Original article

Comparison of The Phagocytic Activity Of Gingival Crevicular Fluid (GCF) Neutrophils At Healthy Sites With Periodontally Diseased Sites.

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ABSTRACT

Aim and Objectives: The purpose of this study was to compare the GCF neutrophils function with the following objectives:1) To assess the total GCF neutrophils count in Healthy and Chronic Periodontitis patients. 2) To assess the percentage of phagocytic capacity of GCF neutrophils in Healthy and Chronic Periodontitis patients.

Method and Materials: Total thirty subjects (17 females and 13 males) in the age range 20 to 57 years were recruited for the study. Two groups with 15 subjects each were designated as Group 1 (Clinically Healthy), and Group 2 (Chronic Periodontitis) respectively, according to the plaque index, Papillary Bleeding Index and diagnosis was based on their periodontal status assessed clinically and radiographically.2 µl of GCF samples was collected from Group 1 and Group 2 subjects. Total neutrophil count and the neutrophils with ingested bacteria was calculated in both the groups.

Results: The mean total GCF neutrophil counts was significantly greater in Group 2 (9027.33 ± 404.16 per cu.mm) as compared to Group 1 (4046.66 ± 324.29 per cu.mm)with positive Pearson's coerrelation coefficient test between neutrophil count and clinical parameters. However, the percentage of GCF Neutrophils phagocytosing the bacteria in Group 2 (59.66 ± 4.25) was significantly lower than the phagocytic activity of GCF Neutrophils from Group 1 (75.26 ± 4.11).

Conclusion: Neutrophil count and its phagocytic activity is altered in chronic periodontitis as compared to healthy subjects, increasing the susceptibility of individual to disease. Therefore, GCF neutrophils count could be used to assess the disease activity as a simple chair-side diagnostic tool.

Introduction

Periodontitis is an inflammatory reaction to a microbial infection associated with dental plaque that results in tissue loss. It is classified as: Chronic periodontitis: An infectious disease resulting in inflammation within the supporting tissues of the teeth, progressive attachment loss and bone loss. Aggressive periodontitis: Comprises of group of rare, severe, rapidly progressive forms of periodontitis often characterized by an early loss of teeth.

Periodontal diseases are best considered as the outcome of an imbalance in the host parasite interaction. The extent and severity of disease depends on the interaction between the pathogenic challenge and host response. The narrow balance between periodontal homeostasis and disease depends upon an

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Figure no. 1.Colour Coded Calibrated volumetric Micro capillary pippetes (10 µ*l*)
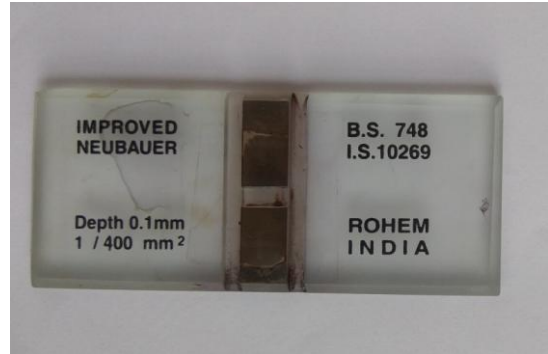

figure. No. 2. GCF Collection

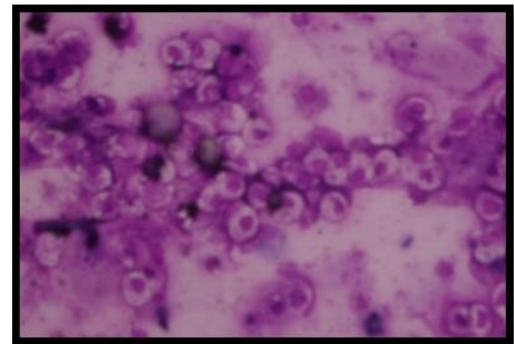Figure No. 3.Heamocytometer (Improved Neubauer's Chamber)

Figure No. 4.Neutrophils With Phagocytosed Bacteria

appropriate qualitative and quantitative response of host defense mechanisms to the infection of the periodontal tissue. Inadequate or excessive host response can lead to tissue destruction and such an outcome may be largely specific to the individual. Studies which were done on host responses in periodontal diseases have pointed out clearly that the polymorphonuclear leukocytes (PMNs) were the key protective cells, which under normal circumstances, limited the pathology which was caused by periodontal micro- organisms[1].

The presence of neutrophils in the gingival crevice serves to protect the periodontal tissue against microbial attack by phagocytosing the microorganisms or by releasing lysozomal enzymes into the crevicular environment extracellularly.[2] Patients with PMN defects, either quantitative or qualitative, are

associated with increased susceptibility to rapid and often severe periodontal destruction. The location of PMNs at the plaque interface, their phagocytic activity and signs of lysozomal enzyme release give morphological evidence that these cells, may on one hand, protect the tissue from bacterial attack but on the other hand, may induce tissue damage and increased inflammation via release of lysozomal enzymes. Thus, high numbers of subgingival leucocytes could possibly indicate an active periodontal lesion.[3]Subgingival leucocyte counts may be useful in identifying sites with active periodontal disease. This is possible if a correlation is established between the clinical measures of disease activity and GCF neutrophil levels. Therefore, present study was undertaken to compare the GCF neutrophils function with the following objectives: 1) To assess the total GCF neutrophils count in Healthy and Chronic Periodontitis

PARAMETERS	Group 1	Group 2	*p*value
PI	0.7 ± 0.25	3.06±0.47	*p*< 0.001**S**
PBI	0.4 ± 0.5	2.34±0.18	*p*< 0.001**S**

Table – 1 :Mean PI and Mean PBI in Group 1 and Group 2 (Mean ± SD)   S: Significant

PARAMETERS	Group 1	Group 2	*p*value
PPD	0.73 ± 0.25	4.76 ±0.99	*p*< 0.001**S**
CAL	00 ± 00	5.06±1.35	*p*< 0.001**S**
REC	00 ± 00	0.3 ± 0.41	*p*< 0.001**S**

Table -2:Mean Probing pocket depth (PPD), Mean clinical attachment loss (CAL) and Gingival recession (REC) in Group 1 and Group 2 (Mean ± SD; in mm)

Parameters	Group 1	Group 2	*p*value
Total Neutrophil Counts (per cu.mm)	4046.66 ± 324.29	9027.33 ± 404.16	*p*< 0.001**S**
Phagocytosed Neutrophil Counts (%)	75.26 ± 4.11	59.66 ± 4.25	*p*< 0.001**S**

Table – 3: Comparison of Total Neutrophil Counts and Phagocytosed Neutrophil Counts between Group 1 and Group 2

Sample	Mean ± SD	Correlation'r'
PPD	4.76 ±0.99	0.97
Neutrophil Count	9027.33±404.16	
PPD	4.76 ±0.99	-0.95
Phagocytic activity	59.66±4.25	
CAL	5.06±1.35	0.97
Neutrophil Count	9027.33± 404.16	
CAL	5.06±1.35	-0.93
Phagocytic activity	59.66± 4.25	

Table 4 :Correlation of clinical parameters with Neutrophil Count and phagocytic activity in Chronic Periodontitis Patients (Pearson's Correlation Coefficient Test)

patients. 2) To assess the percentage of phagocytic capacity of GCF neutrophils in Healthy and Chronic Periodontitis patients.

Materials And Methods

This study was conducted in the a) Department of Periodontics and Implantology, Sharad Pawar Dental College and Hospital, and b) Department of General Pathology, Jawaharlal Nehru Medical College; Sawangi (Meghe), Wardha, Maharashtra, India. Total thirty subjects (17 females and 13 males) in the age range 20 to 57 years were recruited for the study. Informed written consent was obtained from all the subjects and ethical clearance was obtained from the ethical board of this institution. Two groups with 15 subjects each were designated as Group 1 (Clinically Healthy), and Group 2 (Chronic Periodontitis) respectively, according to the plaque index (Turkesy–Gilmore–Glickman Modification Of Quigley-Hein 1970)[4], Papillary Bleeding Index (Muhleman HR, 1974)[5] and diagnosis was based on their periodontal status assessed clinically and radiographically.

Group 1- Healthy Subjects

1.   Subjects with clinically healthy periodontium with no evidence of disease, Showing no attachment loss (CAL), probing pocket depth (PPD) < 3 mm, PI and PBI scores < 1.

Group 2 –Generalized Chronic Periodontitis

1.   Age 30 -35 years or more

2.   Amount of microbial deposits is consistent with the severity of periodontal tissue Destruction

3.   Generalized severe alveolar bone loss with multiple horizontal and vertical osseous defects radiographically.

4.   Disease usually affects many teeth with no rapid progression of the disease

Subject inclusion criteria:

1. Systemically healthy patients.

2. Subjects with varying degree of periodontal disease. (Chronic periodontitis).

3. No invasive periodontal therapy in the past six months.

Subject exclusion criteria

1. Systemic diseases like diabetes mellitus.

2. Pregnant subjects.

3. Smokers and alcoholics.

4. Presence of disease with possible effects on the immune system like chronic infection or cancer.

5. Treatment with any drugs that might alter PMN number or function.

6. Use of any antibiotics during the study period or in the recent past.

7. Presence of carious lesion or any kind of mucosal ulceration.

Collection of GCF and Analysis:

The subjects were asked to gargle their mouth vigorously with water to cleanse the teeth of loosely adherent debris. Samples of GCF were obtained from any healthy site by placing color coded, calibrated, volumetric, micro capillary pipettes (Sigma-Aldrich, Inc.) (Fig. 1). The test site was dried and isolated with cotton rolls. Volumetric micropipettes were placed extra crevicularly at the entrance of the gingival crevice and for each subject, and the GCF sample collected (Fig. 2). The pipettes that were contaminated with blood or saliva were discarded. $2\ \mu l$ of GCF sample was collected from healthy subjects and whereas for periodontitis site, samples were collected from sites exhibiting severe inflammation and deepest probing depth (>5mm). Test sites, which did not express any volume of GCF and Micropipettes contaminated with blood and saliva, were not included in the study.

Technical processing of the GCF collected from gingival sulcus was carried out by the methods suggested by Murray and Patters[6] as well as Patil and Metgud[7]. The samples were processed to observe and compare the results of 1) Total neutrophil count, and 2) Phagocytosing neutrophil counts.

A total neutrophil count was performed in Group 1 and Group 2 using Hemocytometer (Improved Neubauer's chamber) (Fig. 3), using White blood cell diluting fluid, which contains gentian violet as coloring dye. The counts were performed using conventional formulae of multiplication of average number of cells in WBC Square multiplied by constant factor as suggested by Patil and Metgud[7].

Prior to assessing bacterial phagocytosis by neutrophils, the non-viability of Neutrophils was determined in all solutions of Group 1 and Group 2 by differential counts. The differential counts of the sediments where performed after incubating Trypan blue dye solution for 3 to 5 minutes. The smears were prepared of the sediments and the cells without Trypan blue staining were considered non-viable. It was expressed in percentage in differential count and per ml values were calculated by formulae.

Phagocytosed neutrophil counts were performed in Group 1 and Group 2 samples. The neutrophil was defined as the phagocytosed one when the cytoplasm of the Neutrophils show blue stained bacteria in it as a phagolysosomes (Fig. 4). It was performed on the smears of the sediments and percentage of it was carried out as a differential count on Giemsa stain similar to the method described by Hung et al[8]. One hundred cells were counted in each preparation and the phagocytosed cells were expressed as percentage.

Statistical analysis

The means and standard deviation (Means + SD) values were calculated for all the clinical parameters. Student's unpaired t-test was used to compare data between two groups. The level of significance "*p*" value at 95% confidence interval was calibrated as: Non significant (NS): *p*> 0.05, Significant (S): *p* < 0.001. Further, Pearson's correlation coefficient test was used for correlation of clinical parameters (PPD, CAL) with Neutrophil Count and phagocytic activity in chronic periodontitis patients. The data was analyzed using Statistical Package for the Social Sciences (SPSS 11.0).

Results

A total of 30 subjects with 17 males and 13 females were selected for the study. Selected individuals were divided into two groups i.e. periodontally healthy group (Group 1: 15 subjects; 7 males, 8 females), and chronic periodontitis group (Group 2: 15 subjects; 10 males, 5 females), aged between 20-57 years with a mean age of 37.76 ±12.78 years.

The mean PI and PBI scores for Group 1 was 0.70 ± 0.25 and 0.04 ± 0.5, for Group 2 was 3.06 ± 0.47 and 2.34 ±0.18 respectively. The difference in PI and PBI score between two groups when compared was found to be statistically significant (*p*< 0.001) (Table-1).

The mean PPD, CAL and Gingival Recession (REC) for Group 1 was 0.73 ± 0.25 mm, 0.0± 0 mm and 0.0 ± 0 mm and for Group 2 was 4.76 ± 0.99 mm, 5.06 ± 1.35 mm and 0.3 ± 0.41 mm respectively. There was statistically significant difference noted in all the clinical parameters recorded between Group 1 and Group 2 (*p*< 0.001) (Table no.2).

All the samples in each group showed the presence of neutrophils. The mean of total neutrophil count in Group 2 was 9027.33 ± 404.16 per cu.mm, which was significantly greater than in Group 1 (4046.66 ± 324.29 per cu.mm) (*p*< 0.001) (Table no.3). The results suggest that the number of GCF neutrophils increased from health to periodontitis in all the samples. Also, when comparison of clinical parameters i.e PPD and CAL was done with Neutrophil Count by using Pearson's Correlation coefficient test in chronic periodontitis patients, a highly positive correlation was observed suggesting that, with increase in PPD and CAL, neutrophil count increases in GCF (Table no. 4).

The percentage of neutrophils with bacterial phagocytosis in Group 1 was 75.26 ± 4.11 and in Group 2 was 59.66 ± 4.25. Significantly reduced phagocytic activity was noted in Group 2 as compared to Group 1 (*p*< 0.001) (Table no.3). When neutrophil phagocytic activity was correlated with PPD and CAL, negative correlation was observed, i.e, with increase in the PPD and CAL, phagocytic activity appeared to be reduced in chronic periodontitis patients (Table no. 4).

Discussion

The present study examined the total neutrophil counts and in vitro phagocytic activity of GCF neutrophils in healthy and periodontitis subjects. In our study we have taken only healthy and periodontitis subjects into consideration since gingivitis is a reversible process, which is more dependent on the oral hygiene and plaque, control unlike periodontitis, which is an irreversible process. Hence, for a real picture of the actual ongoing disease process, the collection of GCF from a periodontitis patient is any day better since periodontitis is the chronic inflammatory infectious disease that undergoes periods of activity and quiescence[9].

There are various methods, such as enzymatic activity and microbiologic testing, that have evaluated periodontal disease activity, but they are cumbersome

and have low clinical applicability. Efforts to find simple, easy methods to evaluate disease activity are elusive[10]. At present the most commonly used diagnostic tool is periodontal probing but it's a one dimensional measurement of a three dimensional space. Also, an error of 1 mm will result in 50% error, with the biggest advantage being speed of execution and immediacy of interpretation as compared to other microbiologic or immunologic methods. Periodontal probing provides clinical information regarding pocket depth and configuration, but periodontal pockets go through periods of exacerbation and quiescence. Periods of quiescence are characterized by reduced inflammatory response and little or no loss of bone and connective tissue attachment and the opposite, in periods of activity. Thus, it is important to know current disease activity, which will have an implication on treatment options. These considerations suggest that the advantage of probing though acceptable and irreplaceable in routine periodontal practice is deficient when disease activity is to be evaluated. Hence alternate measures to assess periodontal disease activity can be used based on indicators of inflammatory process[11]. The GCF from periodontitis patients only can give a clearer view of the underlying pathophysiology and GCF neutrophils count could be used to assess the disease activity as a simple chairside diagnostic tool.

There are a number of ways of collecting GCF for neutrophil estimation. The use of Styroflex strips, might not give accurate results, due to the clumping of the cells[12]. The washing method suggested by Skapski and Lehner in 1976[13], and by Salonen and Paunio in 1991[14] has a shortcoming that the dilution factor cannot be determined accurately and thus not an ideal method.Periopaper is considered as one of the method

for collection of GCF; however, no studies so far have validated alternative methods for collecting GCF, specifically with respect to their absorption capacity and to the accuracy of measurements in comparison with Periopaper[15,16]. In present study, colour coded capillary micropippetes was used to collect GCF samples since it is one of the novel methods of collecting GCF sample without the disadvantages of the above mentioned methods. There is lesser contamination with blood and saliva and GCF collection can be easily done by extra crevicular method. The main advantage of this is the collection of unstimulated GCF which can give us the near perfect picture of what is actually going on under the sulcular epithelium. Capillary micropippetes also reduces the operational cost (no requirement of Periotron for measuring GFC collected) with ease of use by direct measurement of the amount of GCF collected.

Results of this study showed a greater presence of PMNs in Periodontal disease as compared to the healthy subjects (Table no. 2). Also, this was verified by a positive correlation seen between the PPD, CAL and neutrophil counts in samples from chronic periodontitis patients (Table no. 4). Increase in PMNs count in GCF neutrophils from the samples of chronic periodontitis patients could be attributed due to increase surface area of ulcerated epithelium and hence increase in the migration of PMN leucocytes through the ulcerated epithelium[17]. Using neutrophil counts in the GCF to evaluate the periodontal disease activity has been used in earlier studies and has shown a positive correlation with the probing pocket depth.[18,19]

This study reported a highly significant decrease in the GCF PMN activity in Chronic Periodontitis patients as compared to controls [Table 2]. Also, there was

negative correlation was observed when neutrophil phagocytic activity was compared with the clinical parameters (PPD and CAL), i.e, with increase in PPD and CAL, phagocytic activity of neutrophil decreased (Table no. 4). This lower phagocytic capacity of neutrophils in periodontitis has also been reported by other researchers.[20, 21, 22, 23]

When investigating the phagocytosis, two lines of reasoning can be defined: the direct action of bacteria in phagocytosis and the effects of host parasite interactions in phagocytosis. The mechanisms for the deficient response of phagocytes have not been explained yet; however some hypotheses can be suggested. It is known that many periodontal pathogens develop particular strategies for subverting the mechanisms of phagocytosis[24]. Among the mechanisms of immunosuppression, the role of LPS has been considered. Studies have indicated that the LPS of P. gingivalis appears to be antagonists of the toll-like receptor-4, thus competing with LPS of other species to couple with that receptor. This is a possible mechanism of deficiency of the innate immune system of the host, since recognition of the bacterial pathogens identified by the toll-like receptor-4 would be blocked[25]. The recruitment of neutrophils and macrophages in mice infected with Aa and lacking the toll-like receptor-2 led to the reduction in the influx of these cells in the peritoneal cavity. Infection with Aa in this experimental model caused a significant decrease in the cytokine and chemokine levels and reduction in the phagocytic capacity of neutrophils and monocytes, in addition to alveolar bone loss[26].

Conclusion

Within the limitations of the study, it can be concluded that, though there was increased in GCF neutrophil counts in chronic periodontitis patients, the phagocytic activity was reduced as compared to healthy subjects. Furthermore, it can be inferred that altered neutrophil function could severely weaken the host defense, resulting in the initiation and progression of periodontal disease.Thus, individual susceptibility, which is an additional and important modifying factor in the pathogenesis of periodontal disease, can be used as a diagnostic marker of periodontal disease activity and can be accomplished by this simple non-invasive method.

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