the Journal of CONTEMPORARY DENTAL PRACTICE at www.thejcdp.com
Volume 6 Number 4 November 15, 2005 Fall Issue

Alteration of Cyclic Nucleotides Levels and Oxidative Stress in Saliva of Human Subjects with Periodontitis

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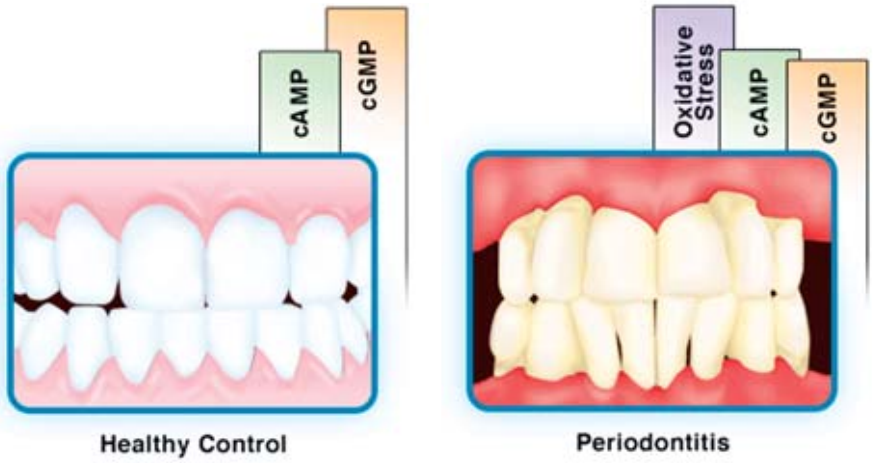


Abstract

Experimental findings suggest a protective role for cyclic nucleotides against induction of oxidative stress in saliva. Oxidative stress is a major contributor to the pathogenesis of inflammatory diseases. This study was conducted to evaluate salivary oxidative stress along with cGMP and cAMP levels in periodontitis subjects. cAMP and cGMP are second messengers that have important roles in salivary gland functions. Unstimulated whole saliva samples were obtained from periodontitis patients and age- and sex-matched healthy individuals. Saliva samples were analyzed for thiobarbituric reactive substances (TBARS) as a marker of lipid peroxidation, ferric reducing ability (total antioxidant power, TAP), and levels of cAMP and cGMP. Concentrations of cAMP and cGMP were reduced in the saliva of patients with moderate and severe periodontitis. Saliva of patients with severe periodontitis had higher TBARS and lower TAP than control subjects. The presence of oxidative stress and lower levels of salivary cGMP and cAMP in periodontitis are in association with disease severity.

Keywords: Oxidative stress, periodontitis, human saliva

Citation: Mashayekhi F, Agha-hosseini F, Rezaie A, Zamani MJ, Khorasani R, Abdollahi M. Alteration of Cyclic Nucleotides Levels and Oxidative Stress in Saliva of Human Subjects with Periodontitis. J Contemp Dent Pract 2005 November;(6)4:046-053.

Introduction

Periodontitis is a chronic inflammatory reaction to subgingival bacteria, inducing irreversible destruction of periodontal tissues and tooth loss. Periodontitis, affecting 7-15% of the adult population, is a multifactorial disease in which the existence of pathogenic bacteria is necessary but not sufficient. Under the influence of several behavioral, environmental, and genetic factors, the host immunologic and inflammatory response is the critical determinant of susceptibility to the disease. The magnitude and quality of host response and inflammatory mediators are not completely understood in periodontitis.[1, 2]

Research on saliva is a dynamic field. Saliva has proven its value for diagnostic purposes, especially those effects produced by drugs[3], because saliva can be collected in a safe and patient-friendly way requiring no special training. Improved technology also makes it possible to diagnose virtually everything by using blood tests. Whole saliva is a mixture of gingival fluid and the secretions of the major and minor salivary glands. The composition of saliva reflects the nature and amplitude of the host response to a periodontal microbial challenge. Therefore, determination of saliva constituent levels represents a putatively reasonable approach to the evaluation of a patient's risk for disease occurrence, intensity, or prognosis.[4, 5] It is well-known saliva has considerable antioxidant capacity, and oxidative stress may happen as a consequence of lipid peroxidation and impaired capacity of saliva antioxidant power.[6, 7] Our recent studies have confirmed significant antioxidant roles for cAMP and cGMP in saliva.[8, 9] cAMP and cGMP are second messengers that control salivary gland functions. There is good evidence oxidative stress resulting from increased levels of reactive oxygen species or decreased antioxidant power of the body contributes toxicologically in the pathogenesis of inflammatory diseases.[6, 11, 12] Therefore, we conducted this study to evaluate the association between salivary oxidative stress and cyclic nucleotides in subjects suffering from periodontitis.

Materials And Methods

Materials

Phosphate buffer, 2,4,6-tripyridyl-s-triazine (TPTZ), 2-thiobarbituric acid (TBA), 1,1,3,3-tetraethoxypropane (malonedialdehyde, MDA), trichloroacetic acid (TCA), and n-butanol were purchased from Merck Chemical Company (Germany). cAMP and cGMP determination kits (Immunoassay Kits, Quntikine®) were provided by R&D Systems GmbH, (Germany).

Methods

This case-control study was conducted in a public oral medicine clinic at Tehran University of Medical Sciences (TUMS). The subjects included 24 (12 male, 12 female) patients in stages of early, moderate, and advanced periodontitis during the period February through July 2004. The age range of the subjects was 25 to 55 years. Eight age- and sex-matched healthy controls were chosen from the clinic staff and students who did not have clinical signs of periodontal ligament destruction. Prior to any procedure, written informed consent was obtained from all subjects. Smokers and patients on medications for other diseases were excluded from the study. The diagnosis and classification of patients was based on the criteria of the American Dental Association[13] for diagnosis of periodontal diseases. This included clinical (bleeding following probing, pocket depths, grade of furcation invasion areas, and the tooth mobility class) and radiographic (bone loss, radiographic furcations grade, and crown to root ratio) findings. Patients with periodontitis were classified by early, moderate, and advanced categories. All measurements and samples were taken before starting periodontal treatment, and the patients underwent no periodontal therapy for at least six months prior to sampling. Whole unstimulated saliva was collected over a five-min period from subjects with instructions to allow saliva to pool in the bottom of the mouth and drain into a collection tube, when necessary. Prior to analysis, the saliva was centrifuged at 4000 g for 10 min at 4°C. The supernatant fraction was then aliquotted into storage vials and kept at -80°C until required for analysis. The salivary flow rate was calculated by dividing the volume collected by time and was found without difference among healthy and periodontitis subjects (mean of 0.36 vs. 0.35 ml/min). The protocol of this study was approved by the TUMS Medical Ethics Committee.

Total Antioxidant Power (TAP) Assay

The total antioxidant power (TAP) of saliva was determined by measuring its ability to reduce

Fe^{3+} to Fe^{2+} using the FRAP test.[14] Briefly, in this test, the medium is exposed to Fe^{3+} and the antioxidants present in the medium produce Fe^{2+} as a result of antioxidant activity. The reagent included a 300 mmol/liter acetate buffer with a pH of 3.6 and 16 ml of $C_2H_4O_2$ per liter of buffer solution along with 10 mmol/liter of TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mmol/liter HCL, 20 mmol/liter $FeCl_3$ $6H_2O$. The working FRAP reagent was prepared as required by combining 25 ml of acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml $FeCl_3$ $6H_2O$ solution. Ten µl of H_2O-diluted sample was then added to 300 µl freshly prepared reagent and warmed at 37°C. The complex between Fe^{2+} and TPTZ gives a blue color with absorbance at 593 nm.

Lipid Peroxidation Assay

The method based on the reaction of MDA (malonedialdehyde) as the end product of the oxidation of polyunsaturated fatty acids and its concentration in the medium is an established measure of lipid peroxidation extent. In this test the reaction of MDA with TBA (thiobarbituric acid) creates a complex which is determined spectrophotometrically, while lipid peroxidation in samples are assessed in terms of thiobarbituric acid reactive substances (TBARS) produced.[15] Briefly, the samples were diluted by buffered saline (1:5) and 800 µl of TCA (28% w/v) was added to 400 µL of this mixture and centrifuged in 3000 g for 30 min. The precipitation was then dissolved in sulfuric acid and 600 µl of the mixture was added to 150 µl of TBA (1% w/v). The mixture was then incubated for 15 min in a boiling water bath. Following incubation, 4 ml of n-butanol was added, the solution centrifuged, cooled, and the absorption of the supernatant was recorded in 532 nm using a UV-160-A Shimadzu double beam spectrophotometer (Japan). The calibration curve of a 1,1,3,3-tetraethoxypropan standard solutions was used to determine the concentrations of TBA-MDA adducts in samples.

cAMP and cGMP Assays

Concentrations of cAMP and cGMP of saliva were determined by the immunoassay technique using specific enzyme-linked immunoassay kits. It combines the use of a peroxidase-labeled cAMP or cGMP conjugate, a specific antiserum, which can be immobilized on to pre-coated microtitre plates, and a single substrate solution. The assay is based on competition between unlabelled cAMP or cGMP and a fixed quantity of peroxidase-labeled cAMP or cGMP, for a limited number of binding sites on a cAMP or cGMP specific antibody.

Statistical Analysis

Data were analyzed by analysis of variance (ANOVA) followed by Newman-Keuls tests. Differences between experimental groups with P values equal or lower than 0.05 were considered significant.

Results

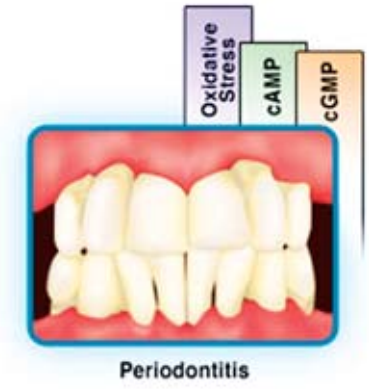
Concentrations of both cAMP and cGMP were found to be decreased ($P<0.01$) in saliva of moderate and severe periodontitis patients in comparison to those of healthy subjects as shown in Figures 1 and 2.

Saliva concentration of TBARS in severe periodontitis patients increased significantly ($P<0.01$) in comparison to those of healthy subjects (Figure 3).

Analysis of TAP revealed the saliva of severe periodontitis patients had lower TAP ($P<0.01$) than the healthy control subjects (Figure 4).

Discussion

Saliva constantly bathes the teeth and oral mucosa acting as an antibacterial solution, an ion reservoir, a lubricant, and a buffer. In addition to these host protective properties, saliva constitutes a first line of defense against free radical mediated oxidative stress.[5, 16] It has been claimed imbalances in levels of free reactive oxygen radicals with antioxidants play a major role in development of oral diseases and



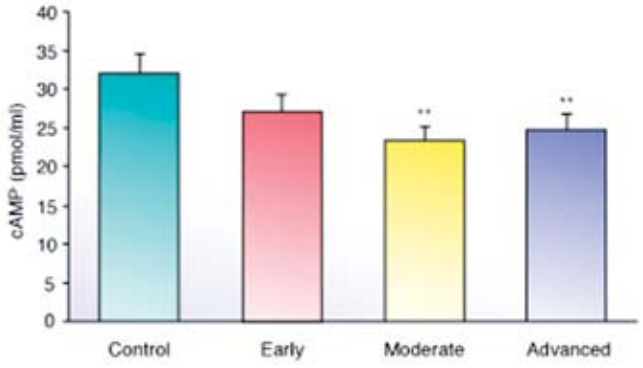


Figure 1. cAMP levels in saliva of periodontitis patients in comparison to healthy control subjects. (Values are expressed as mean±SE of 8 subjects in each group. Periodontitis in patients are categorized as early, moderate, and advanced. **The difference between periodontitis (moderate, advanced) patients and control subjects is significant at P<0.01).

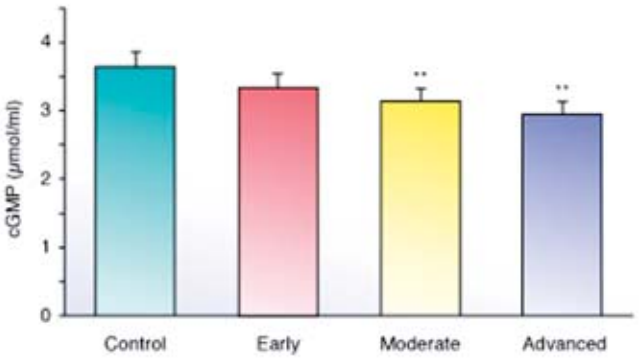


Figure 2. cGMP levels in saliva of periodontitis patients in comparison to healthy control subjects. (Values are expressed as mean±SE of 8 subjects in each group. Periodontitis in patients are categorized as early, moderate, and advanced. **The difference between periodontitis (moderate, advanced) patients and control subjects is significant at P<0.01).

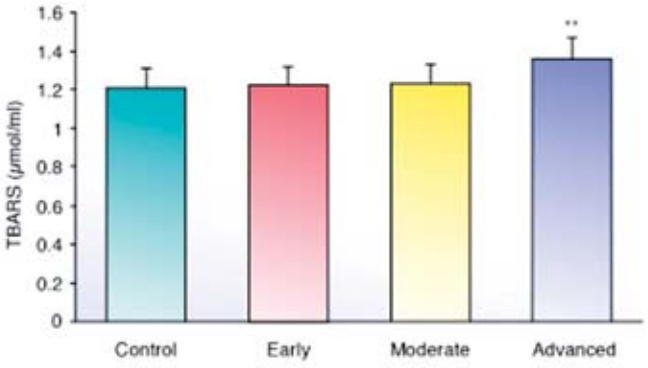


Figure 3. Lipid peroxidation levels in saliva of periodontitis patients in comparison to healthy control subjects. (Values are expressed as mean±SE of 8 subjects in each group. Periodontitis in patients are categorized as early, moderate, and advanced. **The difference between periodontitis (advanced) patients and control subjects is significant at P<0.01).

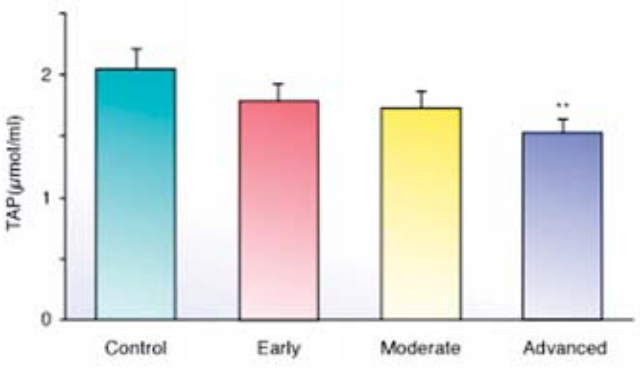


Figure 4. Total antioxidant power (TAP) in saliva of periodontitis patients in comparison to healthy control subjects. (Values are expressed as mean±SE of 8 subjects in each group. Periodontitis in patients are categorized as early, moderate, and advanced. **The difference between periodontitis (advanced) patients and control subjects is significant at P<0.01).

inflammation. In addition there is evidence during gingival inflammation that gingival crevicular fluid adds more inflammatory products such as reactive oxygen species to saliva, providing a blind loop which worsens the situation.[17-19] The antioxidant systems of saliva are highly complex and rich in several antioxidants (i.e., uric acid, glutathione, and ascorbic acid). Thus, investigating individual antioxidants are less representative than measuring total antioxidant capacity (TAC), which holistically shows the total antioxidant power.[20, 21] Among the patients suffering from early, moderate, or advanced periodontitis, only those with advanced disease showed significant decrease in TAP and elevated levels of TBARS as a marker for presence of reactive oxygen species. The existence of oxidative stress in advanced periodontitis and its absence in the early and moderate categories may explain the discrepancies in previous studies. For instance, by using TEAC (Trolox equivalent antioxidant capacity) method for measuring TAP, patients with periodontitis did not reveal any difference in their TAP compared

with healthy subjects[22]; however, this difference had been significant in other studies.[7, 18] None of these studies had classified periodontitis based on its severity, and this might have biased the final results due to different behavior of oxidative stress in periodontitis categories. Supporting the present finding on decreased TAP in patients with severe periodontitis, Brock et al.[23] recently reported both saliva and gingival crevicular fluid antioxidant concentration in periodontitis subjects is lower than that of healthy subjects. They concluded changes of TAP in periodontitis may reflect predisposition to or the results of reactive oxygen species (ROS)-mediated damage which is still unclear. With measurement of saliva lipid peroxidation in the present study, that idea is amplified and indicates the presence of oxidative stress. In support of this concept, Sculley and Langley-Evans[24] found periodontal disease is associated with lower antioxidant capacity and increased protein oxidation in whole saliva. They categorized patients according to periodontitis severity and concluded reduced salivary TAP and increased protein carbonyls were associated with the severity of periodontitis.

Considering above-noted reports and the present findings, the role of oxidative stress in oral inflammation is confirmed and suggests antioxidant agents may have a considerable prophylactic and curative role in severe periodontitis. On the other hand, it should be noted saliva is secreted by two major mechanisms: secretion of acinar cell proteins into saliva is mediated by β-adrenergic receptors and cAMP pathways, while secretion of fluid is induced through muscarinic and α-adrenergic receptor stimulation.[25-27] The cGMP is believed to be a mediator for muscarinic and α-adrenergic-stimulated salivary fluid secretion. An enhanced cellular level of cGMP is accompanied with activation of muscarinic and α-adrenergic receptors.[28] In addition nitric oxide (NO), whose regulatory effects in salivary secretion is evident[29, 30], acts through intracellular cGMP-dependent processes. Production of NO leads to generation of cGMP which opens ion channels to initiate salivary secretion.[31, 32] In rats administration of phosphodiestrase inhibitors (i.e., theophylline and sildenafil), which selectively increase cGMP and cAMP levels by prohibiting their catabolism, has shown significant increase in salivary flow rate, total protein concentration, and epidermal growth factor (EGF) secretion.[33] It is thought these drugs have antioxidant effects in saliva.[8, 9] In the present study patients with periodontitis showed lower levels of cGMP and cAMP compared to the control subjects; however, these decreases were only significant in moderate and advanced groups. Lower cGMP levels in our patients was not surprising as cGMP is the mediator of NO, and previously it has been shown that NO concentration decreases in patients with periodontitis with a direct correlation to its severity.[34, 35] The present finding confirms the protective effect of NO[36] in saliva for oral inflammation along with probable protein and cytokine secretion impairment in periodontitis.

Conclusion

The presence of oxidative stress and lower levels of salivary cGMP and cAMP in periodontitis associated with disease severity suggests antioxidant effects for phosphodiestrase inhibitors (PDIs) in the prophylaxis or treatment of periodontitis. Selective PDIs such as sildenafil for cGMP and theophylline for cAMP or non-selective PDIs such as milrinone for both cGMP and cAMP[37, 38] can be used. However, further animal or human studies on a larger scale are needed to elaborate the efficacy of these drugs.

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