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RESEARCH ARTICLE

"Estimation and Comparison of the Levels of Elastase in Saliva, GCF & Serum of Smokers and Non-smokers with Chronic Periodontitis -A Clinico-biochemical Study"

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Abstract

Objective: This study was done to estimate and compare the levels of Elastase in Saliva, GCF & Serum of smokers and non-smokers with chronic periodontitis. Materials and Method:125 male subjects in the age group of 21-50 years were enrolled and categorized as Group 1, Non-smoking subjects with healthy periodontium Group 2, Smoking subjects with healthy periodontium Group 3, Non-smoking subjects with Chronic periodontitis Group 4, Smoking subjects (<10 Cigarettes/day) with Chronic periodontitis and Group 5, Smoking subjects (>10 Cigarettes/day) with Chronic periodontitis. Plaque Index, Gingival Bleeding Index, Probing Pocket Depth, Clinical Attachment Level, Salivary, GCF and Serum Elastase levels were recorded and evaluated using sandwich ELISA method. Results: The clinical parameters expressed a significant difference between diseased group and healthy group. Elastase levels (Saliva, GCF and Serum) showed significant difference between diseased group and healthy group. In between the diseased group comparison, GCF and Saliva showed Lower Elastase levels in smoking periodontitis group. Whereas, Serum showed higher Elastase levels. Conclusion: Elastase can be used as a reliable biomarker for smokers and non-smokers with chronic periodontitis to denote disease activity.

Keywords: *Periodontitis; Smoking; Elastase; Saliva; Gingival crevicular fluid.*

Introduction

Periodontitis is an irreversible, cumulative and chronic condition causing inflammatory destruction of periodontal tissues and alveolar bone supporting the teeth, initiated and propagated by a local accumulation of bacteria and their metabolic products, and host factors [1].Dental Plaque develops and matures over a period of several weeks, initially developing supragingivally with mainly aerobic bacteria.

Over time, the flora changes from predominantly gram-positive to gram-negative, from facultative aerobes to strictly anaerobic species, with more motile forms present [2]. The destruction of the periodontium is associated with the presence of gram-negative anaerobic bacteria localized in the subgingival region, and includes typically Porphyromonas gingivalis (Pg), Prevotella intermedia (Pi), Actinobacillus actinomycetemcomitans (Aa), and Bacteroides for sythus (Bf) [3]. These bacteria are considered to play a significant role in the pathogenesis of periodontitis and the formation of the periodontal pocket, destruction of the connective tissue, and resorption of the alveolar bone.

While it is the bacterial infection that triggers the destructive process, it is the host's immune response to the bacterial challenge that is responsible for the molecular processes leading to periodontal tissue destruction [4]. Activation of the innate immune system by periodontal pathogens results in an excessive production

of pro-inflammatory cytokines, which plays a major role in the progression of periodontitis.

Furthermore, periodontal pathogens activate the acquired immune system, which further contributes to disease progression [5]. Inflammation is driven and maintained by the network of cytokines and chemokines, which are continuously produced by immune cells. The qualitative profile of cytokines is usually determined by the subsets of CD4+ T lymphocytes, which are also known as T helper (Th) cells. Different subsets of Th cells are characterized by strikingly different cytokine profiles, Th1 cells (IL-1ß, IL-2, IL-12, IFN-γ, and TNF-α), which induce the cellular immune response and Th2 cells (IL-4, -5, -6, -10, and-13), which induce the humoral immune response. Finally, Th17 (IL-17), which promotes rapid recruitment of neutrophils and it is involved in an initial inflammatory response against pathogens and in injuries [6].

The histopathological characteristics of periodontitis include periodontal pocketing, apical migration of junctional epithelium apical to the cemento-enamel junction, loss of collagen fibers subjacent to the pocket epithelium, numerous polymorphonuclear leukocytes in the junctional and pocket epithelium, and a dense inflammatory cell infiltrate with plasma cells, lymphocytes, and macrophages [7].

The primary clinical features of periodontitis include clinical attachment loss (CAL), alveolar bone loss (BL), periodontal pocketing, and gingival inflammation. In addition, enlargement or recession of the gingiva, bleeding of the gingiva following application of pressure, and increased mobility, drifting, and/or tooth exfoliation may occur. With few exceptions, most forms of periodontitis are chronic inflammations that may progress continuously or by bursts of activity [8].

The rate of disease progression and severity of the disease may be influenced by local / systemic conditions that alter the normal host response to bacterial plaque such as Microbial, Genetic, Immunological, Environmental (Smoking and Tobacco use), Acquired risk factors (Diabetes mellitus, Osteoporosis, hematologic disorders, connective tissue disorders), stress and nutritional deficiencies [9].

Smoking is the one of the most important environmental risk factors in the pathogenesis of chronic periodontitis. Risk calculations suggest that 40% of chronic periodontitis cases may be attributed to smoking. Smokers are four times (odds ratio of 4.0) as likely to have chronic periodontitis as non-smokers. Tobacco smoking affects the oral environment, including the gingival tissues and vasculature, the inflammatory response, the immune response and homeostasis and healing potential of periodontal tissues. Clinically, smoking is associated with more attachment loss, bone loss and tooth loss, but paradoxically, less signs of inflammation [10].

Chronic low doses of nicotine causes, sustained peripheral vasoconstriction of the gingival microvasculature which induces a more pathogenic organisms and red complex species in deep periodontal pockets. Components of cigarette smoke results in increased release of pro-inflammatory mediators in periodontal tissue, impaired host defence by altering neutrophil chemotaxis, phagocytosis, granulocyte function and neutrophil respiratory burst which causes oxidative stress in tissues, Along with increased TNF α, PGE_2, Neutrophil collagenase and elastase with decreased subgingival temperature [11]. Also in periodontitis, high levels of proteolytic enzymes can be found both in the gingival tissues and in the sulcus. Connective tissue degradation occurs in periodontal disease as a result of action of proteases.

Neutrophil elastase (NE) is a neutral serine protease stored in the azurophilic granules of granulocytes. This enzyme degrades elastin and several functionally and structurally important molecules of the periodontium including collagen, proteoglycans and basement membrane components. Thus, it has an important role in periodontal pathology [12].Human saliva is known to contain numerous enzymes: phosphatases, esterases, lipases and proteases, etc.

Among the proteases, elastase was the one that reflected more in periodontal destruction. The level of Elastase was found to be a suitable marker for assessing periodontal disease progression [13]. Salivary PMN are known to be derived principally from gingival crevice, with a small

contribution from oral mucosa and the tonsils. There is a weak correlation between the rate of salivary PMN accumulation and gingival inflammation in healthy individuals. GCF flow rate is dependent on vascular permeability and is one of the most important indications of inflammatory changes in gingival tissues.

Several studies have demonstrated a correlation between GCF flow rates along with histologic signs of inflammation. Since smoking may affect MMPs neutrophil degranulation products in the systemic level eventually leading to more severe periodontal tissue destruction and systemic inflammation predisposing to various diseases. Thus keeping into mind the above mentioned facts, this study has been designed to estimate and compare the levels of Elastase in Saliva, GCF & Serum of smokers and non-smokers with chronic periodontitis.

Materials and Methods

This was designed as a, Clinico-biochemical study, after obtaining ethical approval from Institutional Review Board (IHEC/0023/2015), Rajah Muthiah Medical College, Annamalai University, Chidambaram, 125 males with the age group of 21-50 years were enrolled. Complete case history and clinical examination was done for all participants using mouth mirror, explorer and William's periodontal probe and they were categorized into five groups, 25 subjects in each group.

Group 1 -25 subjects

Non-smoking subjects with healthy periodontium (PD ≤3mm, BOP <10% and no loss of attachment)

Group 2 -25 subjects

Smoking subjects with healthy periodontium (PD ≤3mm, BOP <10% and no loss of attachment)

Group 3-25 subjects

Non-smoking subjects with Chronic periodontitis (PD≥4mm, BOP ≥50% and with loss of attachment)

Group 4-25 subjects

Smoking subjects (< 10 Cigarettes/day) with Chronic periodontitis (PD≥4mm, BOP ≥50% and with loss of attachment)

Group 5-25 subjects

Smoking subjects (> 10 Cigarettes/day) with Chronic periodontitis (PD≥4mm, BOP ≥50% and with loss of attachment)

A written informed consent was obtained from all the participants after explaining the purpose of the study. Male Smokers and non-smokers with 20-50 years with a healthy periodontium or with periodontitis were included in the study. All Females, and subjects with the presence of <22 permanent teeth, former smokers and subjects with any systemic disorders or history of periodontal therapy within 6 months were excluded from the study.Clinical examination was done and periodontal clinical parameters (Plaque index, Gingival bleeding Index, Probing pocket depth and clinical attachment) were recorded.

Sample Collection

From all the above mentioned groups of the study population, blood, saliva and gingival crevicular fluid were collected.

- Five millilitres of venous blood were drawn from the ante-cubital vein for the biochemical assay by using disposable needle and syringe following standard protocol. The collected blood samples were transferred into a closed vial and allowed to clot at room temperature. Serum preparation was done by centrifuging the clotted sample at 2500 rpm for 10 mins to separate serum from blood. The sera extracted were separated, frozen and stored immediately at -40°C.

- The subjects were asked to rinse their mouth with water prior to saliva collection and 5millilitres of saliva was collected by draining or spitting method and then expectorated into sterile test tube. The samples were then centrifuged at 3000 rpm for 10 Minutes and aliquoted and stored in plastic vials at -20°C.

- GCF samples were harvested using absorbent paper strips (by Intracrevicular method). Three filter paper strips approximately 2mm wide and 7mm long were inserted side by side in the buccal crevice and left in position for 30 seconds. The central drying strip was then removed and immediately replaced by the collecting strip measuring 2mm wide and 7mm long.

The two drying strips, which acted as a dam to wall of fluid from the remainder of the crevice were later removed and discarded. The filter paper containing gingival crevicular fluid was then transferred to a sterile Eppendorf tube containing 2ml of saline. Samples contaminated with blood or saliva was discarded. Pooled GCF samples were then transferred to biochemical lab for the estimation of elastase levels.

Quantification of Elastase was carried out using the sandwich immune assay. The reagents and standards were prepared according to the manufacturer guidelines. This assay employs an antibody specific for Human Neutrophil Elastase coated on well plate. All the samples and reagents used were brought to room temperature (20-250C).

50µl of each standard and sample were added into each well. Wells were covered and were incubated for 2 hours and then Washed five times with 200 µl of Wash Buffer manually. Invert the plate each time and decant the contents, hit 4-5 times on absorbent material to completely remove the liquid. 50 µl of prepared Biotinylated Human Elastase Antibody was added to each well and incubated for 1 hour at room temperature. 50µl of prepared streptavidin was added to each well and incubated for 30 minutes. 50µl of chromogen substrate Tetramethylbenzidine was added to each well and incubated for 7mins or until the optimal blue color density has developed. 50µl of 0.5 N hydrochloric acid (stop solution) was added to each well and color change was observed from blue to yellow. The intensity of color was measured at 450nm immediately [14].

Results

Paired sample't' test was applied to find out the statistical difference between the clinical parameters and Elastase activity in between the groups. For Clinical parameters, comparisons were made in between the groups for Plaque scores, Gingival bleeding scores, Probing pocket depths and Clinical attachment levels (Table.1).

Table 1: Descriptive statistics of periodontal clinical parameters (PI, GBI, PPD, CAL) -group wise

	Plaque Index		Gingival Bleeding Index		Probing Pocket Depth		Clinical Attachment Level	
GROUPS	**Mean**	**P value**	**Mean**	**P value**	**mean**	**P value**	**Mean**	**P value**
G1 vs G2	0.328 vs 0 .811	0.000	8.317 vs 6.766	0.000	2.121 vs 2.577	0.004	0.000 vs 0.000	-
G1vs G3	0.328 vs 1.860	0.000	8.317 vs 74.020	0.000	2.121 vs 5.582	0.000	0 .000 vs 5.946	0.000
G1 vs G4	0.328 vs 2.235	0.000	8.317 vs 71.629	0.000	2.121 vs 5.860	0.000	0.000 vs 5.548	0.000
G1vs G5	0.328 vs 2.097	0.000	8.317 vs 70.064	0.000	2.121 vs 5.728	0.000	0.000 vs 5.273	0.000
G2 vs G3	0.811vs 1.860	0.000	6.766 vs 74.020	0.000	2.577 vs 5.582	0.000	0.000 vs 5.946	0.000
G2vs G4	0.811 vs 2.235	0.000	6.766 vs 71.629	0.000	2.577 vs 5.860	0.000	0.000 vs 5.548	0.000
G2 vs G5	0.811 vs 2.097	0.000	6.766 vs 70.064	0.000	2.577 vs 5.728	0.000	0.000 vs 5.273	0.000
G3 vs G4	1.860 vs 2.235	0.011	74.020 vs 71.629	0.142	5.582 vs 5.860	0.217	5.946 vs 5.548	0.163
G3 vs G5	1.860 vs 2.097	0.140	74.020 vs 70.064	0.027	5.582 vs 5.728	0.539	5.946 vs 5.273	0.029

G4 vs G5	2.235vs 2.097	0.194	71.629 vs 70.064	0.204	5.860 vs 5.728	0.646	5.548 vs 5.273	0.109

The results of the comparison in the clinical parameters showed significant difference between diseased group and healthy group. In **Plaque Score** comparison, all groups showed a statistical significance at P=0.00, except G_3 vs G_5 and G_4 vs G_5.In **Gingival bleeding** score comparison, all groups showed a statistical significance at P=0.01, except G_3 vs G_4 and G_4 vs G_5.**Probing Pocket depth** comparison, showed a statistical significance at P=0.05, except G_3 vs G_4, G_3 vs G5 and G4 vs G_5. In **Clinical Attachment Level** comparison, all groups showed a statistical significance at P=0.05, except G_3 vs G_4 and G_4 vs G_5.

When Elastase activity was compared between groups, **Salivary and GCF Elastase** showed statistically significant difference between all the groups except G_4 vs. G_5, In contrast **Serum Elastase** activity showed significant difference between all the groups. (Table.2)

Table 2: Descriptive statistics of Salivary, GCF and Serum Elastase -group wise

	Salivary Elastase		**Gcf Elastase**		**Serum Elastase**	
GROUPS	**Mean**	**P value**	**Mean**	**P value**	**mean**	**P value**
G1 vs G2	26.62 vs 32.88	0.000	26.62 vs 32.88	0.000	119.79 vs 170.72	0.001
G1vs G3	26.62 vs 102.82	0.000	26.62 vs 102.82	0.000	119.79 vs 441.59	0.000
G1 vs G4	26.62 vs 50.43	0.000	26.62 vs 50.43	0.000	119.79 vs 810.36	0.000
G1vs G5	26.62 vs 51.71	0.000	26.62 vs 51.71	0.000	119.79 vs 922.08	0.000
G2 vs G3	32.88 vs 102.82	0.000	32.88 vs 102.82	0.000	170.72 vs 441.59	0.000
G2vs G4	32.88 vs 50.43	0.000	32.88 vs 50.43	0.000	170.72 vs 810.36	0.000
G2 vs G5	32.88 vs 51.71	0.000	32.88 vs 51.71	0.000	170.72 vs 922.08	0.000
G3 vs G4	102.82 vs 50.43	0.000	32.88 vs 50.43	0.000	441.59 vs 810.36	0.000
G3 vs G5	102.82 vs 102.82	0.000	32.88 vs 51.71	0.000	441.59 vs 922.08	0.000
G4 vs G5	50.43 vs 51.71	0.462	50.43 vs 51.71	0.000	810.36 vs 922.08	0.000

Discussion

The presence of active periodontal disease and continuing attachment loss threatens the oral health, comfort and function of the patient. If disease activity can be determined, different therapeutic measures may be fashioned for the individual patient. In order to predict the disease activity, useful diagnostic indicator should indicate the presence or absence of periodontal disease. Elastase is one such serine protease enzyme which reflects the disease activity. Since smoking is one of the major risk factor that

has an direct association with severity of periodontal disease, Our study emphasis the relationship between periodontitis, smoking and Elastase levels. In our study Elastase levels was measured by ELISA (Sandwich technique) where elastase is sand witched by immobilised antibody and a polyclonal antibody specific for human neutrophil elastase which is similar to the study done by Alavi et al [20]. In our study there is increase in enzyme activity from healthy control to disease group which is similar to the study done by Uitto et al [21], where there is increased elastase activity in oral rinse samples from healthy, gingivitis, and varied degree of periodontitis.

Smokers exhibit higher plaque score when compared to non-smokers in both health and diseased group due to inefficient tooth brushing and increased salivary flow leads to increased calculus formation, which is similar to the study done by Maddipati Sreedevi et al [22]. Smokers showed lower gingival bleeding than non-smokers which is similar to the study done by Goultschin et al [23]. due to vasoconstrictive action of nicotine.

There is an increased PPD and CAL level in both smoking and non-smoking chronic periodontitis patients with that of both healthy controls when compared between the smoking and non-smoking chronic periodontitis, there was no significant difference which shows that there was similar disease activity in group C, D & E. Salivary and GCF elastase levels was lower in smoking periodontitis when compared to non-smoking periodontitis group which is similar to the study done by Pauletto et al [24], Alavi et al [20]. Whereas serum elastase levels was higher in smoking periodontitis group which is similar to the study done by Ozaka et al [25]. The lower elastase activity in saliva and GCF of smoking periodontitis group in spite of similar clinical parameters may be due to tobacco which causes vasoconstriction and reduced permeability of blood vessels which inhibits neutrophil migration. Because of this, there was abnormal accumulation of neutrophils and macrophages in the inflamed tissues, which rather migrating via the GCF to the oral cavity, gets accumulated in the periodontal tissues and release their constituents causing increased degradation of connective tissue components.

So lower elastase activity in smoking periodontitis group cannot be mislead as having lower disease activity rather it has more periodontal destruction than non-smokers. So if chronic periodontitis is altered by environmental or systematic factors like smoking, stress or diabetes, it's always best to have both local and systemic sample sources to find out the bio-markers activity.

Limitations

In our study, Former/ ex-smokers were not included, if it has been included, more predictable association between Elastase levels and periodontal destruction would have been determined. Periodontitis severity was not categorised as mild, moderate and severe form.

Conclusion

Elastase can be used as a biomarker for predicting diagnosis and treatment outcome of periodontal therapy but when periodontitis is influenced by systemic or environmental risk factor, it is always best to have a systemic sample source along with local sample source. The future direction of this study is to compare the Elastase activity with microbiological plaque samples.

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