Estimation of salivary tumor necrosis factor-alpha in chronic and aggressive periodontitis patients

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Abstract

Introduction: Periodontitis is a chronic bacterial infection characterized by persistent inflammation, connective tissue breakdown and alveolar bone destruction mediated by pro-inflammatory mediators. Tumor necrosis factor-alpha (TNF-α) is an important pro-inflammatory mediator that produced causes destruction of periodontal tissues. **Objective:** The aim of the study is to estimate the salivary TNF-α in chronic and aggressive periodontitis and control participants and further correlate the levels with clinical parameter such as gingival index (GI), plaque index (PI), probing pocket depth (PPD) and clinical attachment loss. **Materials and Methods:** The study population consisted of 75 subjects age ranging from 25 to 55 years attending the outpatient section of Department of Periodontics, Saveetha Dental College and Hospital. The study groups included Groups 1, 2, and 3 with participants with healthy periodontium (n = 25), generalized chronic periodontitis (n = 25) and generalized aggressive periodontitis (n = 25), respectively. Salivary samples from the participants were used to assess the TNF-α levels using enzyme-linked immunosorbent assay. **Results:** GI and PI were found to be significantly higher in chronic and aggressive periodontitis compared to the controls. The mean TNF-α value in chronic periodontitis patients (12.92 ± 17.21 pg/ml) was significantly higher than in control subjects (2.15 ± 3.60 pg/ml). Whereas, in aggressive periodontitis patients the mean TNF-α (7.23 ± 7.67) were not significantly different from chronic periodontitis or healthy subjects. Among periodontitis participants, aggressive periodontitis subjects exhibited a significant positive correlation between the salivary TNF-α and PPD. **Conclusion:** Salivary TNF-α levels are significantly higher in chronic periodontitis than in healthy subjects, but there was no significant correlation with the clinical parameters.

Keywords: Aggressive periodontitis, chronic periodontitis, cytokines, tumor necrosis factor-alpha

Introduction

Periodontitis is a chronic bacterial infection characterized by persistent inflammation, connective tissue breakdown and alveolar bone destruction. Inflammatory mediators and tissue breakdown products have been frequently detected in gingival tissues, gingival crevicular fluid, serum, and saliva.[1] However, the cytokine secretion profiles are different among various types of periodontitis.[2] Hence, their qualitative and quantitative changes is of therapeutic significance.

Tumor necrosis factor (TNF) is one such pro-inflammatory cytokine that causes periodontal tissue destruction.[3,4] TNF is active in 2 forms: TNF-α and TNF-β. TNF-α is produced by the activated macrophages, neutrophils, keratinocytes, monocytes, and mast cells in response to lipopolysaccharides (LPSs). TNF-β is produced by TH1 subsets of CD4+ T cells that have been activated by antigens or mitogens.[5]

TNF-α has a myriad of actions, mostly pro-inflammatory. Leukocyte recruitment and vascular permeability are facilitated by the stimulated expression of selectins and adhesins by TNF-α.[6] Macrophage induced angiogenesis is also mediated by TNF-α and has a pivotal role in vascular proliferation in the periodontal granulation tissue formation.[7] In response to bacterial LPS, TNF-α triggers osteoclast activation, proliferation and differentiation resulting in bone resorption.[8,9]

Saliva has long been used to assess periodontitis and its role as a diagnostic medium has been a subject of considerable research activity. Proposed markers for the disease include proteins of host origin (enzymes, cytokines, and

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immunoglobulins), phenotypic markers (epithelial keratins), host cells, hormones (cortisol), bacteria and bacterial products.[10] The biomarkers help in identifying susceptible patients and serve as surrogate endpoints for monitoring the end of therapy.[11] It therefore, meets the demands of being an inexpensive and noninvasive collection technique. The aim of the present study is to estimate and compare salivary TNF-α levels in patients with generalized chronic periodontitis, generalized aggressive periodontitis and healthy periodontium and also to assess the correlation of TNF-α with the clinical variables.

Materials and Methods

This cross-sectional study was conducted in the Department of Periodontics, Saveetha Dental College and the biochemical estimation was done in the herbal and Indian Medicine Research Laboratory, Sri Ramachandra Medical College. The study was approved by the Institutional Review Board (Ref. no: 002). A written informed consent was obtained from those who agreed to participate in the study.

The study participants comprised 75 subjects (n = 75; 49 men and 26 women) in three groups of 25 each, who were selected from the outpatient department of Saveetha Dental College. Groups 1, 2 and 3 consisted of participants with healthy periodontium, generalized chronic periodontitis, and generalized aggressive periodontitis, respectively. Inclusion criteria comprised of patients in the age range of 20–55 years with a minimum of 18 teeth. Group 1 patients had a healthy periodontium with no gingival inflammation (gingival index [GI] = 0; pocket depth ≤3 mm and clinical attachment loss [CAL] = 0). Patients were categorized as generalized chronic or aggressive periodontitis based on the American Academy of Periodontology criteria. The periodontitis group had an attachment loss of ≥5 mm and pocket depth of ≥6 mm in at least 30% of the sites. Only those participants who presented with deep pockets with a minimal subgingival plaque and healthy tissue response, free of inflammation were selected for aggressive periodontitis category. Radiographs of aggressive periodontitis patients revealed angular bone loss, especially in the uh molar incisor region. The diagnosis was reconfirmed by two other examiners, and the ambiguous cases were excluded. Exclusion criteria consisted of patients with systemic diseases, pregnant and lactating mothers, patients on medications, and history of periodontal treatment in the last 3 months. Smokers and alcoholics were also excluded. Plaque index (PI), GI, periodontal pocket depth and loss of attachment was measured by a single examiner using a Williams periodontal probe after the salivary sample collection.

Patients were instructed to rinse their mouth with water followed which unstimulated whole expectorated salivary samples were collected into sterile Eppendrofs and stored at −80°C. The assay was carried out with a commercially available enzyme-linked immunosorbent assay kit (human quantitative high sensitivity TNF-α assay by R&D system using ELISA).

Principle of the assay

TNF-α ELISA is a solid phase enzyme amplified sensitivity immuoassay performed on a microtiter plate. The assay uses calibrators and the samples react with the capture monoclonal antibody (MAb1) coated on a microtiter well and with a monoclonal antibody (MAb2) labeled with horseradish peroxide (HRP). After an incubation period, allowing the formation of a sandwich, the microtiter plate is washed to remove unbound enzyme labeled antibody. Bound enzyme labeled antibody is measured through a chromogenic reaction. The chromogenic solution is added and incubated. The reaction is stopped with a stop solution and the microtitre plate is read at 450 nm. The amount of substrate turnover is determined colorimetrically by measuring the absorbance, which is proportional to the TNF-α concentration.

Procedure

The required strips were selected and placed on the holding frame. Sequentially 50 µl of incubation buffer, 200 µl of calibrator, 200 µl of control and 200 µl sample were pipetted into the appropriate wells. They were incubated for 2 h at room temperature on a horizontal shaker set at 700 rpm. The excess liquid was aspirated from each well and the plate was washed thrice with distilled water. Later, 100 µl of calibrator and 50 µl of anti-α HRP conjugate were pipetted into the wells. It was incubated for 2 h on a horizontal shaker set at 700 rpm. The liquid was aspirated from each well and washed again. This was followed by the addition of 200 µl of freshly prepared chromogenic solution tetra methyl benzydine into each well and incubated for 30 min at room temperature on a horizontal shaker set at 700 rpm. Finally, 50 µl of stop solution was pipetted into each well, and the absorbance values read at 450 nm and 490 nm within 3 h.

Statistical analysis

The overall comparison of mean values among the different study groups was done by Kruskal–Wallis one-way ANOVA. Comparison between any two study groups was done by Mann–Whitney U-test followed by Bonferroni correction method. The correlation between TNF-α and the clinical variables in each group was done by Spearman's correlation test.

Results

GI and PI were found to be significantly higher in chronic (Group 2) and aggressive periodontitis (Group 3) compared to the controls (Group 1). However, between the Groups 2 and 3, the former had a significantly higher GI. There were no statistically significant differences in mean periodontal pocket depth and clinical attachment level between Groups 2 and 3 [Table 1].

The mean TNF-α value in Group 2 (chronic periodontitis) patients was significantly higher than in control subjects (Group 1). However, there was no significant difference in TNF-α values between Group 2 and 3 and also between Group 1 and 3 [Table 2]. There was a significant negative correlation between TNF-α and PI among the controls ($r = -0.42$, $P = 0.04$). Among periodontitis participants, only Group 3 (aggressive periodontitis) subjects exhibited a significant positive correlation between the salivary TNF-α and periodontal pocket depth ($r = 0.04$, $P = 0.05$) [Table 3].

Discussion

The results of the present study indicated a significant increase in salivary TNF-α levels in generalized chronic periodontitis patients when compared to periodontally healthy controls ($P < 0.05$). These findings were in accordance with a study by Frodge *et al*.[12] However, chronic periodontitis patients in this study showed higher TNF-α levels (12.92 pg/ml) as compared to the study conducted by Frodge *et al*. (4.33 pg/ml). A possible explanation for the discrepancy might be variations in disease severity between the two study populations. The present study is in concurrence with many other studies conducted earlier. These studies also reported elevated levels of TNF-α in gingival crevicular fluid, serum, gingival tissues, and plasma of chronic periodontitis patients compared to controls. On the contrary, Ng *et al*. reported a very low level of salivary TNF-α in periodontitis patients.[13] Attributable reasons include change in salivary composition due to the use of stimulated saliva and the discrepancy in case selection as their diagnosis was based only on radiographic assessment. Similarly Teles *et al*. and Yousefimanesh *et al*. reported a lack of association between the levels of salivary biomarkers, including TNF-α and periodontal disease status.[14,15] The reasons put forward by them were an excessive dilution of the gingival crevicular fluid containing cytokines or the inhibition of cytokines in the saliva by the putative inhibitors present in the whole saliva. There also existed a difference in the disease severity in the study population between the Teles group (mean probing depth - 3.0 ± 0.6) and the present study (6.43 ± 0.55).

Salivary TNF-α levels of aggressive periodontitis patients were higher but not statistically significant when compared with the healthy controls. A lack of statistical significance could be a result of smaller sample size and reduced gingival inflammation in aggressive periodontitis patients. Sun *et al*. also reported that plasma TNF-α levels were not significantly different between aggressive periodontitis and healthy controls.[16] Aurer *et al*. had also reported that TNF was below the level of detection in the saliva of patients with aggressive periodontitits.[17] Recently, Gümüs *et al*. found that salivary TNF-α levels were significantly higher in aggressive periodontitis group than healthy controls but not so in chronic periodontitis group.[18] The discrepancy could have arised due to the inclusion of both smokers and nonsmokers in their study population. Similarly, few other studies have reported that TNF-α levels were significantly increased in GCF,[19,20] serum,[21,22] gingival tissues[21] and plasma[22] of patients with aggressive periodontitis.

Table 1: Comparison of clinical variables between different study groups

Variables	Group	Mean±SD	Overall *P* value	Significant groups
PI	1	0.29±0.27	<0.0001*	1 versus 2
	2	1.29±0.41		1 versus 3
	3	1.43±0.32		
GI	1	0.28±0.27	<0.0001*	1 versus 2
	2	1.70±0.70		2 versus 3
	3	0.87±0.32		1 versus 3
PD	1	-	<0.0001*	1 versus 2
	2	6.43±0.55		1 versus 3
	3	6.40±0.40		
CAL	1	0.00±0.0	<0.0001*	1 versus 2
	2	6.55±0.56		1 versus 3
	3	6.46±0.40		

*Significant. PD: Probing depth; CAL: Clinical attachment level; GI: Gingival index; PI: Plaque index; SD: Standard deviation

Table 2: Comparison of TNF-α among different study groups

Group	Mean±SD (pg/ml)	Overall *P* value	Significant groups
1	2.15±3.60	<0.0001*	1 versus 2
2	12.92±17.21		
3	7.23±7.67		

*Significant. SD: Standard deviation; TNF-α: Tumor necrosis factor-alpha

Table 3: Correlation between TNF-α and clinical parameters

Variable	Group 1		Group 2		Group 3	
	Correlation coefficient	*P*	Correlation coefficient	*P*	Correlation coefficient	*P*
PI	−0.42	0.04*	−0.15	0.46 (NS)	−0.20	0.33 (NS)
GI	0.01	0.96 (NS)	0.04	0.84 (NS)	0.36	0.08 (NS)
PD	-	-	−0.14	0.50 (NS)	0.40	0.05*
CAL	-	-	−0.16	0.44 (NS)	0.39	0.06 (NS)

*Significant. NS: Not significant; PD: Probing depth; CAL: Clinical attachment level; GI: Gingival index; PI: Plaque index; TNF-α: Tumor necrosis factor-alpha

Comparison of salivary TNF-α levels between chronic and aggressive periodontitis did not reveal any significant difference in the present study. Kurtis *et al*. also reported the similar finding that TNF-α in GCF was not significantly different between chronic and aggressive periodontitis.[19] Though nonsignificant, salivary TNF-α levels were more in chronic periodontitis patients than in aggressive periodontitis. It is reasonable to assume that in chronic periodontitis, where gingival inflammation is an important feature, salivary TNF-α can be a marker. Whereas in aggressive periodontitis where inflammation is not a predominant feature despite tissue destruction, salivary TNF-α may not be a candidate marker. This indicates a probable difference in the pathogenic mechanisms of chronic and aggressive periodontitis that requires further probe.

Among the clinical parameters in the three groups, a significant positive correlation existed only between probing pocket depth (PPD) and salivary TNF-α in aggressive periodontitis group (Group 3). Salivary TNF-α levels showed no correlation with most of the clinical parameters possibly due to the extensive dilution of these markers in the saliva, thereby failing to reflect the minor variations in the clinical parameters. Ikezawa *et al*. reported a significant positive correlation between GCF TNF-α levels with pocket depth in chronic periodontitis patients.[23] Kurtis *et al*. also reported a positive correlation between salivary TNF-α levels and clinical parameters such as PD, CAL, PI, and GI in GCF samples of patients with chronic and aggressive periodontitis.[19] This discrepancy between the salivary TNF-α levels in our study and GCF levels in previous studies may question the usefulness of salivary TNF as an indicator of disease severity. Surprisingly in our study we observed a negative correlation of salivary TNF-α levels with the PI in Group 1 (healthy periodontium). It is well known that microorganisms and LPS products act as potent stimuli to a variety of host cells which subsequently result in the expression of pro-inflammatory cytokines especially TNF-α.[24] But the predominance of Gram-positive organisms in the plaque of healthy patients might have influenced the negative outcome.[25]

Among the various biological media, saliva was chosen in this study since it is readily available, noninvasively collected and contains both locally and systemically produced serum markers which are of significant diagnostic importance. Unstimulated saliva was collected and used in the study, as the salivary composition is altered in stimulated saliva.[26,27] Whole saliva is the mixture of the salivary gland secretion, serum products, GCF, epithelial and immune cells and organisms. This makes the whole saliva compared to gland specific saliva a likely source for identifying unique biomarkers that reflect oral and systemic health changes.[1] Thus, the analysis of whole saliva holds great promise than gland specific saliva, especially while considering the development of a diagnostic test for periodontal diseases. Major limitations posed by the present study were the grouping of patients based on the clinical and radiographic parameters and not on the microbiological assessments. Hence, quiescent and active states of the disease could not be differentiated. Other limitations include a small sample size and the lack of an intervention group.

Elevated levels of TNF-α in chronic periodontitis not only establishes its role in periodontal pathogenesis but serves as a link in the development of cardiovascular diseases (CVDs). Investigations has led to substantial evidence suggesting the role TNF-α plays in intensifying the cardiovascular pathology.[28] Hence, salivary TNF-α and its possible causal relationship between periodontitis and CVD can be used to identify patients at risk.

Conclusion

Salivary TNF-α levels is significantly elevated in chronic periodontitis but not in aggressive periodontitis when compared to healthy subjects. Despite the salivary TNF-α levels being elevated in generalized chronic periodontitis, no correlations with clinical variables was observed. However, in aggressive periodontitis group, PPD correlated with the salivary TNF-α levels.

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Conflicts of interest
There are no conflicts of interest.

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