Acta Odontologica Scandinavica, 2014; 72: 42–47

informa
healthcare

ORIGINAL ARTICLE

Salivary biomarkers: Relationship between oxidative stress and alveolar bone loss in chronic periodontitis

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Abstract

Objectives. Oxidative stress is implicated in the pathogenesis of many systemic and oral diseases such as periodontal disease. The main aim of this study is to explore a possible association between salivary markers of OS and alveolar bone loss. ***Materials and methods.*** The study included 20 patients with chronic periodontitis and 20 controls. Salivary OS biomarkers 8-hidroxy-desoxguanosine (8-HOdG), malondialdehyde (MDA), uric acid, total antioxidant capacity (TAC) and glutathione peroxidase (GPx) were evaluated. Bone loss markers such as C-terminal telopeptide of type I collagen (CTX I), matrix metalloproteinases-8 (MMP-8), osteocalcin and 25-hydroxy vitamin D_3 (25- OH D) were detected in this study. The methods included general biochemical tests and ELISA. ***Results.*** Salivary 8-OHdG, MDA levels were significantly higher in the chronic periodontitis group compared with controls ($p < 0.05$). Salivary activities for uric acid, TAC and GPx were significantly decreased in patients with chronic periodontitis vs controls ($p < 0.05$). Salivary levels for CTX I, MMP-8, 25-OH D and Osteocalcin were significantly higher in the chronic periodontitis group compared to the controls ($p < 0.05$). A significant positive correlation was observed between salivary levels of MDA and CTX I. Significant negative correlations between uric acid and CTX I and between MMP-8 and uric acid have been found. Significant positive correlations were observed between CTX I, MMP-8, 25-OH D, osteocalcin and clinical parameters of periodontal disease. ***Conclusions.*** Important oxidative stress associated with alveolar bone loss biomarkers can be detected in saliva of patients with periodontal disease.

Key Words: *biomarkers, bone resorption, chronic periodontitis, saliva*

Introduction

In living organisms oxidative stress (OS) is defined as an imbalance between excessive reactive oxygen species (ROS) production and antioxidant mechanisms. ROS such as superoxide radical, hydrogen peroxide and hydroxyl radical are products of normal cellular metabolism [1]. Studies show that OS is implicated in the pathogenesis of many systemic diseases including in oral conditions [1,2].

A rapidly growing body of literature has connected the pathogenesis of periodontitis with OS [2]. Periodontal diseases including both gingivitis and periodontitis are the most widespread chronic conditions affecting populations worldwide [3]. Recent data shows that ROS can directly influence fibroblast and osteoblast cells and can decrease collagen production in extracellular matrix producing cells [2]. Recent studies demonstrate that both total antioxidant status and OS index are increased in patients with chronic periodontitis [4]. Another study reports a positive connection between systemic markers of inflammation and OS [5]. Also OS seems to be a mechanism that explains the relationship between periodontal disease and systemic affections like metabolic syndrome [6] or cardiovascular diseases [7].

Saliva is considered the first line of defence against OS. Several key salivary antioxidant mechanisms include uric acid, albumin, ascorbic acid and glutathione [8]. Studies report that salivary antioxidants levels are decreased in patients with chronic periodontitis. Further the end-products of lipid peroxidation malondialdehyde

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(Received 21 February 2013; accepted 2 April 2013)

ISSN 0001-6357 print/ISSN 1502-3850 online
DOI: 10.3109/00016357.2013.795659

(MDA) and DNA oxidation marker 8-hydroxy-2-deoxyguanosine (8-OHdG) are significantly higher in patients with periodontitis [9,10].

Oral fluids are used currently as a new diagnostic fluid for oral and systemic diseases [11,12]. Easily collected and containing local- and systemic-derived biomarkers, saliva may offer the basis for a patient-specific diagnostic test for periodontal disease [11]. Previous studies showed that several markers are expressed in patient's saliva with chronic periodontitis: inflammatory biomarkers (β-glucuronidase, C-reactive protein, IL-6, IL-1β), molecules of connective tissue destruction (α2-macroglobulin, matrix metalloproteinases-8,9,13, aminotransferases) and bone remodelling biomarkers (alkaline phosphatase, telopeptide of type I collagen) [13–20]. Other reports established an association between periodontal disease and some systemic diseases such as type 2 diabetes mellitus and cardiovascular diseases, OS being a possible link [5,21]. Thus, inflammatory biomarkers such as C-reactive protein, TNF-α and myeloperoxidase can be detected in patient's saliva with cardiovascular diseases [22]. Previous studies on the connection between OS and bone resorption are still very limited [23,24].

The main aim of the present study is to explore the relationship between the salivary antioxidant defence system and salivary bone resorption biomarkers present in saliva from patients with chronic periodontitis. More specifically OS biomarkers such as 8-OHdG, MDA and antioxidant systems—uric acid, GPx, TAC will be correlated with bone resorption biomarkers—MMP-8, CTX I, osteocalcin and 25 –OH D from saliva of chronic periodontitis patients.

Materials and methods

Patients

This study was reviewed and approved by the ethics board of the University of Medicine and Pharmacy Carol Davila, Faculty of Dental Medicine. Informed consent was obtained from each participant who agreed to participate voluntarily in this study. The study was carried out in 20 patients with chronic periodontitis (five males and 15 females, with a mean age of 51.26 ± 7.4). Twenty healthy subjects with no gingival inflammation, good oral hygiene and no history of periodontal disease were grouped as controls. All patients included in the study fulfilled the following criteria: absence of any systemic disease; no use of systemic medications like antibiotics within the preceding 3 months, anti-inflammatory drugs or anti-oxidant drugs (especially vitamins A, C, E); no history of tobacco usage or alcoholism. Periodontal status was determined by measuring PD (probing depth), plaque index (PI), bleeding index (GI). GI and PI are expressed as a percentage (%). PI represents the number of plate surfaces reported to the total number of dental surfaces. GI is expressed as the number of reported bleeding surfaces to the total number of dental surfaces. For probing depth the periodontal probe (PCP15; HuFriedy, Chicago, IL) was inserted parallel to the vertical axis of the tooth and 'walked' circumferentially clockwise around each surface of the tooth, to detect the area of deepest penetration. Chronic periodontitis was diagnosed using the following criteria: at least six sites with PD ≥4 mm; bone loss higher than 30% and gingival inflammation. All clinical examinations were performed by one qualified examiner. For bone loss assessment full-mouth radiographs were taken for each patient.

Saliva sampling

The subjects included in this study were told not to eat or drink anything in the morning before collecting the samples. Unstimulated whole saliva was collected into sterile tubes between 9 and 10 a.m. after a single mouth rise with 10 mL of distilled water to wash out exfoliated cells. About 2 mL of unstimulated whole saliva collected was immediately centrifuged at 3000 rpm for 10 min to remove cell debris. The supernatant was kept and stored in small aliquots at 80°C until further analysis. Some biochemical assays were performed in the same day.

Oxidative stress biomarkers

DNA oxidation. We measured salivary 8-hydroxy-2-deoxyguanosine (8-OHdG) using an enzyme-linked immune assay kit (8-hydroxy-2-deoxyguanosine EIA kit, Cayman Chemical Company, Ann Arbor, MI, USA). Samples, controls and standards were pipetted into wells pre-coated with a specific monoclonal antibody and incubated. Following a washing step, the substrate solution was added to the wells. The colour developed is opposite to the amount of the 8-OHdG in the sample. The colour development was stopped and the intensity of the colour was measured using a plate reader (Stat Fax 303 Plus, Awareness Technology Inc., Palm City, FL) at a wavelength between 405–420 nm.

Lipid peroxidation. Malondialdehyde (MDA) is formed by breakdown of lipid hydroxyperoxide. The method used in the study was based on the reaction of MDA with thiobarbituric acid (TBA) by heating to produce a complex that can be determined spectrophotometrically. Further 0.1 mL supernatant was mixed thoroughly with 0.5 mL trichloroacetic acid (20%), 0.05 mL butylated hydroxytoluene and 1.5 mL TBA. The samples were placed in boiling water for 1 h, cooled to room temperature and the

absorbance was measured at 532 nm using a spectrophotometer.

Antioxidant markers

Salivary uric acid analysis. Salivary uric acid analysis was performed using an analysis kit (Uric acid Assay kit, Biosystems, Barcelona, Spain), on automatic analyser EOS BRAVO-Hospitex Diagnostics. The principle of the method for saliva uric acid analysis is the colourimetric reaction with uricase and ascorbate-oxidase and reading the absorbance at 520 nm.

Salivary glutathione peroxidise (GPx) analysis. Salivary GPx analysis was performed using an analysis kit (Glutathione Peroxidase Assay kit, Randox, Crumlin, UK), on automatic analyser EOS BRAVO-Hospitex Diagnostics. GPx catalyses the oxidation of glutathione to cumene hydroperoxide. The absorbance was measured at 340 nm.

Total antioxidant capacity (TAC). Salivary TAC activity was performed using an analysis kit (Total Antioxidant Status-TAS, Randox), on automatic analyser EOS BRAVO-Hospitex Diagnostics. TAC activity in saliva was measured by the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) radical cation (ABTS◦+). The method is based on the ability of antioxidant molecules to quench the long-lived ABTS◦+, a blue–green chromophore with characteristic absorption at 734 nm, in comparison to that of Trolox, a water-soluble vitamin E analogue.

Bone resorption biomarkers

The ELISA method was used for quantitative detection of MMP-8 (Human Total Matrix Metalloproteinases, R&D Systems, Hamburg, Germany), osteocalcin (Human Osteocalcin (1–43) (N-terminal or mid regional and human osteocalcin (1–49) intact, R&D Systems), 25–OHD (25-hydroxy vitamin D3, IDS Immunodiagnostics systems, Boldon, UK) and CTX I (C-terminal telopeptide of type I collagen, TSZ Scientific LLC, Framingham, MA). Samples, controls and standards were pipetted into wells pre-coated with a monoclonal antibody specific for each marker to be detected. Following a washing step, a substrate solution was added to wells. After another washing step, an enzyme-linked monoclonal antibody specific for each assay was added to all wells. After incubation and washing to remove the entire unbound enzyme, a substrate solution which was acting on the bound enzyme is added to induce a coloured reaction product. The intensity of this coloured product was directly proportional to the concentration of target protein present in the samples. The intensity of the colour is directly proportional to the concentration of target protein present in the samples, measured using a microplate-reader at a wavelength between 405–450 nm. In order to avoid salivary flow influence on the acquisition of data, all salivary parameters were normalized to the salivary concentration of albumin. Salivary albumin (Albumin Assay Kit, Barcelona, Spain) was measured by the bromcresol green method.

Table I. Clinical parameters and demographic variables. Demographic and clinical variables are presented for both healthy controls and patients with periodontal disease. Plaque index, Bleeding Index and Probing Depth are significantly different between the two groups (p-value < 0.05).

Variable	Patients (n = 25)	Controls (n = 25)	p-value
Age (years)	51.26 ± 7.4	18.66 ± 2	—
Gender (female/male)	14/11	20/5	—
Smoking status	0	0	—
PI (%)	48 ± 0.21	19 ± 0.60	< 0.05
GI (%)	62.8 ± 0.34	14.6 ± 0.32	< 0.05
PD (>5 mm)	4.41 ± 0.42	0	< 0.05

Statistical analysis

Data distributions were expressed as means, standard deviations (SD), ranges and percentages, as appropriate. The Pearson's correlation coefficient and ANOVA test were used. The data were analysed statistically on the computer using StataIC 11 (StataCorp. 2009. Stata: Release 11. StatisticalSoftware. College Station, TX). A p-value < 0.05 was considered statistically significant.

Results

Clinical data

Patients and healthy controls were investigated for plaque index (PI), bleeding index (GI), probing depth (PD) and smoking status ($p < 0.05$). Results are detailed in Table I. No significant differences were detected for age and gender between the periodontal group and the controls.

Oxidative stress biomarkers

Salivary 8-OHdG, MDA levels were significantly higher in the chronic periodontitis group compared with controls ($p < 0.05$). Salivary activities for uric acid, TAC and GPx were significantly decreased in patients with chronic periodontitis vs controls ($p < 0.05$). Mean values for OS biomarkers from unstimulated whole saliva are presented in Table II.

Bone loss biomarkers

Salivary levels for bone loss biomarkers were significantly higher in the chronic periodontitis group

Table II. Salivary oxidative stress biomarkers. Oxidative stress parameters: total antioxidant capacity, DNA oxidation marker—8-hydroxy-2-deoxyguanosine, lipid peroxidation marker—malondialdehyde and antioxidant parameters: uric acid and glutathione peroxidase. Each data point represents mean values ± standard deviation. Statistical significance was set at p-value < 0.05.

Parameters	Patients	Controls	p-value
8-HOdG (ng/mg albumin)	6.78 ± 1.80	6.46 ± 0.93	<0.05
MDA (nmol/mg albumin)	0.296 ± 0.10	0.25 ± 0.4	<0.05
Uric acid (mg/mg albumin)	2.41 ± 0.265	3.12 ± 0.85	<0.05
TAC (nmol/mg albumin)	0.75 ± 0.16	1.24 ± 0.16	<0.05
GPx (U/mg albumin)	15.81 ± 7.22	28.16 ± 11.95	<0.05

compared to the controls ($p < 0.05$). The results obtained from the two groups are presented in Table III. In the chronic periodontitis group, significant positive correlations were detected between salivary levels of MDA and CTX I. Also significant negatively correlations between uric acid and CTX I and between MMP-8 and uric acid have been found (Table IV). Our results show significant positively correlations between CTX I, MMP-8, 25-OH D, osteocalcin and PD (>5 mm). At the same time no correlation was detected between OS biomarkers and any clinical parameter evaluated at the chronic periodontitis group. The correlations between salivary bone loss biomarkers and clinical parameters are presented in Table V.

Discussion

Periodontitis, the destructive form of periodontal disease, is a non-reversible inflammatory disease affecting supporting tissues of teeth. After its initiation, the disease progresses with loss of collagen fibres and attachment to the cement surface, apical migration of the pocket epithelium and resorption of alveolar bone. Untreated, the disease evolves to progressive bone destruction, leading to tooth mobility and subsequent tooth loss [25]. The inflammatory and immune reactions induced by the bacterial plaque play the main roles in periodontitis pathogenesis [26]. The majority of tissue destruction is considered to be the result of an altered inflammatory/immune response to microbial plaque and involve massive release of neutrophils, reactive oxygen species (ROS) and enzymes [2].

Matrix-metalloproteinases (MMPs) are special host proteinases playing a key role in tissue degradation. Studies [20] show that MMP-8 or collagenase-2 is released during polymorphonuclear leukocytes (PMNs) maturation and is capable of degrading collagen type I, II and III typically found in alveolar bone. As previously reported, MMP-8 is the most prevalent MMP to be found in periodontal tissues [20]. Thus, Mancini et al. [27] demonstrated that patients with periodontitis have an 18-fold increment in MMP-8 levels as compared to normal controls. Another group showed that MMP-8 is markedly elevated in peri-implant sulcus fluid in patients with >3 mm bone loss [28,29]. Taken together MMP-8 can be a potential marker for periodontitis. In the present study MMP-8 levels were significantly increased in chronic periodontitis patients when compared to controls. Also MMP-8 showed an inverse correlation with an important salivary antioxidant

Table III. Bone loss biomarkers. Osteocalcin, 25-hydroxy vitamin D3, matrix metalloproteinase 8, C-terminal telopeptide of type I collagen were measured in both saliva of patients with periodontitis and healthy controls. Data is presented as mean values ± standard deviation. Statistical significance is set at $p < 0.05$.

Parameters	Patient	Controls	p-value
Osteocalcin (pg/mg albumin)	0.40 ± 0.177	0.26 ± 0.85	<0.005
25 –OH D (ng/mg albumin)	22.74 ± 5.45	10.24 ± 4.08	<0.005
MMP-8 (ng/mg albumin)	10.12 ± 2.99	9.69 ± 0.71	<0.005
CTX I (ng/mg albumin)	0.77 ± 0.16	0.41 ± 0.12	<0.005

Table IV. Correlations between bone loss and salivary oxidative stress biomarkers. C- terminal telopeptide of type I collagen correlated negatively with malondialdehyde and positively with uric acid. Uric acid was also found to be in a negative correlation with matrix metalloproteinase-8. Statistical correlation between biomarkers were calculated using Pearson's product-moment correlation coefficient (r-value).

Parameters	r	p
CTX I and MDA	0.43	0.05
CTX I and uric acid		0.09
MMP-8 and uric acid		0.09

Table V. Correlations between bone loss biomarkers and clinical parameters. Probing depth, a key clinical parameter for assessing periodontal disease severity, was found to correlate positively with all assessed bone loss biomarkers. No statistical correlation could be found between probing depth and oxidative stress markers. Statistical correlation (r-value) was determined using Pearson's product-moment correlation coefficient.

Parameters	r	p
CTX I and PD	0.46	0.005
25 OH D and PD	0.4	0.005
MMP-8 and PD	0.36	0.07
Osteocalcin and PD	0.4	0.002

mechanism, uric acid. Clinical results showed that MMP-8 positively correlated with PD.

Osteocalcin has been long linked with rapid bone turnover general conditions such as osteoporosis or multiple myeloma [30]. The connection between osteocalcin levels in oral fluids and periodontitis has also been investigated [31]. In the present study salivary osteocalcin levels were found to be elevated in periodontal patients when compared to respective controls. Moreover, while no correlation could be found between osteocalcin and the antioxidant defence system, the marker showed an expected positive correlation with PD.

C-terminal telopeptide of type I collagen (CTX I) is a collagen-related degradation marker used for measuring bone resorption [32]. There are currently few studies to demonstrate that salivary CTX I differs between patients with periodontitis and healthy controls. Our results demonstrate that CTX I levels are increased in patients with chronic periodontitis and also that there are functional relationships between bone resorption markers and antioxidant levels in saliva. Furthermore, CTX I was positively correlated with PD.

Previous studies have shown that 25-OH D levels in plasma are higher in patients with generalized aggressive periodontitis than in healthy controls and are also positively correlated with gingival bleeding index [33]. Other studies also prove that periodontal therapy reduces systemic and local 25-OH D levels [34]. Our data shows that 25-OH D levels were significantly higher in samples than in controls. Consistent with previous studies the marker correlated with clinical parameters such as PD.

An increasing number of studies report a correlation between periodontal tissue initiation and development and increased levels of ROS. One of most used biomarkers to evaluate oxidative damage in both local and systemic disorders are 8-OHdG and MDA [35]. At the cellular level OS progression starts through membrane lipid peroxidation followed by different cytosolic proteins and ending with DNA oxidation. It has been shown that OS is increased during periodontitis [35]. DNA may be damaged as a result of OS increment; one marker of DNA damage is 8-OHdG, a nucleoside that is excreted in the body fluids. 8-OHdG levels have been correlated with numerous chronic inflammatory diseases including periodontitis [36]. The current data also showed that 8-OHdG levels were increased in periodontal patients. However, the present results could not establish any significant association between 8-OHdG and OS or bone resorption markers. MDA displayed a similar pattern with 8-OHdG.

Antioxidants play key functions and biological effects in many systemic or local diseases induced by OS [37,38]. In our study salivary antioxidant defence systems such as uric acid, TAC or GPx were significantly decreased in patients with chronic periodontitis vs controls. Also statistical correlations could be identified between antioxidant markers and bone loss parameters in saliva.

In conclusion the present study shows that important biomarkers of OS and bone resorption can be detected in saliva. Moreover, a functional relationship could be established between these salivary markers. In the light of recent developments in the field [39,40] one possible step forward of the present study would be to assess the inter-relationships between salivary markers of inflammation, OS and bone loss in patients with periodontitis.

Acknowledgements

This study was supported by the Sectorial Operational Programme Human Programme Human Resources Development (SOP HRD), financed from the European Social Fund and by the Romanian Government under the contract number POSDRU/6/1.5/S/S17.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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