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Estimation of GCF and salivary aminopeptidase levels in periodontal health and disease: A Clinico Bio-Chemical study

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ABSTRACT

Background The purpose of this study is to observe the diagnostic potential of chromogenic substrate (Benzoyl (DL) Arginine p-nitranilide, BAPNA) for detection of bacterial enzymatic activity in gingival crevicular fluid and saliva of health and disease associated periodontal sites.

Methods, Forty patients underwent a clinical periodontal examination and were then classified into 4 groups depending on periodontal index score. The GCF and Saliva samples from each group were the quantitatively evaluated for aminopeptidase activity by using Benzoyl (DL) Arginine p-nitranilide (BAPNA) as substrate., **Results,** The mean of aminopeptidase level of GCF shows an increase from group I to group IV(These values run in parallel with the values of clinical index i.e., more severe the inflammation, higher the index score, and higher is the aminopeptidase level (with the mean of 0.00032 in group I to 0.06883 in group IV). ,In case of the mean values of aminopeptidase levels of saliva showed an increase from group I to group II and a decrease in group III and group IV., **Conclusion,** As the severity of disease increases, there is a significant increase in aminopeptidase levels suggesting that there is a direct relationship between aminopeptidase levels in gingival crevicular fluid and periodontitis.

Keywords: Chronic periodontitis, aminopeptidase–biochemical marker, endotoxins, and tissue destruction.

INTRODUCTION

Periodontitis is an infectious disease characterized by a destructive inflammatory process that affects the supporting tissues of the teeth. Experts agree that human periodontitis is initiated and perpetuated by a small group of predominantly gram negative anaerobic or microaerophilic bacteria colonizing the subgingival area. At the 1996 World Workshop on Clinical Periodontics, it was stated that most human periodontitis is caused by Porphyromonas gingivalis, Tannerella forsythia and Aggregatibacter actinomycetemcomitans.[31]

Bacteria initiate inflammation in various ways. Bacterial products are chemotactic for neutrophil, activate the plasma proteinase cascade system, trigger mast cells to release biogenic amines, and stimulate inflammatory cells and resident tissue cells to form cytokines, platelet activating factor and prostanoids.

Gingival crevicular fluid seems a promising potential medium for the detection of early changes which indicate the onset of disease. It provides a medium for evaluating the

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relationship between pathogenic bacteria and host factors for development and progression of inflammatory periodontal disease.

Since bacteria are the initiators of periodontal disease it seems sensible to consider bacteria as indicators of disease activity. A considerable number of bacterial products including endotoxin, hydrogen sulphide, butyric acid, propionic acid, and a number of enzymes can be monitored in plaque, GCF and saliva. [11]

Since enzymes are present in bacterial cells, PMNs and lysosomes of host tissue cells, the choice of specific bacterial products is limited. Choice of very specific substrates, specific inhibitors, and working at specific pH optima, however, make it possible to differentiate between bacterial and mammalian enzymes.

Microscopic and microbiological approaches have been attempted in recent years to diagnose and monitor periodontal conditions in humans. Being time consuming, however, makes them inappropriate for routine use. Hence the detection of selected bacterial enzymatic reactions directly in plaque, GCF or saliva associated with periodontal disease may therefore be an easy and efficient diagnostic method. [37]

The present study is an attempt made to observe the diagnostic potential of chromogenic substrate (Benzoyl (DL) Arginine p-nitranilide, BAPNA) for detection of bacterial enzymatic activity (aminopeptidase) in gingival crevicular fluid and saliva of health and disease associated periodontal sites.

MATERIALS AND METHODS

SUBJECT SELECTION

Patients attending the clinics at Department of Periodontics, S.D.M. College of Dental Sciences & Hospital, Sattur, Dharwad were invited to participate in the study. Written informed consent was obtained from each subject participating in the study. Complete medical histories were obtained after selection. The study was conducted for a period of three months.

The selection criteria included patients with clinical evidence of gingival inflammation and destruction. The exclusion criteria included history of any periodontal treatment in the past 6 months, history of antibiotic intake or oral antiseptic therapy for at least 4-6 weeks, history of any other systemic disorder which could alter the course of disease.

All the subjects underwent a clinical periodontal examination and were classified into 4 groups depending on periodontal index score.

1. Group 1- control group, 10 patients (5 males and 5 females) - No signs of gingival inflammation were seen. Periodontal scores ranged from 0 to 0.2.
2. Group II: 10 patients (6 males, 4 females) - Periodontal scores ranged from 0.3 to 0.9.
3. Group III: 10 patients (4 males, 6 females) - Periodontal scores ranged from 1.0 to 2.0.
4. Group IV: 10 patients (7 males, 3 females) - Periodontal scores ranged from 2.1 to 5.0 and above.

SITE SELECTION AND GCF SAMPLING

GCF samples were taken from multiple sites of each patient. These sites were selected after checking for the bleeding on probing.

Subjects selected were seated upright in the dental chair under proper lighting conditions. They were asked to gargle vigorously with a glass of sterile water to cleanse the tooth of loosely adherent debris. The test site was dried and isolated with cotton rolls. Volumetric micropipettes were placed extracrevicularly. A standardized volume of 2 microlitres was collected with the help of a plunger, dispensed into a microfuge tube and analyzed.

Test sites not expressing any volume of fluid and micropipettes contaminated with blood and saline were excluded from the study.

COLLECTION OF SALIVA

Unstimulated saliva was collected from the patients, using a needle less syringe for aspiration. It was dispensed into a microfuge tube and analyzed.

GCF AND SALIVA ANALYSIS

The GCF collected was diluted to l00ìl by adding 50mM tri-HCl buffer pH 7.6 and vortexed briefly on a cyclohomogenizer at 21000xg for 20 minutes at 4°C (In a high-speed refrigerated centrifuge Kubota 6800).

Saliva sample collected from the patients was allowed to settle for 30minutes at 4°C. 50ml of the saliva was measured and transferred to a sterile microfuge tube. The volume was made up to l00ml by diluting with 50mM tri-HCl acid buffer pH 7.6. Samples were c1arified by centrifugation as described for GCF.

The clarified clear supernatant solution was used for the quantitative estimation of aminopeptidase activity using Benzoyl (DL) Arginine p-nitranilide (BAPNA) as substrate (Kakade et al). The assay mixture consisted of diluted 100ìl of GCF/saliva, 700ìl of BAPNA substrate. This reaction mixture was incubated for 30 minutes in a water bath maintained at 30°C. The reaction was arrested by addition of 200ml of 30% acetic acid after 30 minutes. The amount of paranitranilide released was measured by determining the absorbance at 410nM against a suitable blank in a double beam U.V.V is spectrophotometer. The blank contained all the components except the GCF or saliva.

STATISTICAL ANALYSIS

The results of this study were analyzed by applying student 't' test to assess the significance.

RESULTS

SUBJECT DEMOGRAPHICS

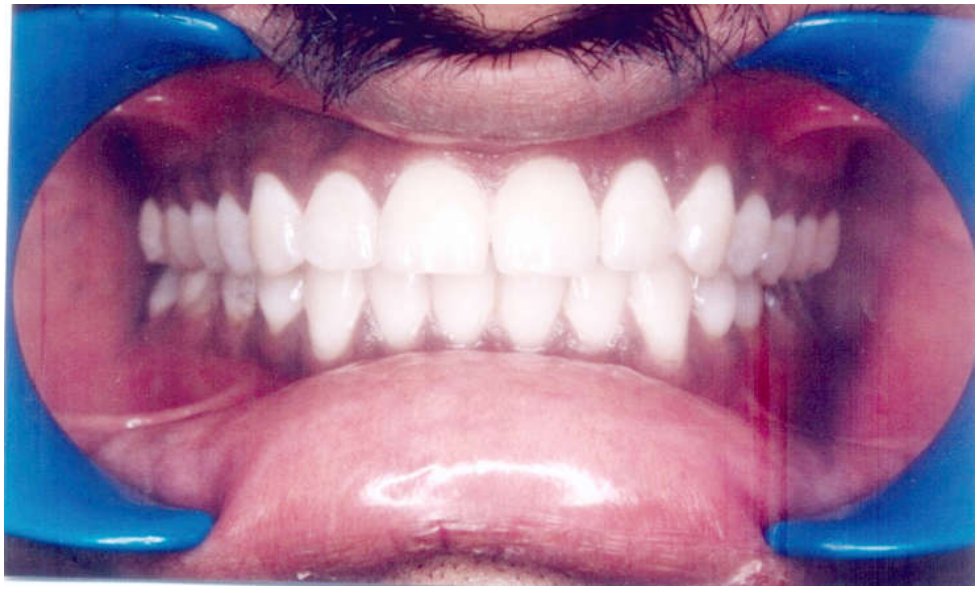
A total of 40 patients (22 males and 18 females) ranging in age from 20 – 45 years, were involved in the study.

AMINOPEPTIDASE LEVELS IN GCF AND SALIVA

GROUP I (NORMAL)-10 PATIENTS

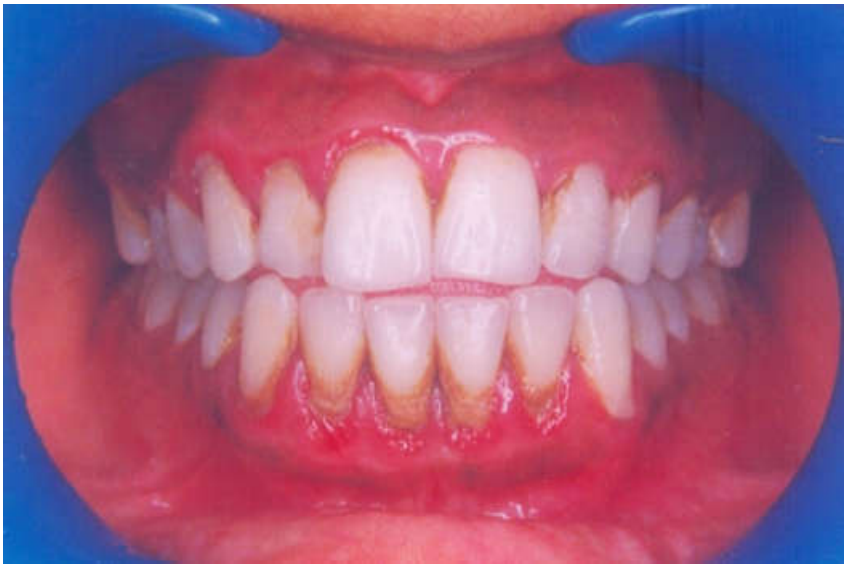
8 out of 10 patients did not show any aminopeptidase level in the GCF collected

Photograph Of An Individual Belonging To Group I



each. The mean and standard deviation were 0.00032 and 0.00068 respectively. The aminopeptidase levels in the saliva ranged from 0.008 to 0.0070 for all 10 patients. The mean and standard deviation were 0.0317 and 0.0171 respectively.

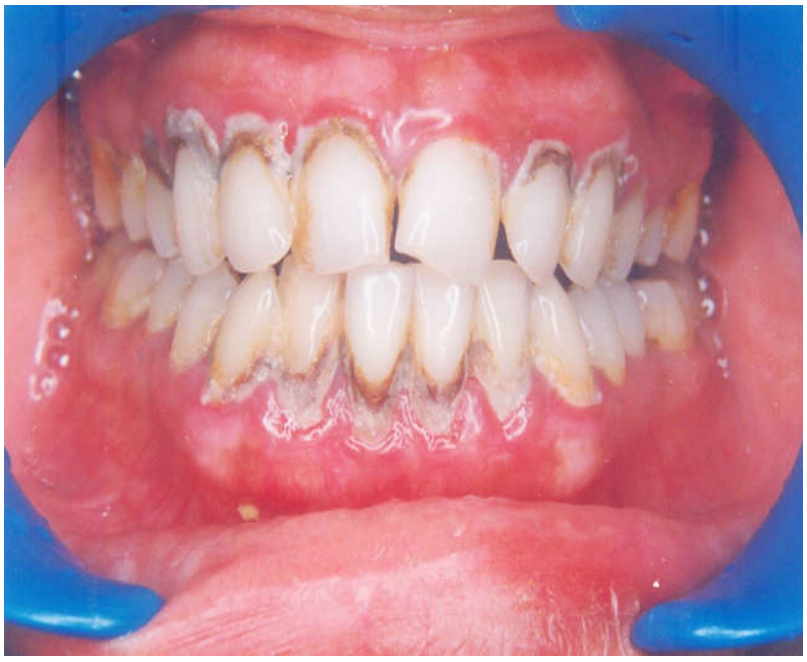
Photograph of an individual belonging to group II



GROUP II: 10 PATIENTS

Aminopeptidase levels ranging from 0.007 to 0.060 were recorded in GCF of all individuals with mean and standard deviations 0.0242 and 0.0158 respectively. The aminopeptidase levels in saliva ranged from 0.010 to 0.161 with mean and standard deviation 0.0626 and 0.0506 respectively.

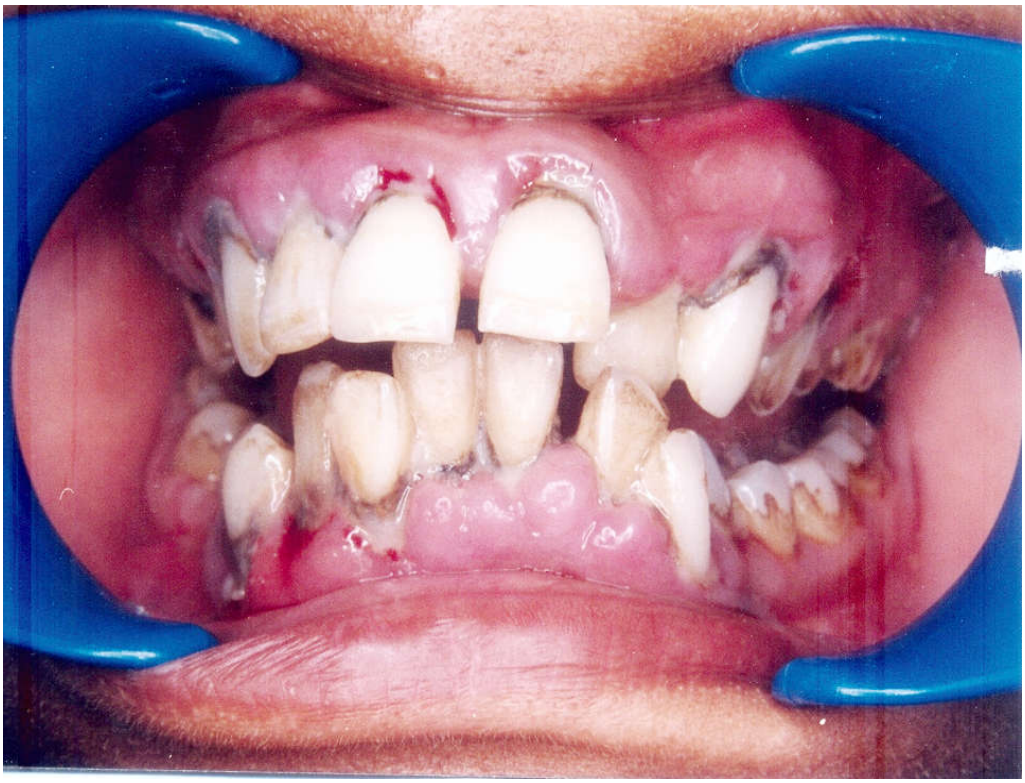
Photograph of an individual belonging to group III



GROUP III: 10 PATIENTS

The aminopeptidase levels in GCF ranged from 0.021 to 0.060, with mean and standard deviation 0.0367 and 0.0136 respectively. The saliva showed aminopeptidase levels ranging from 0.002 to 0.098, with mean and standard deviation of 0.057 to 0.0298 respectively.

Photograph Of An Individual Belonging To Group Iv



GROUP IV: 10 PATIENTS.

The aminopeptidase levels in GCF ranged from 0.041 to 0.139, with mean and standard deviation 0.0688 and 0.0367 respectively.

The saliva showed aminopeptidase levels ranging from 0.000 to 0.151, with mean and standard deviation 0.049 and 0.0452 respectively.

Comparison of Clinical Index (Russell's) with Aminopeptidase levels in GCF and Saliva between different groups

The mean values obtained from aminopeptidase levels in GCF and saliva in the different groups were subjected to statistical analysis by applying student't'test.

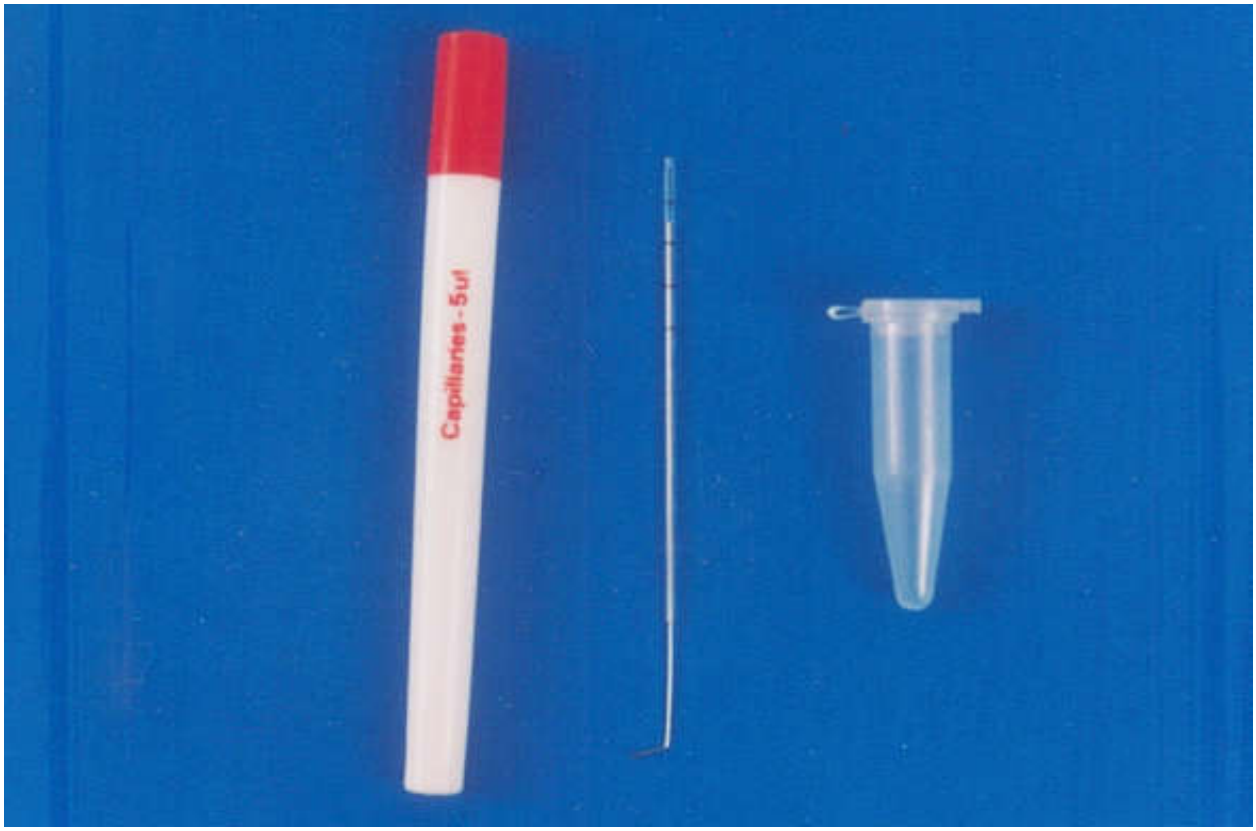
In the computed results of aminopeptidase levels in GCF, when group I was compared to group II, group III and group IV, it was found to be highly significant (P<0.00l). In case of aminopeptidase levels in saliva, when group I was compared to group II and group III, it was found to be significant (P<0.05).

When aminopeptidase levels in GCF of group II was compared to group III and group IV, it was found to be significant (P<0.10 and P<0.05).

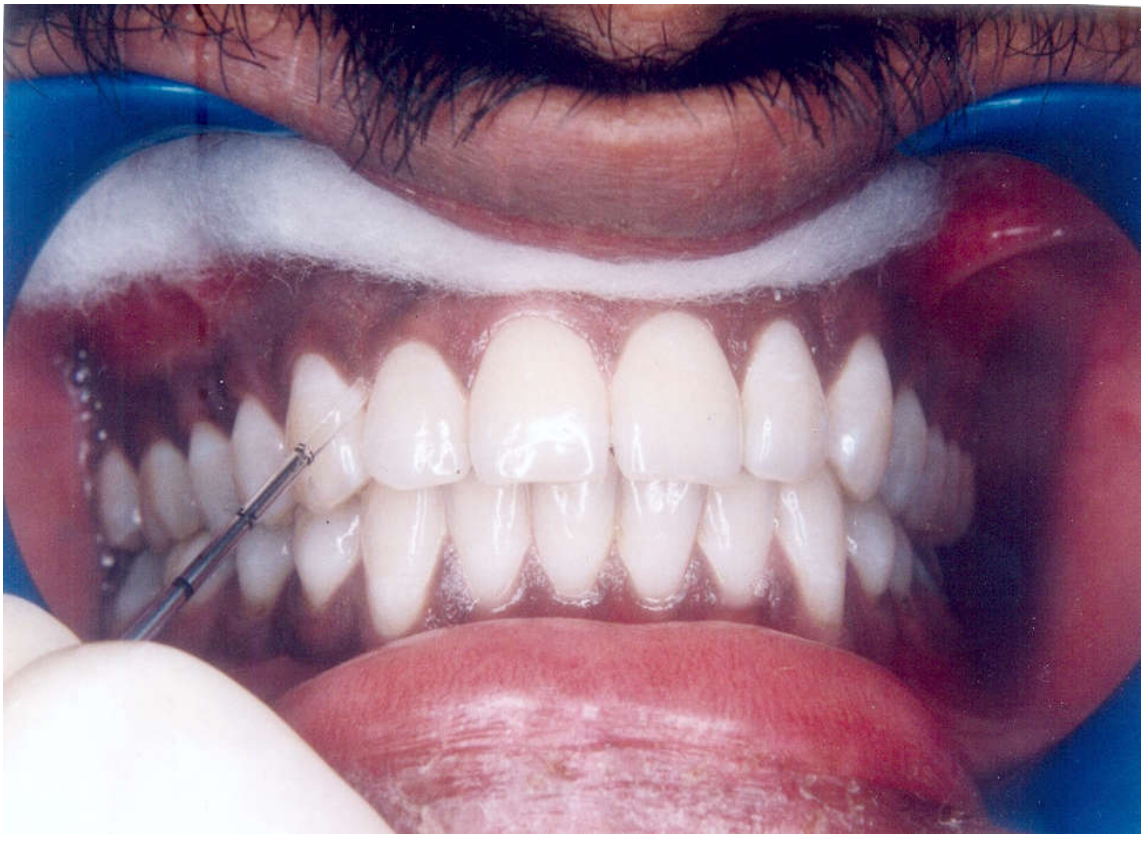
The aminopeptidase levels in saliva of group II compared to group III and group IV, was found to be insignificant (P>0.05). In case of group IV it was insignificant (P>0.05).

When aminopeptidase levels in GCF of group III was compared to group IV, it was found to be significant (P<0.05). The aminopeptidase levels in saliva of group III when compared to group IV was found to be insignificant (P>0.05).

Photograph of microcapillary pipettes and microcentrifuge



Photograph showing collection of GCF with microcapillary pipette



Photograph of electronic physical balance

Photograph of spectrophotometer

Photograph of computerised centrifuge

The mean of aminopeptidase levels of GCF show an increase from group I to group IV. These values run parallel with the values of clinical index i.e., greater the inflammation, higher the index score, and higher is the aminopeptidase level. In case of mean values of aminopeptidase levels of saliva, an increase was seen from group I to group II and a decrease in group III and group IV.

DISCUSSION

The current understanding of the pathogenesis of periodontal disease suggests that periodontal tissue is destroyed by the modulation of host defense by bacterial products. They stimulate the host inflammatory process to release cytokines and enzymes capable of destroying host tissue. The microbial composition associated with gingivitis apparently releases material that results in a local tissue reaction of inflammation.[31]

Studies from several microbiological laboratories demonstrated periodontopathic bacteria to be characterized by their enzymatic activities (produce extracellular lytic enzymes). It is intriguing that the suspected principal pathogens in periodontal disease are among the most proteolytic organisms in the dental plaque microbiota, which elaborate an assay of proteases including collagenase, trypsin like enzymes.[8] So, these bacterial enzymes and the enzymes of tissue origin may have diagnostic potential for detecting periodontal disease activity. It is also possible that these enzymes might be detected in saliva.[8]

In the present study, the aminopeptidase levels in GCF and saliva were compared with clinical index (Russell's). The results clearly showed that, as the severity of clinical inflammation increases, the aminopeptidase level in GCF also increases. In case of saliva, the aminopeptidase levels increased in group II and a decrease in mean values of group III and group IV was evident.

In group I (normal), 8 out of 10 patients did not show any aminopeptidase level in the gingival crevicular fluid, indicating that the enzyme producing organisms activity is absent in periodontally healthy sites. The remaining patients did show a very slight amount of aminopeptidase level. This could be due to presence of sub clinical inflammation of enzyme activity by some amount of periodontal microorganisms. This finding coincides with the study of Syed et al and Ando.K[37] who found a weaker activity at healthy sites.

In group II i.e. mild inflammation all the patients showed a slight increase in the aminopeptidase levels suggesting aminopeptidase enzyme activity by periodontal pathogens. This finding coincides with the conclusion of the study of Syed et al.[37] that the onset of gingivitis would correlate with the increase in the enzymatic activities.

In group III i.e. moderate periodontitis, there was a significant increase in the aminopeptidase level due to the increased activity of periodontal pathogenic process.

In group IV i.e. severe periodontitis with deep pockets, a few showed very high levels of aminopeptidase levels and some showed a significant increase in levels of the enzyme due to an increase in quantity and quality of subgingival pathogens. These findings coincide with the studies done by Syed et al., Ando K,[37]and also supports the findings obtained by the study done by Nakamura and Slots on aminopeptidase activity which showed to cleave many types of N-terminal amino acid residues and contribute to inflammatory periodontal disease due to conversion of Kellidin - 10 to Bradykinin. A positive correlation has also been established between severity of periodontitis and Bradykinin activity. The aminopeptidase also has preference for proline bonds suggesting that collagen fragments may be attached.[32] When the saliva from the different groups of individuals was subjected to the analytical procedure to estimate the aminopeptidase enzyme activity,

the mean value showed an increase in group II, III and IV.

In group I (normal) all the individuals showed a range from 0.008 - 0.043 of aminopeptidase level. In group II i.e. mild periodontitis all the patients showed an increase in the levels of aminopeptidase when compared to the group I individuals. This might be due to the contributing factors for enzyme activity from supra gingival plaque microorganisms. This findings coincide with the study done by Zambon et al, [12] showed a marked increase of salivary enzymatic activity when compared with the periodontal health.

In case of group III and group IV, the aminopeptidase levels were decreased. But when these groups were compared to the group I, the enzyme activities were found to be slightly more. This decreased level of the enzyme in group III and group IV might be due to the reduction of enzyme activities contributed by the supragingival microbiota and also due to the difference in quantity and quality of microbiota as mentioned by Zambon et al.[42]

Periodontal pockets are chronic inflammatory lesions and as such are constantly undergoing simultaneous destruction and repair. The destructive changes are characterized by degenerative changes. The reparative changes are characterized by the tissue repair. [4]

In recent years, culture methods to detect and quantify presumptive periodontopathogens have been used successfully in many research projects as well as clinical trials, but their routine use for the rapid diagnosis of periodontal disease is time consuming, cumbersome and hence impractical. So the chromogenic substrates for the rapid enzymatic characterization of clinically important bacterial and of oral bacterial isolates, which can be hydrolysed by the enzymatic activity of pathogens due to the activity of enzyme aminopeptidase might simplify the microbiologic procedures.[25]

CONCLUSION

The results of this study show that, as severity of periodontitis increases, the aminopeptidase level in gingival crevicular fluid also increases. However, this does not hold good for saliva.

From the results obtained, it can be concluded that as the severity of disease increases, there is a significant increase in aminopeptidase levels suggesting that there is a direct relationship between aminopeptidase levels in gingival crevicular fluid and periodontitis. Aminopeptidase in gingival crevicular fluid may be used as a potential

Table No.1: Table showing Aminopeptidase levels in GCF of patients *Absorbance units per 1ìl of GCF collected

PATIENT	AMINOPEPTIDASE LEVEL*			
	GROUP 1	GROUP 2	GROUP 3	GROUP 4
1	0.000	0.025	0.056	0.081
2	0.0015	0.031	0.036	0.139
3	0.0017	0.031	0.023	0.078
4	0.000	0.060	0.048	0.078
5	0.000	0.015	0.060	0.031
6	0.000	0.033	0.031	0.083
7	0.000	0.017	0.034	0.097
8	0.000	0.007	0.032	0.0113
9	0.000	0.007	0.027	0.049
10	0.000	0.016	0.020	0.041

Table 2: Table showing Aminopeptidase levels in saliva of patients *Absorbance units per 25ìl of saliva collected

PATIENT	AMINOPEPTIDASE LEVEL*			
	GROUP 1	**GROUP 2**	**GROUP 3**	**GROUP 4**
1	0.030	0.161	0.098	0.151
2	0.070	0.060	0.063	0.092
3	0.043	0.044	0.083	0.062
4	0.032	0.076	0.057	0.053
5	0.008	0.142	0.076	0.044
6	0.023	0.024	0.081	0.042
7	0.041	0.041	0.047	0.011
8	0.017	0.026	0.042	0.021
9	0.022	0.042	0.002	0.014
10	0.031	0.010	0.081	0.000

Table 3: Mean and standard deviation of Aminopeptidase levels in GCF and Saliva in group 1,2,3,4.

GROUP	SAMPLE SIZE	MEAN	STANDARD DEVIATION
		IN GCF	
I	10	0.00032	0.00068
II	10	0.0242	0.0158
III	10	0.0367	0.0136
IV	10	0.0688	0.0367
		IN SALIVA	
I	10	0.0317	0.0171
II	10	0.0626	0.0506
III	10	0.057	0.0298
IV	10	0.0452	0.0452

Table 4: Comparison of Aminopeptidase levels in GCF and Saliva between Groups 1 & 2

GROUP	NO. OF INDIVIDUALS	AMINOPEPTIDASE LEVELS		T VALUE	DF	P VALUE	SIGNIF-ICANCE
		Mean	S. Devia				
			IN GCF				
I	10	0.00032	0.00068	4.5301	18	<0.001	HS
II	10	0.0242	0.0158				
			IN SALIVA				
I	10	0.0317	0.0171	1.1356	18	<0.10	S
II	10	0.0626	0.0506				

Table 5: Comparison of Aminopeptidase levels in GCF and Saliva between Groups 1 & 3

GROUP	NO. OF INDIVIDUALS	AMINOPEPTIDASE LEVELS		T VALUE	DF	P VALUE	SIGNIF-ICANCE
		Mean	S. Devia				
			IN GCF				
I	10	0.00032	0.00068	7.3807	18	<0.001	HS
III	10	0.0367	0.0136				
			IN SALIVA				
I	10	0.0317	0.0171	2.2092	18	<0.05	S
III	10	0.057	0.0298				

Table 6: Comparison of Aminopeptidase levels in GCF and Saliva between Groups 1 & 4

GROUP	NO. OF INDIVIDUALS	AMINOPEPTIDASE LEVELS		T VALUE	DF	P VALUE	SIGNIF-ICANCE
		Mean	S. Devia				
			IN GCF				
I	10	0.00032	0.00068	5.3616	18	<0.001	HS
IV	10	0.0688	0.0367				
			IN SALIVA				
I	10	0.0317	0.0171	1.0739	18	>0.05	NS
IV	10	0.049	0.0452				

Table 7: Comparison of Aminopeptidase levels in GCF and Saliva between Groups 2 & 3

GROUP	NO. OF INDIVIDUALS	AMINOPEPTIDASE LEVELS		T VALUE	DF	P VALUE	SIGNIF-ICANCE
		Mean	S. Devia				
			IN GCF				
II	10	0.0242	0.0158	1.7989	18	<0.10	S
III	10	0.0367	0.0136				
			IN SALIVA				
II	10	0.0626	0.0506	0.2861	18	>0.05	NS
III	10	0.057	0.0298				

Table 8: Comparison of Aminopeptidase levels in GCF and Saliva between Groups 2 & 4

GROUP	NO. OF INDIVIDUALS	AMINOPEPTIDASE LEVELS		T VALUE	DF	P VALUE	SIGNIF-ICANCE
		Mean	S. Devia				
			IN GCF				
II	10	0.0242	0.0158	3.3487	18	<0.05	S
IV	10	0.0688	0.0367				
			IN SALIVA				
II	10	0.0626	0.0506	0.6014	18	>0.05	NS
IV	10	0.049	0.0452				

Table 9: Comparison of Aminopeptidase levels in GCF and Saliva between Group 3 & 4

GROUP	NO. OF INDIVIDUALS	AMINOPEPTIDASE LEVELS		T VALUE	DF	P VALUE	SIGNIF-ICANCE
		Mean	S. Devia				
			IN GCF				
III	10	0.0367	0.0136	2.4605	18	<0.05	S
IV	10	0.0688	0.0367				
			IN SALIVA				
III	10	0.057	0.0298	0.4433	18	>0.05	NS
IV	10	0.049	0.0452				

biochemical marker for assessment of periodontal disease activity. Although there is a difference in aminopeptidase levels in saliva of different group individuals, it is insignificant to be a potential biochemical marker.

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