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NEUTROPHIL ELASTASE ACTIVITY IN PERIODONTAL DISEASE SECONDARY TO DIABETES MELLITUS

Biochemistry

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ABSTRACT

Diabetic patients often suffer from periodontal disease with progressive periodontal destruction. Bidirectional relationship exists between Diabetes mellitus and periodontal disease. Screening and evaluation of periodontal risk in every diagnosed diabetic patient is required which can be executed by availability of an biomarker for periodontal tissue destruction. The aim of this study was to identify the presence of Neutrophil Elastase activity as enzymatic biomarker of periodontal tissue destruction in patients of Diabetes mellitus associated with Periodontitis. Neutrophil Elastase activity was estimated in saliva, GCF and serum in healthy subjects and patients with Diabetes mellitus, Periodontitis and Diabetes associated periodontitis. Enzyme activity was compared with clinical parameters like Probing pocket depth, Plaque Index and Gingival Index. It was maximally raised in diabetic cases with periodontitis. Enzyme activity was found to be highest in crevicular fluid compared to saliva and serum. However serum Neutrophil Elastase correlated significantly with clinical indices. Neutrophil Elastase may be employed on routine basis as a chair side test for screening and diagnosis of patients with periodontitis in diabetics.

KEYWORDS

Type-2 Diabetes mellitus, Periodontitis, biomarkers, Neutrophil Elastase

INTRODUCTION

Diabetes mellitus is a metabolic disease reflected by chronic hyperglycemia arising due to disturbances in carbohydrates, fat and protein metabolism. It is a condition resulting from defective insulin secretion or insulin action or both. Defective Insulin metabolism is the key mediator. Insulin is synthesized in beta cells of islets of pancreas. Their autoimmune destruction leads to complete loss of insulin secretion ending in to Type I Diabetes mellitus. However in Type 2 diabetes insulin resistance is the metabolic defect. As the hyperglycemia appears gradually and often without symptoms patients with type 2 diabetes can remain undiagnosed for years Diabetes is a silent disease although slowly developing in dwelling macro and micro vascular complications. Long-term diabetic oral complications includes micro vascular diseases like Xerostomia, greater susceptibility of oral tissues to trauma, more opportunistic infections (e.g., candidiasis), greater accumulation of plaque, greater risk of caries, delayed healing, greater susceptibility to periodontal disease and greater risk of developing periodontal abscesses when periodontitis is present. [1]

Periodontitis is a "low grade infection of gums" capable of developing a "low grade systemic inflammation" with an ability to influence the general systemic health. Very often, course of periodontal disease is modified by the systemic disorder of patients. The systemic disorder exerts the effect in a generalized manner and so also affects the occurrence and management of the periodontal conditions. Diabetes is a risk factor for development of periodontal disease and there is a significant progressive periodontal destruction in diabetic patients. It is proposed that periodontal disease should be considered as a component of metabolic syndrome [2]

The association between periodontal disease and diabetes has been explored in several studies over the years, and it is generally accepted that periodontal disease is more prevalent and more severe in people with diabetes than in non-diabetic persons. Indeed, the periodontal signs and symptoms are now recognized as the "sixth complication" of diabetes. [3]

Awareness of diabetic patients with regards to oral care and hygiene may be strengthened. This may further increase the possibility of early screening and diagnosis of periodontal disease. Availability of easy to perform chair side tests for screening of periodontal disease may help.

The purpose of the study was to identify the presence of Neutrophil Elastase activity as an enzymatic biomarker of periodontal tissue destruction and to correlate levels in the three oral fluids i.e. saliva, Gingival Crevicular Fluid (GCF) and serum with the severity of periodontal disease in diabetic patients.

Aim: To detect the efficacy of Neutrophil Elastase activity as an enzymatic biomarker of periodontal tissue destruction, aiding in diagnosis of periodontitis in patients with Diabetes mellitus.

Objectives:

1. To estimate and compare the levels of Neutrophil Elastase in saliva, GCF and serum in healthy subjects and patients with Diabetes mellitus, Periodontitis and Diabetes associated periodontitis.
2. To assess whether this biomarker is a suitable indicator of clinical indices of periodontitis
3. To study the association of diabetes mellitus with periodontal disease using Neutrophil Elastase activity as means of diagnostic tests.

MATERIALS AND METHODS:

Subject selection:

The study was conducted at the Department of Biochemistry, MGM Medical College Navi Mumbai and Periodontology OPD of MGM Dental College, Navi Mumbai. Ethical clearance was obtained from the ethical committee of the institute, and a written informed consent was obtained from all the participants prior to the study.

A total of 40 subjects were equally categorized into four groups: Group-I (10 Healthy individuals), Group-II (10 Type-2 Diabetes without Periodontitis), Group-III (10 Periodontitis without Type-2 Diabetes) and Group-IV (10 Type-2 Diabetes with Periodontitis).

INCLUSION CRITERIA:

1. Patients under diabetic treatment or had been diagnosed diabetes mellitus for at least last one year.
2. Patients diagnosed clinically as having periodontitis.
3. Not having any other systemic diseases.
4. Not having any history of diabetic complications like neuropathy, nephropathy, retinopathy etc.
5. Not undergone any periodontal treatment since last one year.
6. Willingness to participate in the study.

EXCLUSION CRITERIA:

1. Age less than 30yrs.
2. History of systemic disease with high liver enzyme activities
3. Medical therapy apart from antidiabetic treatment
4. Patients with diabetic complications such as neuropathy and retinopathy.
5. Smokers and ex-smokers.

The periodontal involvement was confirmed when presence of hard deposits or inflammation or bleeding on probing was observed.

Clinical parameters:

The relevant clinical history was recorded for all the patients. A careful oral examination was carried out with the help of mouth mirror and graduated periodontal probe and following clinical parameters were recorded.

1. **Probing Pocket Depth** (mm): It is the distance between the base of the pocket and the gingival margin. This distance is measured with a William's graduated periodontal probe held parallel to the vertical axis of the tooth and walking it circumferentially around each surface of each tooth. The area of deepest penetration is recorded for each individual tooth.
2. **Plaque Index:** Plaque Index was assessed by Sillness and Loe, 1967 scoring criteria.

Criteria for plaque index

0 – No plaque in the gingival area
1 - A film of plaque adhering to the gingival margin and adjacent area of the tooth. The plaque may be recognized only by running a probe across the tooth surface.
2 - Moderate accumulation of soft deposits within the gingival pocket and on tooth, gingival margin and / or adjacent tooth surface which can be seen by the naked eye.
3 - Abundance of soft deposits within the gingival pocket and / or on the gingiva and adjacent tooth surface

The plaque index score for a tooth was obtained by totaling the scores of all the four surfaces and dividing it by the number of surfaces examined. The plaque index score per person was obtained by adding the plaque index scores of each tooth and dividing by the number of teeth examined. Scores range from 0 to 3.

Rating	Scores
Excellent	0
Good	0.1 – 0.9
Fair	1.0 – 1.9
Poor	2.0 – 3.0

3. Gingival Index: Gingival Index was assessed by Sillness and Loe, 1963 scoring criteria.

Criteria for gingival index

0 - Normal gingiva
1 - Mild inflammation, slight change in colour, slight oedema, no bleeding on probing
2 - Moderate inflammation, redness, oedema, glazing and bleeding on probing
3 - Severe inflammation, marked redness, oedema, ulceration and tendency to bleed spontaneously

Totaling the scores of each scoring unit and dividing it by 4 gives the gingival index score for a tooth. Totaling the scores of each tooth and dividing by the number of teeth examined provides gingival index score per person.

Gingival scores	Condition
0.1 - 1.0	Mild gingivitis
1.1 - 2.0	Moderate gingivitis
2.1 - 3.0	Severe gingivitis

Sample collection and preparation:

1. **Collection of Saliva:** Approximately 1.0 ml of non-stimulated saliva samples were collected into sterile Eppendorff tubes which were homogenized by gentle mixing and centrifuged at 3000 rpm for 10 min. 5 µL of supernatant was then transferred to another sterilized Eppendorff tube containing 250 µL of phosphate buffer (pH-7) and stored at -20°C until it was analyzed for salivary enzymes.
2. **Collection of blood:** Venous blood was collected under strict aseptic conditions in fluoride bulb for plasma and in plain bulb for serum. Plasma was collected after an overnight fast and two hour after meal for the fasting and postprandial blood glucose estimation. Serum was used for enzyme assays.
3. **Collection of Gingival Crevicular fluid:** The subjects were seated comfortably in an upright position in the dental chair, with adequate lighting condition. The sites were chosen from maxillary teeth to avoid contamination with saliva. Supra gingival plaque and calculus were removed with hand instrumentation prior to the collection of GCF. The calibrated microcapillary tubes (0- 5- 10 µL range) were placed extracrevicular at the mesiofacial, distofacial, or midfacial surface of the tooth. A standardized volume of 5 µL of GCF was collected. Microcapillary tubes contaminated with blood were discarded.

The collected GCF was immediately transferred with a jet of air pressure from the capillary tube into the sterilized microcentrifuge (Eppendorff) tube containing 250 µL of phosphate buffer (pH-7) and stored at -20°C until it was analyzed.

Neutrophil Elastase Assay:

The activity of free elastase in the sample was assessed with a chromogenic substrate specific for Neutrophil elastase: N-methoxy-succinyl-Ala-Ala-Pro-Val p-nitroanilide (Sigma-Aldrich). 100 µl sample and 100µl 0.2 M Tris-HCl buffer (pH 8.0) were placed in a well of a 96-well plate. Then 10 µl 5 mM substrate dissolved in 1-methyl-2-pyrrolidinone (Sigma-Aldrich) was added. The optical absorption of the hydrolysis product, p-nitro aniline, was read with a ELISA Reader at 405 nm after 24 h incubation at 37°C.The NE concentration in the samples was calculated from a standard curve of elastase from human leukocytes (Sigma-Aldrich) and is expressed in units/ml (one unit releases one nanomole of p-nitrophenol/s from BOC-L-alanine pnitrophenyl ester at pH 6.5 at 37°C). [4-7]

RESULTS

Table No.1: Descriptive Statistics for Neutrophil Elastase in all Fluids

Parameter	Group	Salivary Enzymes	GCF Enzymes	Serum Enzymes
Neutrophil Elastase	Control Group	3.90	9.80	2.410
	Diabetes	6.80	14.70	2.840
	Periodontitis	46.70	92.60	2.452
	Diabetes + Periodontitis	49.70	111.20	4.136

Table No. 2: Multiple Comparisons of **Salivary Enzymes** between control and study groups *(Using Tuckey's POST HOC Test)*

Parameter	Group	Group	P-value
Salivary Neutrophil Elastase	Control Group	Diabetes	.966
		Periodontitis	< 0.010**
		Diabetes with periodontitis	< 0.010**
	Diabetes	Periodontitis	< 0.010**
		Diabetes with Periodontitis	< 0.010**
	Periodontitis	Diabetes with Periodontitis	.963
GCF Neutrophil Elastase	Control Group	Diabetes	.987
		Periodontitis	< 0.010**
		Diabetes with Periodontitis	< 0.010**
	Diabetes	Periodontitis	< 0.010**
		Diabetes with Periodontitis	< 0.010**
	Periodontitis	Diabetes with Periodontitis	.584
Serum Neutrophil Elastase	Control Group	Diabetes	0.776
		Periodontitis	1.000
		Diabetes with Periodontitis	0.003**
	Diabetes	Periodontitis	0.825
		Diabetes with Periodontitis	0.032*
	Periodontitis	Diabetes with Periodontitis	0.003**

*: p value ≤ 0.05: fairly significant; **: p value ≤ 0.01: highly significant

Table No.3: Correlation between Clinical Parameters and Biochemical Enzymes in all Fluids (n = 20)

	PPD		PI		GI	
Parameter	CC	p-value	CC	p-value	CC	p-value
Salivary Elastase	0.026	0.91	-0.183	0.44	-0.311	0.18
GCF Elastase	0.236	0.32	-0.038	0.87	0.087	0.72
Serum Elastase	0.505	0.02*	0.295	0.21	0.456*	0.04*

CC: Correlation Coefficient; *: p vlue ≤ 0.05: fairly significant; **: p value ≤ 0.01: highly significanta

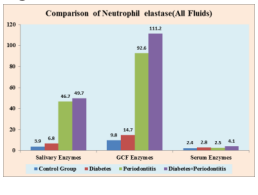

Fig 1.

DISCUSSION

Systemic diseases affect the homeostasis of oral cavity. Diabetes mellitus serves as a risk factor for development of periodontal disease with progressive periodontal destruction. Periodontal disease is a silent disease as most of the times signs and symptoms are unnoticed by the patient. Dental treatment is sought late, when almost significant destruction of periodontium has already been occurred and left unattended. Therefore examining the oral cavity for risk or progression of periodontitis in diabetic case is highly crucial.

Conventional dental diagnostic procedures have limitation to assess the current disease status. Also these conventional diagnostic techniques lack the capacity to identify highly susceptible patients who are at risk for future breakdown. [4] Advances in diagnostic research in oral and periodontal disease are moving towards methods whereby periodontal risk can be identified and quantified by objective measures by so called biomarkers. [8] Therefore we have attempted to study the levels of Neutrophil Elastase in all the three fluids that are saliva, gingival crevicular fluid as well as serum and correlated them with clinical parameters, in order to establish a suitable biomarker for periodontitis.

Neutrophil elastase degrades several proteins including elastin, collagen, fibrinogen, haemoglobin, ground-substance components of connective tissue, and proteoglycans. In inflammation its release into the gingival tissue and/or into the GCF could contribute to tissue damage.[9]

A longitudinal study demonstrated that this enzyme might serve as a predictor for future clinical attachment loss, can reflect the risk and the clinical status of periodontal lesions, and can serve to monitor disease activity. [10]

In our study there was marked increase in salivary Neutrophil elastase levels in study group than in control group as seen in table no .1. Mean Neutrophil Elastase in saliva was highest in patients of diabetes associated periodontitis (49.70 ±16.60). When salivary Neutrophil Elastase levels were compared between all four groups the enzyme levels did not differ statistically for diabetic patients and healthy individuals. Also no significant difference was noted in the levels between group IV (diabetes with periodontitis) and group III (periodontitis). Whereas the levels showed highly significant difference within the rest groups ($p < 0.01$) as can be seen from table no.2.

GCF Neutrophil elastase levels in study groups increased significantly as compared to control group (table no .1). Also as seen in table no .2, when GCF Neutrophil Elastase levels were compared between all four groups, control group and diabetes group showed no difference. Also the enzyme levels did not differ statistically between the patient having diabetes associated periodontitis and patients with periodontitis alone. But we observed highly significant difference in the levels between the rest groups ($p < 0.01$) as can be seen from table no.2.

Neutrophil Elastase in Serum was highest in Group IV i.e. diabetes with periodontitis (4.1 ± 1.6) as seen in table no.1. When serum Neutrophil Elastase levels were compared between all four groups, group IV showed highly significant differences in the elastase levels when compared with control group ($p < 0.01$) and with group III i.e. periodontitis ($p < 0.01$). Whereas Neutrophil Elastase levels in diabetic patients showed fairly significant difference with those found in patients having diabetes associated periodontitis ($p < 0.05$) as seen in table no. 2.

Thus in our study Neutrophil Elastase levels were high in periodontitis group and Diabetes with periodontitis group both in saliva and GCF as compared to serum. GCF levels were highest of all the three fluids as seen in table no. 1 and fig no.1

We observed a significant correlation between serum elastase levels and clinical parameters including probing pocket depth and plaque index. Amongst all fluids' elastase, serum elastase had the highest correlation ($p = 0.023$) with PPD. Also serum elastase correlated significantly with GI ($p = 0.043$) Thus serum elastase can be considered as marker for identifying periodontitis in diabetic patients.

Impaired polymorphonuclear leukocyte (PMN) function such as chemotaxis, adherence, and phagocytosis has been found in patients with diabetes, suggesting that this PMN dysfunction could lead to impaired host resistance and infection [11]Our finding are consistent with those of Alpagot et al [9] who demonstrated that GCF Neutrophil elastase activity is a risk indicators for periodontal disease in patients with diabetes mellitus and concluded that the periodontal condition of diabetes patients was worse than the periodontal condition of systemically healthy subjects.

Palcanis et al. (1992) compared GCF elastase levels against various other measures of disease activity including CAL, probing depth, bleeding on probing (BOP), gingival inflammation score, plaque index, and subtractive radiography. Thirty patients were examined over a 6-month period; the study demonstrated that GCF elastase levels are significantly higher in sites with active chronic periodontitis in comparison to inactive sites. When the standard test used to detect disease consisted of a combination of radiographic bone loss and loss of attachment levels, the elastase test showed sensitivity and specificity values of 82% and 66%, respectively. [12]

Jin *et al.*, and Söder *et al.* studied relationships between the Neutrophil elastase activity and PGE2 level in gingival crevicular fluid on one hand, and the presence of periodontal pathogenic bacteria on the other hand, in patients with untreated periodontitis. They demonstrated that a local immune response to periopathogens varies depending on intensity of the inflammatory response measured by elastase and PGE2 levels in gingival crevicular fluid. [13]

CONCLUSION:

Oral cavity fluids act as mirror for molecular activities occurring in periodontal disease. They act as better diagnostic medium compared to blood as increased level of Neutrophil Elastase activity was observed in saliva and GCF compared to serum with highest activity in GCF. Preventive and palliative care towards oral and dental ailments may also be prioritized along with eye, foot and kidney care during the management of Diabetes mellitus.

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