH. The samples were placed in boiling water for 15 min, cooled to room temperature, and the absorbance was determined at 532 nm.

Assay of 8-OHdG levels

Saliva samples were centrifuged at 10000 Å-g for 10 minutes, and levels of 8-OHdG in the supernatant were determined using a competitive ELISA kit (8-OHdG Check, Highly Sensitive 8-OHdG

Check, Japan Institute for the Control of Aging, Shizuoka, Japan). The determination range was 0.125 to 200 ng/ml.

Statistical analyses

Differences in clinical parameters and age between healthy controls and chronic periodontitis group were analyzed by an unpaired t test. Differences in SOD and GPx activities, and 8-OHdG and MDA levels between groups were analyzed by the Mann-Whitney’s U-test. Correlations between variables were determined by Spearman’s rank test. A value of P<.05 was considered to be significant. All values are expressed as mean ± standard deviation. For these procedures, SPSS for Windows (version 13.0) was used.

RESULTS

Clinical findings

The main values of clinical parameters listed in Table 1. All clinical parameter scores in chronic periodontitis group were significantly higher (P<.001) than in healthy controls (Table 1). There was no significant difference between the mean ages of the two groups (P>.05).

Laboratory findings

Mean results of SOD and GPx activities, and 8-OHdG and MDA levels in whole saliva of periodontally healthy controls and subjects with periodontitis are shown in Table 2. Salivary levels of 8-OHdG and MDA were significantly higher in chronic periodontitis group compared to the controls (P<.001) (Table 2). The SOD and GPx activities in whole saliva of periodontitis patients were significantly lower when compared to the controls (P<.05) (Table 2).

Correlations

In the chronic periodontitis group, significant positive correlations were observed between salivary levels of 8-OHdG and MDA, and between salivary SOD and GPx activities (P<.001) (Table 3). Additionally, there were significant negative correlations between salivary levels of 8-OHdG and both salivary SOD and GPx activities as well as between salivary levels of MDA and both salivary SOD and GPx activities (P<.001) (Table 3). The correlations between salivary oxidative damage markers and clinical parameters, and between salivary antioxidant enzymes activities and clinical parameters were not statistically significant (P<.05) (data not shown). Similarly, no statistically

Table 1. Age and clinical parameters in chronic periodontitis group and controls.

Parameter	CP Group (n=30)	Controls (n=30)
AGE	45.3±0.97	42.7±12.4
PD	4.13±0.44*	1.98±0.56
CAL	4.07±0.74*	0.00±0.00
GI	1.61±0.52*	0.55±0.24
PI	1.12±0.38*	0.43±0.21

Data are mean±SD

* asterix indicates statistical difference (P<.001)

Table 2. Laboratory findings in chronic periodontitis group and controls.

Markers	CP Group (n=30)	Controls (n=30)
8-OHdG(ng/ml)	4.24±1.78*	1.26±0.77
MDA(nmol/ml)	7.35±1.45*	5.41±1.13
SOD(U/ml)	3.24±0.57†	4.02±0.72
GPx(U/l)	74.20±26.96†	90.80±23.62

Data are mean±SD

† asterix indicates statistical difference (P<.05)

* asterix indicates statistical difference (P<.001)

significant correlation between salivary markers and age was observed ($P<.05$) (data not shown).

DISCUSSION

It is widely agreed that, increased generation of ROS may cause toxic effects by oxidative damage of macromolecules, such as proteins, lipids and DNA. Oxidative damage of biomolecules can theoretically contribute to disease development. Reaction of ROS with purine and pyrimidine bases of DNA will generate several products; 8-OHdG is one major product of nucleotide oxidation in DNA.²⁴ Salivary 8-OHdG levels were intensively studied in several oral pathologies, including patients with periodontitis,²⁵ Sjogren’s syndrome²⁶ and oral cancer.²⁷ ROS can also attack polyunsaturated fatty acids and induce formation of lipid peroxidation products. MDA is a stable end product of peroxidation of lipids by ROS.²⁸ Previously, increased salivary MDA levels were detected in patients with periodontitis,²⁹ complex regional pain syndrome³⁰ and recurrent aphthous ulceration.³¹ Both 8-OHdG and MDA are good oxidative damage indicators for harmful effect of ROS on DNA and lipid molecules.

In present study, we found that salivary 8-OHdG and MDA levels were significantly higher in periodontitis patients than healthy controls. This result confirms findings of limited previous studies.^{25,29,32-35} In addition, we also observed a significantly positive correlation between salivary 8-OHdG levels and salivary MDA levels in patients with periodontitis. It is suggested that intracellular aldehydes, such as 4-hydroxynoneal (HNE) or MDA, can directly react with DNA.³⁶ These aldehydes can also react with some amino acids in DNA repair proteins and destroy protein function.³⁷ Therefore, lipid peroxidation products may reduce DNA repairing capacity. Although some researchers suggested that 8-OHdG formation may be related to lipid peroxidation.^{38,39} Wong et al³⁹ concluded a strong association between oxidative DNA

damage and lipid peroxidation in different young and old rat tissues. If so, the positive correlation between salivary 8-OHdG levels and salivary MDA levels in this study may reflect an effect of lipid peroxidation on DNA damage. On the other hand, salivary 8-OHdG and MDA levels had not significant correlations with clinical periodontal parameters and age in group of periodontitis patients. This result indicates that both salivary 8-OHdG and MDA levels are not directly related with disease severity and patient’s age.

Our study also focused on salivary antioxidant activities and its relationship with salivary oxidative damage biomarkers and clinical periodontal parameters in patients with periodontitis. Latest data show that the antioxidant capacity decreases and certain oxidative stress biomarkers increase in periodontitis patients.⁴⁰⁻⁴³ In this study, we found that salivary SOD and GPx activities were significantly lower in periodontitis patients than healthy controls, and a significant positive correlation between each other in periodontitis patients. Also we observed significant negative correlations between salivary SOD activity and salivary oxidative damage biomarkers (8-OHdG and MDA levels), and between salivary GPx activity and salivary oxidative damage biomarkers (8-OHdG and MDA levels). However, we could not find any significant correlations between salivary antioxidant capacities and periodontal status. The function of SOD is to remove damaging ROS from cellular environment by catalyzing the dismutation of two O_2^- to H_2O_2 .¹⁴ GPx reduces H_2O_2 and/or lipid hydrogen peroxides by the oxidation of reduced glutathione or s-nitrosoglutathione.¹⁷ Therefore, the balance between ROS and antioxidant mechanisms is likely to be important in periodontal pathogenesis, and imbalance can be caused by increased ROS and inhibited antioxidant mechanisms and/or decreased antioxidant capacity in diseased subjects.

Table 3. Correlations among laboratory findings in chronic periodontitis group.

	GPx	8-OHdG	MDA
SOD	0.704*	-0.481*	-0.501*
GPx		-0.505*	-0.543*
8-OHdG			0.617*

* asterix indicates statistical difference ($P<.001$)

CONCLUSIONS

Within the limitations of this study, we concluded that patients with periodontitis demonstrated higher oxidative DNA and lipid damage biomarkers and lower antioxidant enzymes activities in saliva than healthy subjects. We suggested in this study that higher salivary 8-OHdG and MDA levels and lower salivary antioxidant activities seemed to reflect increased oxygen radical activity during periodontal inflammation. However, increased oxidative stress in periodontal microenvironment may not directly effect on severity of destructive periodontal diseases. Further investigations are needed to clarify the role of oxidative stress in pathogenesis of destructive periodontal disease.

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