Salivary Interleukin-6 - A Pioneering Marker for Correlating Diabetes and Chronic Periodontitis: A Comparative Study

Abstract

Background: Periodontitis is a chronic inflammatory disease which is multifactorial. Diabetes mellitus (DM) is one of the major systemic factors to influence the severity of chronic periodontitis. Numerous inflammatory markers are produced in the course of the disease which is secreted in saliva too. This study evaluates the salivary concentrations of interleukin-6 (IL-6) in periodontitis patients with type 2 diabetes. Materials and Methods: Whole saliva samples were collected from eighty patients who were further divided into four groups; healthy (control group; n = 20), untreated periodontitis (UPD; n = 20), DM (n = 20), and UPD + DM (n = 20 groups). Salivary IL-6 concentrations were determined by standard enzyme-linked immunosorbent assay. Results: Results show that the UPD patients with and without DM exhibited higher concentrations of salivary IL-6 than the control group and diabetes groups. Further, the salivary IL-6 was correlated with glycosylated hemoglobin A levels in patients with diabetes. Conclusion: Concentration of salivary IL-6 was elevated in patients with periodontitis with and without diabetes. Thus, salivary IL-6 levels can be considered as an important biomarker in the diagnosis of periodontitis and diabetes.

Keywords: Diabetes mellitus, glycosylated hemoglobin A, interleukin-6, periodontitis, saliva

Introduction

Periodontitis is a chronic inflammatory disease characterized by inflamed gingiva, bleeding on probing, increased probing pocket depth, clinical attachment loss, pus discharge, and resorption of alveolar bone. Periodontitis is multifactorial in origin and anaerobic, Gram-negative organisms being the bacterial cause. Environmental, behavioral, and systemic factors also influence the onset, progression, and severity of periodontitis. Diabetes mellitus (DM) is a metabolic disorder manifested by elevated levels of glucose in the blood and influence the progression and severity of periodontitis. The relationship between periodontal disease and DM is two way, meaning both can influence the severity of the other. Both diabetes and periodontitis stimulate the release of proinflammatory cytokines. The microbial challenge provokes an inflammatory response in the periodontal tissue that involves a network of cytokines functioning in synergy. Interleukin-6 (IL-6) is secreted by macrophages in response to inflammation and is involved in recruitment and apoptosis of leukocyte and T-cell activation. IL-6 and its soluble receptor induce bone resorption, either by increasing the receptor activator of nuclear factor κ ligand (RANKL) or by directly inducing the formation of osteoclast. There is ample evidence from the literature to substantiate the elevated levels of salivary IL-6 at the periodontally infected sites from diabetic patients proving the systemic influence of diabetes on periodontium. Thus, by evaluating the salivary IL-6 level, the risk and severity of periodontitis in type 2 diabetic patients can be predicted. In the future, the salivary IL-6 levels can be used as an important biomarker for the diagnosis, prognosis, and to predict the treatment outcomes of periodontitis. Hence, this study aims to evaluate the salivary levels of IL-6 in Type II diabetes and periodontitis patients and to assess the levels of glycosylated hemoglobin A (HbA1c) in diabetic and chronic periodontitis (CP) patients.

Materials and Methods

The study was performed at the Department of Periodontology and Oral Implantology in...

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Access this article online
Website: www.ijdr.in
DOI: 10.4103/ijdr.IJDR_167_14

How to cite this article: Balaji A, Chandrasekaran SC, Subramanium D, Fernz AB. Salivary Interleukin-6 - A pioneering marker for correlating diabetes and chronic periodontitis: A comparative study. Indian J Dent Res 2017;28:133-7.
at Sree Balaji Dental College, Chennai. The study was reviewed and approved by the Ethical Committee of Bharath University of Chennai. Eighty patients were divided into 4 groups. All participants were informed about the procedure and an informed consent was taken. Before the examination, a thorough medical and dental history was taken.

**Study population**
A total of 100 patients, both male and female, were screened and 80 patients were recruited who met the inclusion criteria. Patient’s age group was between 35 and 65 years. Subjects were divided into four groups: control group \((n = 20)\) - systemically and periodontally healthy subjects, untreated periodontitis (UPD) group \((n = 20)\) – subjects who were systemically healthy and clinically diagnosed with untreated CP. DM group \((n = 20)\) - subjects with type II diabetes and a healthy periodontium. UPD + DM group \((n = 20)\) - subjects with Type II diabetes and clinically diagnosed with untreated CP.

**Inclusion criteria**
- Presence of a minimum of 15 natural teeth (excluding third molars)\(^3\)
- Criterion for a periodontitis - presence of proximal attachment loss \(\geq 3\) mm in two or more nonadjacent teeth\(^4\)
- Dental treatment not undergone for past 6 weeks.

**Exclusion criteria**
- Smoking within the past 5 years
- Pregnancy or lactation
- Use of antibiotics or periodontal treatment in the preceding 6 months
- Concomitant medical therapy, except for a diabetic condition
- Other inflammatory conditions
- Major diabetic complications (retinopathy, nephropathy, neuropathy, and atherosclerosis).

**Study protocol**
A detailed medical history and informed consent were taken from all the subjects. Clinical examination was done using mouth mirror and probe. The salivary samples collected were analyzed for IL-6 levels by enzyme-linked immunosorbent assay (ELISA) method. Glycemic level of the subjects was estimated by HbA1c measurement. The data obtained were subjected to statistical analysis.

**Saliva sample collection**
Nonstimulated expectorated whole saliva was collected approximately 3 ml from each patient, into sterile tubes according to Navazesh method.\(^5\) Patients were refrained from eating, drinking, and oral hygiene for 2 h before saliva collection. Saliva samples were placed on ice immediately and aliquoted before freezing at \(-80^\circ\)C. Samples were thawed and analyzed.

**Salivary interleukin-6 analysis**
Salivary concentration of IL-6 was determined using an ELISA using Human Quantikine ELISA kit.

**Glycosylated hemoglobin A measurement**
HbA1c levels were analyzed for the metabolic assessment in DM group and in patients with diabetes and periodontitis. HbA1c was measured and expressed as percentages. Normal range of HbA1c test was <6%.

**Reagents for estimating interleukin-6**
Reagents used were antihuman IL-6 wash buffer, 2 vials of recombinant human IL-6, 30 ml of 0.09% sodium azide as preservative for sample, 15 ml of 5x concentrated buffer, 2 vials of biotinylated antihuman IL-6 hP streptavidin concentrate, 8 \(\mu\)l \(\times 30000\) concentrated horseradish peroxidase-conjugate streptavidin, 3,3’,5,5’ tetramethylbenzidine (TMB) one-step substrate reagent, 12 ml of TMB in buffered solution, and 8 ml of 2M sulfuric acid as stop solution.

**Enzyme-linked immunosorbent assay procedure**
All reagents, samples, and standards were brought to room temperature before use [Figure 1].

100 \(\mu\)l of standard or sample was added into appropriate wells and covered to incubate for 2.5 h at room temperature or overnight at 4°C with gentle shaking. Followed by addition of 100 \(\mu\)l prepared Biotin antibody to each well and incubation done for 1 h at room temperature. To which 100 \(\mu\)l prepared Streptavidin solution was added and incubated for 45 min at room temperature. Followed by addition of 100 \(\mu\)l, TMB one-step substrate reagent to each well and incubation done for 30 min at room temperature. The reaction was ended by adding 50 \(\mu\)l stop solution to each well and read at 450 nm immediately.

**Results**
Eighty subjects in this study were divided into four groups: control group who were systemically and periodontally healthy, UPD group, type 2DM patients with healthy periodontium, and UPD + DM group subjects with type 2 DM and untreated CP. Clinical examination was done after collection of saliva by Navazesh method\(^6\) and salivary concentrations of the samples assessed by Human Quantikine IL-6 ELISA.
Table 1 shows the mean values of optical density of IL-6 among the four groups. On comparison, IL-6 value is found to be highest among uncontrolled type 2 diabetes and periodontitis group and least among the healthy controls. Concentration of IL-6 is found to be increased in subjects with UPD and diabetes patients with healthy periodontium. For the rejection of null hypothesis, ANOVA has been used.

Figure 2 illustrates the optical density values plotted across the various groups. The peak value of IL-6 concentrations was found to be in the UDP + DM group, followed by DM group, UDP group, and least the control group. This proves the influence of type 2 DM and CP on the bone resorption. HbA1c value depicting the glycemic levels of the subjects was estimated for DM and DM + UDP group and both the groups showed >6% revealing the poor glycemic control. DM + UDP group had HbA1c values greater than the other. This elucidates the two-way relationship of DM and CP.

**Discussion**

Saliva is used as a diagnostic tool in medicine and dentistry because of its ease of collection and containing both microbial and host response mediators. Quantifying biomarkers in saliva serve as a useful tool to predict an individual’s susceptibility to periodontitis. Costa et al. (2010).

The present study has analyzed the salivary concentration of proteins associated with periodontal disease and the results demonstrate the differentially expressed IL-6 levels in all the four groups, showing the influence of diabetes and periodontitis on severity of bone destruction. Even though there is evidence supporting that the periodontal infection represents a complication that may be involved in altering the systemic physiology of alveolar bone, conflicts remain as to whether or not diabetes really augments the risk of periodontal disease and if so, how far the diabetic condition can affect the periodontal health need to be studied. IL-6 is a proinflammatory cytokine that dictates the transition from acute to chronic inflammation by changing the nature of leukocyte infiltrate (from polymorphonuclear neutrophils to monocytes/macrophages), exerts stimulatory effects on T- and B-cells, and induces antibody formation, favoring a chronic inflammatory response (Scheller et al. 2005.[8,9]).

In addition, IL-6 stimulates osteoclast activity and bone resorption inducing osteoclastic production of downstream effectors such as RANKL (Cronstein BN 2007).[10] Periodontitis and diabetes are chronic inflammatory diseases that increase inflammatory IL-6 levels which reflects in saliva. Periodontitis triggers systemic and local immune-inflammatory response by significantly increasing the expression of IL-6 which further contributes to bone loss by inducing bone resorption, either by increasing RANKL or by directly acting on osteoclast formation in periodontitis.[11] In DM patients, the chronic hyperglycemic state results in AGE formation and AGE-RAGE interaction induces the expression of proinflammatory cytokine IL-6 and the abnormal increase in the cytokine levels induced by AGE exacerbate the inflammatory response.

In our study, we have found significantly elevated concentrations of IL-6 in the saliva of patients with periodontitis with or without diabetes (DM + UPD and UPD groups). The result was in accordance with the host modulatory effect of IL-6 on the modulation of periodontitis. Geivelis et al. 1993[12] conducted a study to determine whether the amount of IL-6 is greatest common factor correlated with periodontal clinical measures. He estimated IL-6 levels using ELISA procedure and the findings suggest that IL-6 may be a useful indicator of periodontal disease suggesting the association of IL-6 with the severity of periodontal disease. Duarte et al. 2007[13] evaluated whether the biochemical changes associated with type 2 diabetes modulate the expression of IL-6 in periodontitis patients and found higher IL-6 levels in patients with diabetes and periodontitis compared to nondiabetics depicting the diabetes modulated expression of IL-6 in periodontal patients. Studies done by Campus et al. 2005,[14] Jansson et al. 2006,[15] and Cutler et al. 1999[16] have also established type 2 DM as an important risk factor.
for periodontal disease. National Health and Nutritional Examination Survey, 1988[17] conducted a comprehensive study to evaluate the prevalence of diabetes in periodontitis patients and found two-fold increased prevalence of diabetes in periodontitis patients. The effect of onset and duration of diabetes on periodontal status was studied by numerous authors and the studies done by Loe et al. 1993[18] and Firatli 1997[16] have shown positive correlations between the onset and duration of diabetes on periodontal disease, while the studies done by Bacic et al. 1988[19] Rosenthal et al. 1988[20], Hove and Stallard et al. 1970[21] have shown no correlation between the onset and duration of diabetes on the periodontal status. Firatli 1997[16] conducted a case–control study to determine the relationship between DM and clinical periodontal status and found positive correlation between the duration of diabetes and periodontal disease. Bacic et al. 1988[19] investigated using the CPITN system, the periodontal treatment needs in diabetic patients and the possible effects of the duration and control of diabetes on periodontal status in diabetic patients and found no differences in the periodontal condition relating to the duration and control of diabetes. HbA1c test is used to monitor the glycemic control in diabetes patients and it measures the amount of glucose irreversibly bound to hemoglobin molecule (Hb).Scannapieco et al. 2007[22] and Lim et al. 2007[23] studied the relation between the markers of glycemic control and severity of periodontal disease in 181 subjects aged 21–65 years and found that diabetic patients with good to moderate control with HbA1c <8% displayed significantly lower percentage of sites with bleeding on probing and probing depth >5 mm compared with diabetic subjects with poor glycemic control with HbA1c level >8% confirming poor glycemic control as a significant risk factor for periodontitis. Collin et al. 1988[23] found that HbA1c levels deteriorated in type 2 DM subjects with severe periodontitis compared with diabetes subjects without periodontitis. In the current study, we have found a significant correlation between HbA1c levels and IL-6 concentration in patients with periodontitis and DM compared to the other groups. This result was consistent with the studies done by Lim et al. 2007[23] that showed a positive association between poor metabolic control and periodontitis. We have extensively studied the relationship between IL-6 and diabetic status. When the multiple comparisons were done in this study in CP, DM, CP + DM, and healthy controls, it showed P < 0.01 which is statistically significant. The inflammatory marker IL-6 shows its highest activity among patients with CP + DM which coexists with the study done by Costa et al. 2010[7]. In our study, we found an increase in HbA1c concentration which is more prevalent in CP group presenting with uncontrolled DM than in diabetic group proving the effect of IL-6 on glycemic control. More further studies are needed which may be helpful in designing more mechanistic approach to minimize the negative effect of type 2 DM on the periodontal destruction.

Summary
Eighty patients were selected for study and were divided into four groups: Healthy group, diabetic group, UPD group, and patients with diabetes and periodontitis. Patients who have been smoking within the past 5 years and the patients who were under antibiotics and the patients who were under periodontal treatment in the previous 6 months and also the patients having major diabetic complications such as retinopathy and nephropathy and with any other inflammatory conditions except diabetes were excluded from the study. Nonstimulated whole expectorated saliva was collected by Navazesh method[5] and the salivary concentrations of IL-6 are determined by ELISA and HbA1c levels were assessed in diabetics with and without periodontitis. Probing depth, clinical attachment level, bleeding on probing, and suppuration were also recorded in all subjects.

Conclusion
In the present study, when the salivary levels of IL-6 were assessed by ELISA, there was a significantly elevated level of IL-6 in DM + UPD group than the DM group, depicting the host modulatory role of IL-6 in these patients. When the HbA1c levels were assessed, we have found increased HbA1c levels in the DM + UPD groups than the diabetic groups depicting the inflammatory role on glycemic control. Even though IL-6 is an inflammatory mediator that stimulates osteoclast activity and bone resorption in periodontitis, several other inflammatory and immune mediators which modulate the periodontal destruction should be considered in the future studies. Local and systemic conditions, periodontitis, and diabetes, respectively, may change the expression of salivary proteins and should be taken into account in the future studies.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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