Estimation of Tumor Necrosis Factor-Alpha in the Gingival Crevicular Fluid of Poorly, Moderately and Well Controlled Type 2 Diabetes Mellitus Patients with Periodontal Disease - A Clinical and Biochemical Study

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Abstract

Purpose of the study: To study the association between tumor necrosis factor-alpha **(**TNF-α) levels and the severity of periodontal disease in type 2 diabetes patients.

Methodology: Ninety patients with type 2 diabetes mellitus were selected. They were divided into three main groups based on their glycaemic control and further into subgroups of those having sites with gingivitis or periodontitis. In all the participants, blood and gingival crevicular fluid (GCF) samples were collected for analysis of glycated haemoglobin and TNF-α respectively. The statistical analysis was carried out using ANOVA and Bonferroni tests.

Results: The periodontitis sites showed higher levels of TNF-α as compared to gingivitis sites. The poorly controlled diabetes group showed higher levels of TNF-α as compared to the other two groups; the difference amongst the groups being statistically significant.

Interpretation and Conclusion: Poor glycaemic control is associated with elevated TNF-α in the GCF. Hyperglycaemia contributes to a heightened inflammatory response, which may explain the association between poor glycaemic control and periodontal destruction.

Key words: Diabetes mellitus, gingival crevicular fluid, periodontal disease, glycated haemoglobin, tumor necrosis factor alpha.

Introduction

Diabetes mellitus (DM) is a highly prevalent metabolic disorder and it constitutes a global public health burden. DM is associated with a wide range of complications that increase the morbidity and mortality in the affected individuals.[1] DM can be classified as type 1, type 2, gestational diabetes mellitus and other specific types. The five well known complications of DM are- Retinopathy, Nephropathy, Neuropathy, Macrovascular disease and Altered wound healing. Periodontal disease is considered as the sixth complication of diabetes mellitus.[2] Patients with type 2 diabetes mellitus (T2DM) have a greater incidence and severity of periodontal disease than those without diabetes. Hyperglycaemia has been thought to play a role in periodontal disease incidence and prevalence. Longitudinal studies have shown that patients with relatively good glycaemic control are less prone to periodontal destruction.[1]

It is well known that DM is a clinical syndrome characterized by hyperglycaemia. In the hyperglycaemic state, numerous proteins and matrix molecules undergo a nonenzymatic glycosylation, resulting in production of advanced glycation end products (AGEs). AGEs play a central role in the classic complications of diabetes. AGEs accumulate in all the tissues of the body, including the gingiva and interact with their receptors present on the cell surface called the receptor for AGE (RAGE), bringing about various pathological changes. These changes are different in different cells, for example, AGE-RAGE interaction in the endothelium brings about increased vascular permeability whereas the AGE-RAGE interaction in the macrophages causes increased release of pro-inflammatory cytokines like tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β).[3]

It is generally accepted that much of the periodontal destruction observed in periodontitis is host mediated through the release of pro-inflammatory cytokines like TNF-α.[4] Thus it is plausible to suggest that elevated TNF-α expression in patients with DM maybe related to the increased severity of periodontitis in these patients. It is speculated that TNF-α is an important candidate molecule in the two-way relationship between diabetes mellitus and periodontal disease. Due to the relative paucity of studies on the Indian population, this study is being undertaken to test the hypothesis that levels of TNF-α is directly proportional to the degree of periodontal destruction among T2DM patients. The study was thus carried out to understand the effect of TNF-α level in the severity of periodontal disease in type 2 diabetes patients and to estimate the level of TNF-α in the GCF of poorly, moderately and well controlled type 2 diabetes patients with gingivitis and periodontitis.

Material and methods

The study population consisted of ninety T2DM patients from the out-patients of the Department of Periodontics, D A Pandu Memorial R V Dental College. The age of the participants ranged from 36 years to 73 years and participants of both sexes were included.

The inclusion criteria were patients suffering from T2DM, patients with periodontal disease who had not received any periodontal therapy for the last 6 months, patients with at least 3 teeth per quadrant and patients consenting to participate in the study. The exclusion criteria were patients with type 1 diabetes mellitus, pregnant women and lactating mothers, patients suffering from haemoglobinopathies, bleeding and clotting disorders, patients who smoked and patients with systemic conditions that required antibiotic prophylaxis.

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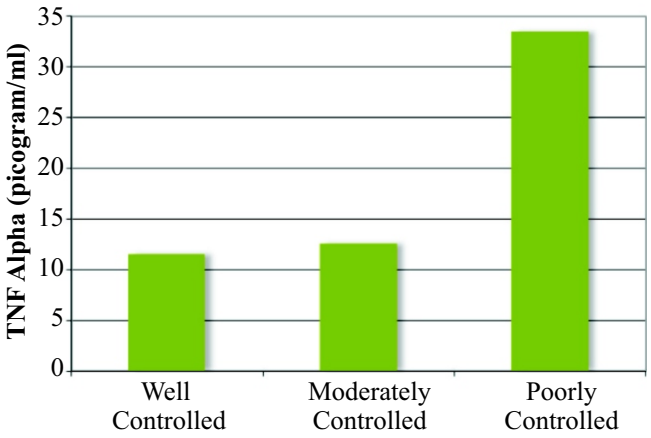
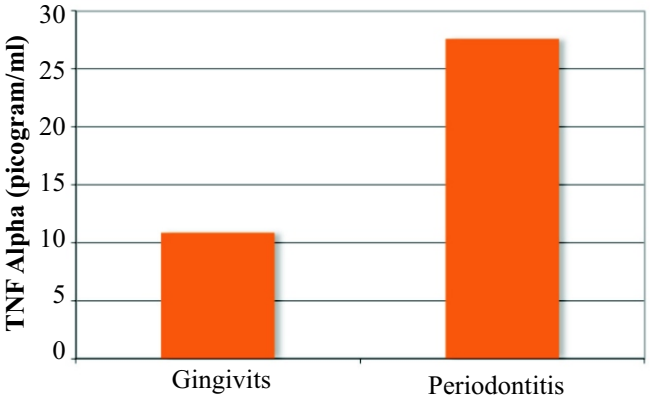
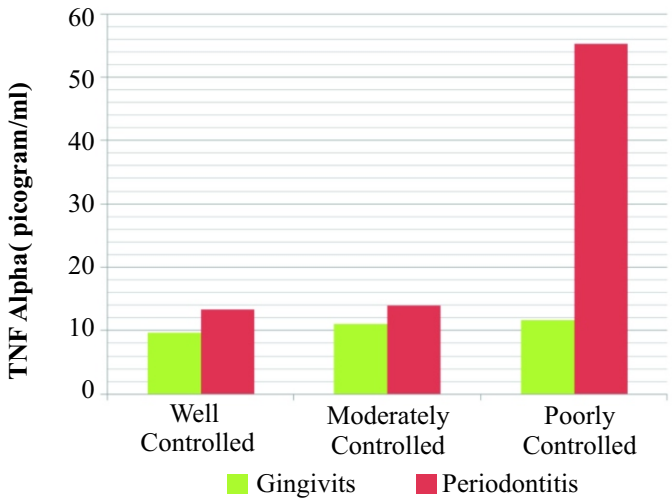


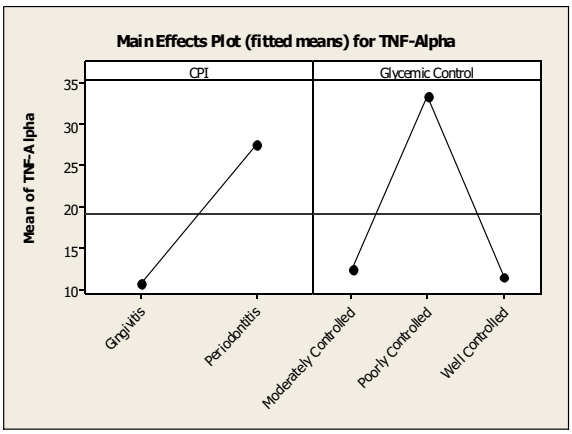
Graph 1: Mean TNF-α level (pg/ml) in the three study groups based on glycaemic control



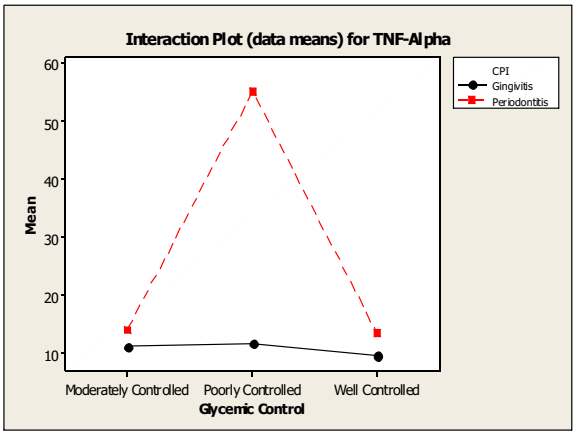
Graph 2: Mean TNF-α level (pg/ml) in gingivitis and periodontitis



Graph 3: Mean TNF-α level (pg/ml) in all the six study groups



Graph 4: Main effects plot for TNF- α



Graph 5: Interaction plot for TNF-α

The ethical clearance for the study was obtained from the ethical committee of D A Pandu Memorial R V Dental College. The participants were explained about the study and a written consent was obtained from each of the participants. The data for all subjects was recorded in a previously prepared clinical proforma.

Blood samples were obtained from the subjects for the estimation of glycosylated haemoglobin levels (HbA_{1c}). The subjects were classified into three groups of 30 subjects each, based on their HbA_{1c}, according to the guidelines by the American Diabetes Association (2003)[2] - Group I- well controlled diabetes: 6-7% HbA_{1c} ; Group II-

Glycaemic Control	n	Mean	Std Dev	Min	Median	Max
Well Controlled	30	11.52	3.31	3.16	11.86	19.60
Moderately Controlled	30	12.56	6.74	5.18	12.15	42.71
Poorly Controlled	30	33.47	35.33	8.72	14.11	125.25

Table 1: Mean TNF-α level (pg/ml) in the three study groups based on glycaemic control

CPI	n	Mean	Std Dev	Min	Median	Max
Gingivitis	45	10.82	5.50	3.16	9.72	42.71
Periodontitis	45	27.56	29.92	8.81	14.61	125.25

Table 2: Mean TNF-α level (pg/ml) in gingivitis and periodontitis sites

Glycaemic Control	Gingivitis					Periodontitis				
	Mean	Std Dev	Min	Median	Max	Mean	Std Dev	Min	Median	Max
Well Controlled	9.64	2.87	3.16	9.72	13.79	13.41	2.61	8.97	13.08	19.60
Moderately Controlled	11.11	8.94	5.18	8.81	42.71	14.02	3.09	8.81	13.94	22.11
Poorly Controlled	11.71	2.16	8.72	12.23	15.22	55.24	39.57	12.29	45.12	125.2

Table 3: Mean TNF-α level (pg/ml) in all the six study groups

Source		Sum of Squares (SS)	Mean SS	F	p-value
CPI	1	6304.736	6304.736	22.587	<0.001*
Glycaemic Control	2	9200.689	4600.345	16.481	<0.001*
CPI * Glycaemic Control	2	8077.372	4038.686	14.469	<0.001*
Error	84	23447.231	279.134	---	---
Total	90	80161.909	---	---	---

* denotes significant difference

Table 4: ANOVA calculation

Dependent Variable: TNF-Alpha (picogram/ml)

Multiple Comparisons

Bonferroni

(I) Glycemic Control	(J) Glycemic Control	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Well Controlled	Moderately Controlled	1.03987	4.313805	1.000	-11.57792	9.49819
	Poorly Controlled	-21.94943	4.313805	.000	-32.48749	-11.41138
Moderately Controlled	Well Controlled	03987	4.313805	.000	-9.49819	11.57792
	Poorly Controlled	-20.90957	4.313805	.000	-31.44762	-10.37151
Poorly Controlled	Well Controlled	21.94943	4.313805	.000	11.41138	32.48749
	Moderately Controlled	20.90957	4.313805	.000	10.37151	31.44762

Based on observed means

The mean difference is significant at the .05 level

Table 5: Bonferroni calculations

moderately controlled diabetes: 7-8% HbA_{1c} ;Group III-poorly controlled diabetes: >8% HbA_{1c}.

The subjects were further classified into sub-groups (n=15) of those having sites with gingivitis or periodontitis based on their Community Periodontal Index (CPI) score as Class A- those with gingivitis sites (having CPI scores of 1 or 2) and Class B- those with periodontitis sites (having CPI scores of 4 and a loss of attachment of 3 mm or more). Thus, a total of six groups were formed, each containing 15 subjects.

GCF samples were collected from all the subjects. The site for collection of GCF was dried and isolated with cotton rolls. Supragingival calculus if present, was removed using universal scaler (U15/30). Samples of GCF were obtained from predetermined sites by placing calibrated, volumetric, microcapillary pipettes with a 0-5 µl range (obtained from Sigma Aldrich co., St. Louis, Missouri, USA). The micropipettes were placed extracrevicularly at the entrance of the gingival crevice. 2-3 µl of GCF was collected from each subject.

The Enzyme Linked Immunosorbent Assay (ELISA) procedure was carried out using a commercially available ELISA kit for human TNF-α (IM1121, Immunotech, Beckman Coulter company, France). The TNF values were obtained by reading the absorbance at 405nm.

The statistical analysis was done using a commercially available software programme (SPSS V13). The ANOVA (Analysis of Variance) and Bonferroni tests were used for statistical analysis.

Results

The subjects were divided into 3 groups based on their glycaemic control as those with well-controlled, moderately controlled and poorly controlled diabetes mellitus. Graph 1 and Table 1 shows the distribution of TNF-α in the above mentioned groups. It can be inferred that the mean TNF-α values increases with the decline in glycaemic control, that is, higher levels of TNF-α can be seen in individuals with poorly controlled diabetes mellitus (33.47 ± 35.33 pg/ml) followed by moderately controlled diabetes (12.56±6.74 pg/ml) and well controlled diabetes (11.52 ± 3.31 pg/ml).

The subjects in each group were further divided into those having gingivitis or periodontitis based on their CPI score. Graph 2 and Table 2 show the distribution of TNF-α in the above mentioned groups. From Graph 2 and Table 2, it can be inferred that higher levels of TNF-α were present in sites with periodontitis (27.96±29.92 pg/ml) as compared with sites having gingivitis (10.82±5.50 pg/ml).

By comparing all the six groups, from Graph 3 and Table 3, it can be inferred that mean levels of TNF-α was highest in the periodontitis patients with poorly controlled diabetes (55.24±39.57 pg/ml) and lowest levels in gingivitis patients with well controlled diabetes (9.64 ±2.87 pg/ml).

Using the statistical technique of Factorial ANOVA, it was noticed that there was a significant difference between the two periodontal conditions gingivitis & periodontitis ($p< 0.001$). It was also observed that there was a significant difference between the mean TNF-α levels of the three different glycaemic control groups ($p<0.001$). Also, the interaction (joint effect) of periodontal status and glycaemic control on TNF-α levels is found to be statistically significant ($p<0.001$). Table 4 represents the ANOVA table.

In the main effects plot, higher mean TNF-α was noticed in patients with periodontitis compared to patients with gingivitis and this difference was found to be statistically significant ($p<0.001$). Among the glycaemic control groups, higher mean TNF-α level was noticed in poorly controlled group followed by moderately controlled and well controlled groups (Graph 4).

In order to find out among which pair of glycaemic control groups there exists a significant difference, multiple comparisons test was carried out using Bonferroni method. It was noticed that a significant difference existed in the mean TNF-α levels between well controlled and poorly controlled diabetes groups ($p<0.001$). Also, statistically significant difference was noticed between mean TNF-α levels in the moderately controlled and poorly controlled diabetes group ($p<0.001$). However, no statistically significant difference was observed in the mean TNF-α levels between the well controlled and moderately controlled diabetes groups ($p>0.05$). Table 5 represents the multiple comparisons made through the Bonferroni method.

It was noticed that mean TNF-α levels were higher in patients with periodontitis compared to patients with gingivitis in all the three different glycaemic control groups. Patients with periodontitis having poor glycaemic control are found to have the highest mean TNF-α in the GCF followed by patients in moderately controlled and well controlled diabetes groups. In patients with gingivitis, higher mean TNF-α is found in poor glycaemic control group followed by moderately controlled and well controlled diabetes groups.

Graph 5 represents the interactions plot between glycaemic control, periodontal status and mean TNF-α level. From the plot, it can be inferred that the difference in the mean TNF-α level in gingivitis and periodontitis in the well controlled and moderately controlled diabetes groups is very minimal as compared to the poorly controlled diabetes group where the difference in mean TNF-α levels in gingivitis and periodontitis is large.

Discussion

Diabetes mellitus and periodontal disease share a two-way relationship. Patients with type 2 diabetes mellitus have a greater incidence and severity of periodontal disease than those without diabetes mellitus. Hyperglycaemia has been thought to play a role in periodontal disease incidence and prevalence. Patients with relatively good glycaemic control are less prone to periodontal destruction in longitudinal studies.[1]

TNF-α plays an important role in the host mediated destruction of the periodontal tissues. TNF-α is significantly elevated in diseased periodontal sites demonstrating inflammation and during periods of active disease/ tissue destruction.[4]

The aim of this study was to understand the effect of TNF-α level in the severity of periodontal disease in type 2 diabetes patients. For this purpose, the level of TNF-α was estimated in the GCF of well controlled, moderately controlled and poorly controlled type 2 diabetes patients with periodontal disease.

The composition of GCF is the result of interplay between the bacterial biofilm and cells of the periodontal tissues.

Analysis of the special constituents in the GCF is indicative of local cellular metabolism that clearly reflects the current periodontal status.[5] The added advantage in GCF collection is that, it is a non invasive procedure as compared to gingival tissue biopsy. Also, Gorska et al., have failed to show a corelation between cytokine expression in the serum and clinical parameters. The authors have stated that the use of blood/serum samples for determination of cytokines is a useless method.[6] Thus, in the present study the levels of the pro-inflammatory cytokine were assessed in the GCF as the composition of GCF reflects the changes occurring in the local periodontal environment.

The results obtained in this study show that higher TNF-α is found in the GCF of subjects with poorly controlled diabetes mellitus and periodontitis, in comparison with the other two groups with better glycaemic control or gingivitis. The highest TNF-α values were seen in the periodontitis sub-group of the poorly controlled diabetes group (55.24± 39.57 pg/ml) and the lowest TNF-α values were seen in the sites having gingivitis in the well controlled diabetes group (9.64±2.87 pg/ml).

On comparing TNF-α level in poorly controlled and well controlled diabetes groups and poorly controlled and moderately controlled diabetes groups statistically significant results were observed; whereas statistically significant results were not obtained when comparing TNF-α levels in the moderately controlled and TNF-α levels in the well controlled diabetes groups. The reason for this could be the extremely high levels of TNF-α in the GCF of individuals with poorly controlled group (33.47± 35.33 pg/ml) as compared to that in the moderately controlled (12.56±6.74 pg/ml) and well controlled groups (11.52±3.31 pg/ml). The high levels of TNF in the poorly controlled group could be attributed to increased AGE accumulation in these subjects.

It has been reported that increased AGEs levels were identified in the gingival extracts from diabetic patients compared to non-diabetic controls and that serum AGEs are significantly associated with deterioration of periodontitis.[8] Thus it can be assumed that with worsening of glycaemic control there is more accumulation of AGEs and hence there is release of more TNF-α.

Many hypotheses have been put forth to explain the increase in inflammatory cytokines in diabetic individuals. These include among others- the presence of a hyper-responsive monocytic trait and elevated glucose levels that may directly or indirectly lead to a heightened inflammatory response.[9]

A hyper-responsive monocytic trait has been proposed by Salvi et al., as an explanation for elevated cytokine levels found in type 1 diabetes patients. This hypothesis holds that a heightened inflammatory response, either as a result of gene polymorphism or hyperglycaemia, causes a heightened monocytic release of inflammatory mediators.[10]

While it is possible that genetic polymorphisms in the production of pro-inflammatory cytokines contribute to diabetes related periodontitis, to the best of our knowledge no specific gene or genotype has been tested so far. This study supports the concept that hyperglycaemia can influence inflammatory mediators in the GCF and does not rule out the possibility that a hyper-responsive monocytic trait is also present in type 2 diabetes patients.

An alternative hypothesis to the hyper-responsive monocytic trait theory is that elevated glucose levels may directly or indirectly lead to a heightened inflammatory response. Acute and chronic hyperglycaemic models have been studied. Ex vivo stimulation of monocytes of human volunteers with solutions high in glucose resulted in increased TNF-α and IL-6 release.

In their study on the plasma levels of TNF-α in patients with type 2 diabetes and chronic periodontitis, Engbretson et al., have reported a positive corelation between plasma TNF-α values and periodontal severity.[7] The authors reported the mean TNF-α value in the plasma to be 2.61±3.50 pg/ml. These values are much lesser than that observed in the current study in which the mean TNF-α levels in the GCF of subjects with well controlled diabetes is 11.52± 3.31 pg/ml (lowest among all three groups). The reason for this could be that higher levels of the cytokine maybe found on high concentrations at and around the site of periodontal disease activity, i.e., in the gingival sulcus /pocket area. Thus the GCF would harbour higher amounts of TNF-α when compared to serum/plasma.

The elevated TNF-α observed in our study may offer a plausible explanation for the increased incidence and severity of periodontal disease in patients with diabetes. Elevated TNF-α brings about more periodontal destruction. This explains the difference that is observed in the TNF-α level in sites with gingivitis and those with periodontitis. It can be inferred that glycaemic control plays an important role in determining the amount of TNF-α release. Poor control of diabetes predisposes an individ-ual to higher TNF-α production and thereby more severe periodontal destruction.

Thus, it is expected that TNF-α is a key player in determining the severity of periodontal destruction in T2DM. The current finding of elevated GCF TNF-α in diabetic subjects with poor glycaemic control raises the possibility of the converse; namely that local gingival inflammation may adversely influence the glycaemic control of patients with diabetes. TNF-α from the periodontal environment can enter the systemic circulation and adversely affect insulin sensitivity.[12] The resultant insulin resistance can lead to a worsening in the glycaemic control which can lead to further periodontal breakdown.

The limitation of this study is that the study did not eliminate the confounding influence of obesity. The patients were not identified to be obese or not. This is important as TNF-α is found be higher in obese individuals than in non obese individuals. The reason why obesity was not considered as an exclusion criterion was to reduce the number of variables in determining the relationship between TNF-α, glycaemic control and periodontal status.

Summary and conclusion

In the present study, higher TNF-α was present in the GCF of individuals with periodontitis and in those with poor glycaemic control as compared to those with gingivitis and those with well controlled or moderately controlled diabetes mellitus respectively. The higher level of TNF-α found at sites with periodontitis in subjects with poorly controlled diabetes raises two questions- did the diabetic process contribute to the raise in TNF-α or did the periodontal disease process contribute to the raise in TNF-α? Perhaps,

Found at sites with periodontitis in subjects with poorly controlled diabetes raises two questions- did the diabetic process contribute to the raise in TNF-α or did the periodontal disease process contribute to the raise in TNF-α? Perhaps, periodontal destruction and diabetes have a synergistic effect in elevating the TNF-α level, though at present this link cannot be completely confirmed. It still remains unclear if TNF-α levels influence diabetes severity, or indeed whether circulating TNF-α influences periodontitis severity.

The relationship of poor glycaemic control, periodontal disease and GCF TNF-α requires further study in light of the current findings. Other cytokines are likely to play a role in this regard as well. These findings also open a new arena of possibilities that the elevated TNF-α in the periodontal environment in poorly controlled T2DM could influence the glycaemic control of these patients. Elevated TNF-α from the periodontal environment may enter the systemic circulation and bring about insulin resistance which could lead to further deterioration in the glycaemic control.

Further studies involving larger samples are required to understand the definitive role of TNF-α in periodontal disease and diabetes mellitus.

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