Neutrophil Exodus From The Gingival Crevice - A Novel Method of Quantification Using Durapore Filter Strips: A Cross Sectional Study

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Abstract

Objectives: Neutrophils play a critical role as a part of the innate immune response.Although neutrophils are primarily protective, they release products partly responsible for the destruction seen in periodontal disease.The techniques presently available for counting neutrophils require special equipment and are only semi-quantitative. The aim of the present investigation was to check the efficacy of a single, rapid, non-invasive assay to enable the expedient quantification of oral neutrophils, and utilize the assay to quantify the number of neutrophils in periodontal disease.

Materials and Methods: Forty five subjects were recruited in the study. They were put into three groups based on the Gingival Index and Russell's Periodontal Index as clinically healthy (Group 1), gingivitis (Group 2) and periodontitis (Group 3). GCF samples were collected using a durapore filter and the number of neutrophils counted using an improved Neubaeur's Chamber.

Results: Neutrophils were present in GCF of all the samples. There was statistically significant difference between the neutrophil numbers in all the samples with respect to severity of periodontal disease. The strength of association was the strongest between probing pocket depth and neutrophil counts.

Conclusion: This study demonstrates that it is possible to collect and quantify oral neutrophils by a single, rapid, noninvasive assay using durapore strips.

Key Words: Neutrophils; dental plaque; saliva; gingival crevicular fluid; Millipore filter.

Introduction

Periodontal disease is defined as an inflammatory reaction to a microbial infection associated with dental plaque that, results in tissue loss. Neutrophils or polymorphonuclear leucocytes (PMNs) play a critical role as a part of the innate immune response acting as a first line of defense against these invading microbes.[1] The presence of leucocytes in the oral cavity has attracted interest for many years.The presence of leucocytes in the oral cavity has attracted interest for many years. Calonius in 1958 compared the salivary leucocyte count in patients with healthy and inflamed gingiva and edentulous patients and found that the levels were least in edentulous patients and highest in patients with gingivitis thus suggesting that leucocytes enter the saliva through the gingival sulcus.[2] This was also confirmed by studies done by Schiott and Loe in1970.[3]

In the healthy periodontium of both humans and experimental animals, PMNs have been demonstrated migrating towards or residing within the sulcular and junctional epithelium and within the underlying connective tissue.[4] With plaque accumulation and the development of clinical inflammation there is an increase in the number of leucocytes present in the lesion.[5,6,7] The location of PMNs at the plaque interface, their phagocytic activity and signs of lysosomal 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 lysosomal enzymes. Thus, high numbers ofsubgingival leucocytes could possibly indicate an active periodontal lesion.[8] 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.

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Periodontal disease activity is the period of disease exacerbation which shows bone loss, connective tissue loss and inflammatory response. There are various methods that have evaluated periodontal disease activity like enzymatic activity and microbiological testing but they are cumbersome and have low clinical applicability. Efforts to find simpler and easier methods to evaluate disease activity are still elusive.[9]

The idea of using neutrophil quantification to assess periodontal disease activity and the effectiveness of therapy was first proposed by Raeste& Aura in 1978.[10] The detection of neutrophils in a plaque sample would seem to capture the host response to all of the periodontopathogens. This superiority of neutrophils as a diagnostic tool for periodontal disease can be transferred to the clinical setting. There are studies that have correlated the salivary neutrophils and gingival health and GCF neutrophils and periodontal health.[11,12,13,4,14,15] However difficulties can be encountered during quantification procedures such as aggregation of cells during collection, and when using washingtechniques, by partial loss of solution. Thus the aim of the present study was to check the efficacy of a single, rapid, non-invasive assay to enable the expedient quantification of oral neutrophils, and utilize the assay to quantify the number of neutrophils in periodontal disease.

Materials And Methods

This study was conducted in the Department of Periodontics and Oral Implantology, Sri DharmasthalaManjunatheswara College of Dental Sciences and Hospital, Dharwad, Karnataka, India. Forty five subjects (25 females and 20 males) in the age range 20 to 65 years were recruited for the study. Informed written consent was obtained from all subjects and ethical clearance was obtained from the ethical board of this institution. Three groups with 15 subjects each were designated as Group 1 (clinically healthy), as Group 2 (gingivitis) and Group 3 (chronic generalized periodontitis) respectively, according to the Gingival Index (Loe and Sillness, 1963) and Russell's Periodontal Index (1956).[16,17]

Subject inclusion criteria:

1. Subjects with varying degree of periodontal disease. (Healthy, gingivitis and chronic generalized periodontitis).
2. Subjects who were systemically healthy.
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 and subjects who have undergone non-invasive periodontal therapy.
7. Presence of carious lesion or any kind of mucosal ulceration.

A dental and medical history was compiled for all subjects with an oral examination, including caries assessment. Clinical parameters evaluated included Gingival index (Loe & Silness 1965) at four sites per tooth, Russell's Periodontal Index scores, and measurement of probing depth at four sites per tooth. The same investigator performed all data collection and examinations.

Collection of GCF: The gingiva was dried by air and cotton pellets 1minute before sampling and the area isolated by means of cotton rolls. Prior to GCF sampling, supragingival calculus was removed using sterile curette. A 7mm by 2 mm strip of Durapore® filters with a pore size of 0.22 µm (hydrophilic membrane filtersof polyviniiidene di-fiuoride); was placed at the entrance of the sulcus and left in place for 10 seconds (Fig 1). Pooled volume of GCF was collected for healthy subjects and with gingivitis, whereas for periodontitis site samples were collected from sites exhibiting severeinflammation and deepest probing depth. Test sites which did not express anyvolume of GCF and Millipore papers contaminated with blood and saliva were not included in the study.

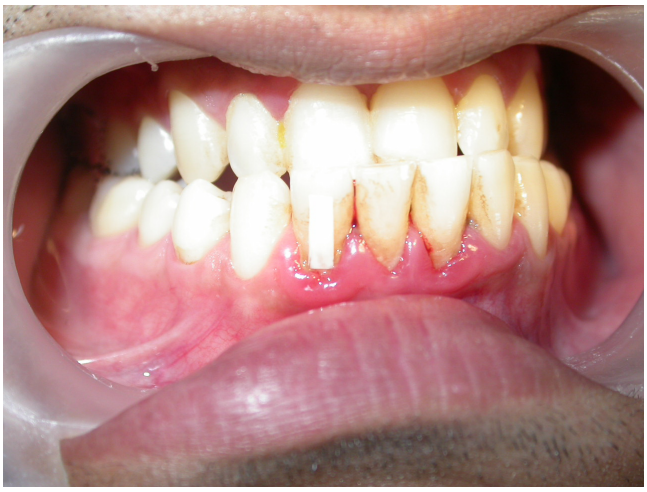

Fig 1: Collection of GCF using Durapore® Filter strip, of dimensions 2mm×7mm

Neutrophil determination:

The strips containing GCF were then inserted and suspended into plastic sealable siliconized tube of polypropylene (Sigma Aldrich, India) containing 40 micro litres (µl) of phosphate buffered saline without calcium (Ca), 3 milli Moles (mM) ethylene diaminetetraacetic acid (EDTA) and 1% bovine serum albumin (BSA), and vortexed for 30 seconds. Twenty

microlitres of this suspension was then withdrawn and stained with 10 ml of Turks solution for ten minutes (Fig 2). Neutrophils were then counted on an improved Neubeur's chamber (Cambridge Instruments Inc., USA) (Fig 3).

Statistical evaluation

The data collected was entered in Microsoft Office Excel Format and statistical analysis was done using Graph pad prism® (Graph pad prism, Graph pad software, Inc. Ver 5.03) One-way analysis of variance (ANOVA) was done to test thesignificant difference between the groups.To determine the correlation between the clinical indices and neutrophil, Carl Pearson correlation analysis along with tests of significance were used.Statistical significance was established at P< 0.05.

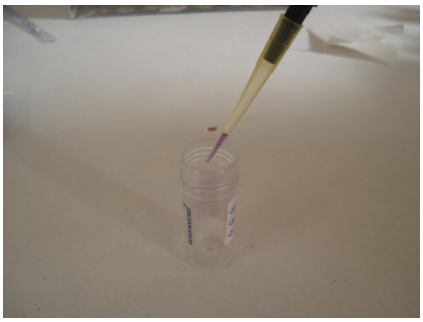

Fig 2: Twenty microlitres of neutrophil suspension withdrawn and stained with 10 ml of Turks solution for ten minutes.

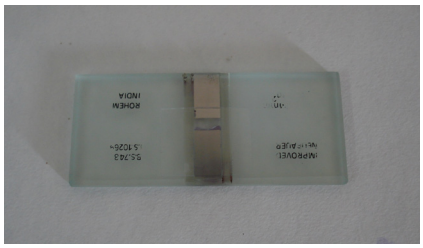

Fig 3: Improved Neubauers chamber

Results

All the samples in each group showed the presence of neutrophils. The highest numbers were in Group 3 and the lowest numbers in Group 1.The number of neutrophils for all the groups for plaque, saliva and GCF is shown in Table 1. One-way ANOVA showed statistically significant difference in the mean neutrophil number in all the groups as shown in Table 2. The results also suggest that the number of plaque, salivary and GCF neutrophils increased from health to gingivitis and to periodontitis in all the samples and it was statistically significant as shown in Figure 1.The clinical indices were correlated with the neutrophil counts and a positive correlation was found between the same as shown in Table 3.

Table 1

Variables	Summary	Groups		
		Healthy	Gingivitis	Periodontitis
Gingival index	Means	0.0650	1.8650	1.7400
	Std.Dev.	0.0272	0.4890	0.4782
Probing depth	Means	1.5100	2.3960	4.2320
	Std.Dev.	0.6535	0.3736	0.8579
Neutrophils	Means	5.6000	13.1000	25.7000
	Std.Dev.	1.4298	2.9609	6.4987

Table 2

SV	DF	SS	MSS	F-value	p-value	Signi.
Between groups	2	2063.4000	1031.7000	58.3492	0.0000	S
Within groups	27	477.4000	17.6815			
Total	29	2540.8000				

Table 3

Variable	neutrophil counts				
	r-value	r^2	t-value	p-value	Signi.
Gingival index	0.7096	0.5035	5.3286	0.0000	S
Probing depth	0.9049	0.8188	11.2470	0.0000	S

Table 4

	Sample	Mean	SD
Health	15	5.6	1.42
Gingivitis	15	13.1	2.96
Periodontitis	15	25.7	6.49

Discussion

The main source of neutrophils in the oral cavity is from those migrating from the gingival sulcus.[2] The results of the present study showed increase in the PMN leucocytes in saliva, GCF as well as in plaque with an increase in severity of periodontal disease. This was verified by a positive correlation seen between the Gingival Index and probing depth and neutrophil counts.This could be attributed to increase surface area of ulcerated epithelium and hence increase in the migration of PMN leucocytes though the ulcerated epithelium.[12] 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.[15] 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.[4] The washing method suggested by Skapski and Lehner in1976, and by Salonen and Paunio in 1991 has a shortcoming that the dilution factor cannot bedetermined accurately and thus not an ideal method.[18,19] The method used in this study is

the one suggested by Andersen and Cimasoni in 1993 and is the most acceptable method for PMN estimation.[15] This requires special millipore filters for the collection of GCF for the analysis of PMN numbers. The previous studies have used only the extracrevicular method of GCF collection and have found good correlation between probing pocket depth and number of neutrophils in GCF inshallow pockets but it failed in deeper pockets. The co-relation analysis in the present study showed strong association between pocket probing depth and PMN numbers in plaque and the strength of correlation was comparable to the that found between PMN numbers in the GCF when sampled intracrevicularly from the site with the deepest probing.This is in tandem with the results of the study by Anderson&Cimasoni in 1993.[15] Despite the tremendous development in microbiologic and immunologic diagnostic markers, most of them failed to show any clinical applicability. Microbiologic markers are fraught with technical difficulties especially when it comes to anaerobic culturing for periodontopathogens and takes time to obtain the results. Collection of gingival biopsy samples for immunologic markers has its own limitations.[20] On the contrary chair side microscopic examination for the quantitative estimation of PMN leucocytes is not plagued by these limitations.

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 1mm 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 reduced amount of bone and little or no loss of bone and connective tissue attachment and the opposite, in periods of activity. Thus it is 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[20]. GCF neutrophils could be used to assess the disease activity provided they could be correlated with the probing pocket depth.

Further studies could be directed to develop a chair side color changing agent similar to a disclosing agent that stains neutrophils in plaque which could help screen and monitor periodontitis subjects. Clinicians can use the plaque neutrophils to check the disease activity in subjects on supportive periodontal therapy. This could be further developed for screening of aggressive periodontitis subjects who have quantitative neutrophil abnormality.

Conclusion

This study demonstrates that it is possible to collect and quantify oral neutrophils by a single, rapid, noninvasive assay using duraporestrips. Neutrophils are found in higher numbers in GCF with increased severity of periodontal disease, a finding that reflects the inflammatory nature of the disease process. GCF neutrophils positively correlated with probing pocket depth.

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