Original Article

Quantification of oral neutrophil counts in periodontal health and disease utilising a standardised oral rinse

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Abstract

Background and Objectives: Polymorphonuclear neutrophils are the most abundant leukocytes in humans and are key host cells in defence against invading microorganisms. The oral neutrophil count may be an indicator of the periodontal health status, which correlates with the severity of periodontal disease. This study attempts to quantify orogranulocytes utilising an oral rinse and to assess the usefulness of this method in evaluating the oral inflammatory load much the same way the circulating neutrophils are used to screen for patients with infection in extra-oral sites.

Methods: A total of 125 participants were divided into five groups with 25 subjects in each group. The groups consisted of healthy, gingivitis, mild periodontitis, moderate periodontitis, and severe periodontitis. Participants were asked to rinse with 10 mL of 0.9% saline for 30 s and to expectorate. Samples were centrifuged at 2000 RPM for 10 min. The supernatant removed was suspended in 5 mL of Hanks's balanced salt solution. One millilitre of this suspension was mixed with 4 µL of acridine orange. A 10 µL aliquot of this suspension was then assessed on a haemocytometer, and the oral PMNs were counted using fluorescence microscopy.

Results: The mean number of oral neutrophils (100,000 cells/mL) was the lowest in the healthy group and increased in ascending order across the different groups with the highest for severe periodontitis group.

Conclusion: The oral neutrophil counts increased with the severity of periodontal inflammation. This is an easy, safe, reliable, and non-invasive method of quantification of oral neutrophils.

Keywords: Fluorescence microscopy, oral neutrophil count, periodontitis

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INTRODUCTION

Polymorphonuclear neutrophils (PMNs) are the key host cells in defence against invading microorganisms and play a role in both acute and chronic inflammation.[1] Neutrophils in the oral cavity enter through the gingival sulcus and function to control the oral bacteria.[2]

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It is estimated that 30,000 PMNs transit through periodontal tissues every minute and that their presence in the gingival crevicular fluid is physiologic.[3,4] The rate at which they migrate through the gingival sulcus into the oral cavity [the orogranulocytic migratory rate (OMR)] is increased in the presence of gingival inflammation.[4,5] The

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OMR also correlates with increased pocket depth and the gingival index.[6]

This approach can be adapted in the future, which may enable dentists to screen for oral infections using oral neutrophil levels. This study is an attempt to quantify orogranulocytes utilising an oral rinse and to assess the usefulness of this method in evaluating the oral inflammatory load.

MATERIALS AND METHODS

The study was conducted, after institutional ethical committee approval (Reference number 014/09), on 125 patients drawn from the Departments of Periodontics, Annoor Dental College and Hospital, Muvattupuzha, Kerala, India. The subjects in the age group of 16–69 years were divided into five groups with 25 participants in each group. The five study groups consisted of healthy, gingivitis, mild periodontitis, moderate periodontitis, and severe periodontitis.

Patients were selected at random attending the Department of Periodontics, and orogranulocyte evaluation was performed in the Department of Oral Pathology and Microbiology, Annoor Dental College, Kerala. Informed consent was obtained from the participants prior to the study.

The participants had no history of scaling and root planing 3 months prior to the study. Periodontal pockets and bleeding upon probing (six sites per tooth) were recorded for each patient, and the subjects were divided into five groups (25 per group) based on the criteria by Mancini *et al*.[7]

Patients with systemic disease, visible oral pathosis, ulceration of the soft tissues, or caries that could affect neutrophil function were excluded from the study.

METHODOLOGY

The participants were first asked to rinse with 10 mL of 0.9% saline (0.9% sodium chloride; w/v Baxter Pvt. Limited, Tamil Nadu, India) for 30 s and asked to expectorate into a 15 mL calibrated test tube. Each of them was then evaluated for pocket depth and gingival scoring. The selected subjects were allotted to one of the five groups based on their gingival index scores and number of periodontal pockets [Table 1].

The collected salivary oral rinses were processed within 5 hours of collection. Rinse samples were centrifuged (KEMI Centrifuge, India) at 2000 RPM for 10 min. The supernatant was removed using a 5 mL pipette, and the pellet was suspended in 5 mL of Hank's balanced salt solution (HIMEDIA, Mumbai, India). One millilitre of this suspension was removed, and 4 μL of Acridine Orange (AO; Sigma Aldrich, USA) was added to the cells. AO is a fluorescent nucleic acid marker, which allows neutrophils to be distinguished from other cells using fluorescence microscopy. A 10 μL aliquot of this suspension was then assessed on a haemocytometer (Improved Neubauer ROHEM, India), and the oral PMNs (oPMNs) were counted visually using fluorescence microscopy (Lawrence and Mayo Trinocular Microscope, India). Neutrophils were counted in 16 grids from each corner of the haemocytometer for each sample. A C-MOS Camera (Lawrence and Mayo, India) was used to capture the image of the cells, and IS- Capture image software was used to transfer it to a laptop. The total cell concentration/mL was calculated using the formula[3]

Cell concentration per mL = total cell count in 4 squares × 2500 × dilution factor.

RESULTS

The values of different study groups were subjected to statistical analysis (SPSS version 22.0.0.0). One-way analysis of variance (ANOVA) and Chi-square test of association are used for analysis. A significant difference in mean value of age ($F(4, 120) = 29.566$, P-value < 0.001) was obtained among the five groups. The mean age is found to be the lowest for the healthy group and the highest for severe periodontitis group [Table 2].

No significant association (p-value = 0.165) was found in gender and among the study groups [Table 3 and Graph 1].

The mean oral neutrophil counts of different groups in our study were 7.96×10^5 cells/mL for control, 2.92×10^6 cells/mL for gingivitis, 3.23×10^6 cells/mL for mild periodontitis, 3.24×10^6 cells/mL for moderate periodontitis, and 11.85×10^6 cells/mL for severe

Table 1: Diagnostic criteria

Group	Diagnosis	Diagnostic criteria
1	Healthy	No pocket with probing depth ≥5 mm; gingival index per tooth ≤1
2	Gingivitis	No pocket with probing depth ≥5 mm; gingival index per tooth >1
3	Mild periodontitis	Five or fewer pockets with probing depth ≥5 mm
4	Moderate periodontitis	Five or more, <20, pockets with probing depth ≥5 mm
5	Severe periodontitis	Twenty or more pockets with probing depth ≥5 mm

Table 2: Descriptive statistics of age among the groups and one-way ANOVA of age among the groups

Diagnosis	*n*	Mean	Std. Deviation	Std. Error	Comparisons	Sum of Squares	df	Mean Square	*F*	Sig.
Control	25	27.72	7.368	1.474	Between Groups	11044.912	4	2761.228	29.566	0.000
Gingivitis	25	41.960	12.119	2.424	Within Groups	11207.040	120	93.392		
Mild Periodontitis	25	49.840	9.353	1.871						
Moderate Periodontitis	25	51.920	10.575	2.115						
Severe Periodontitis	25	52.920	8.154	1.631						
Total	125	44.872	13.396	1.198		22251.952	124			

Table 3: Chi-square test of association of gender among the groups

	Male	Female	Total	Chi-square	Df	*P*
Control	9	16	25	6.494	4	0.165
Gingivitis	11	14	25			
Mild Periodontitis	7	18	25			
Moderate Periodontitis	13	12	25			
Severe Periodontitis	15	10	25			
Total	55	70	125			

periodontitis. Our results showed an increasing count from healthy to severe periodontitis group. A significant difference in the mean value of number of oral neutrophil count (100,000 cells/mL) (F (4, 120) = 19.554, *P*-value < 0.001) was observed among the five groups [Table 4].

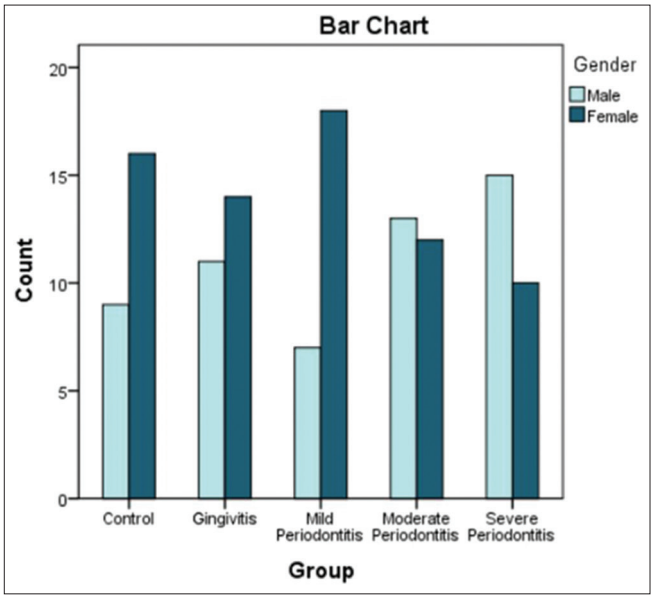
The mean value of number of oral neutrophils was found to be highly significant (p = 0.000) among healthy and severe periodontitis, gingivitis and severe periodontitis, mild periodontitis and severe periodontitis, and moderate periodontitis and severe periodontitis [Table 5 and Graph 2].

DISCUSSION

The human mouth has a persistent bacterial presence that is kept under control by a continual influx of neutrophils from the adjacent periodontal tissues.[8] The release of toxic products by neutrophils is thought to be relatively responsible for the destruction seen in periodontal disease. OMR is greater in the presence of gingival inflammation and also correlates with increased pocket depth and the gingival index.[8]

Klinkhamer in 1968 put forward the technique of rinsing the mouth with hypertonic saline and examining the collected samples. It was appealed that this technique had several advantages.[5] The oral rinses used for quantification of oral neutrophils include 0.9% saline and phosphate-buffered saline solution. Earlier studies used 0.2 M sodium chloride. In the present study, we used 0.9% saline oral rinse to assess the oral neutrophil counts in periodontal health and disease.

In a study by Raeste *et al*., 1978,[2] the OMR was determined with sequential mouth rinse sampling in periodontitis



Graph 1: Bar chart for gender

patients and controls. The results indicated that the OMR shows the presence of oral inflammation and proposed that this measure can be used as a laboratory test.[2]

Calonius in 1958 revealed higher rates of PMN migration into the oral cavity in the presence of gingival inflammation, and the lowest oPMN numbers have been reported after tooth extractions.[9]

In the present study, the mean number of oral neutrophils (100,000 cells/mL) is the lowest for healthy group and the highest for severe periodontitis group. Also, there is a significant difference in mean value of number of oral neutrophils (100,000 cells/mL) when all other groups are compared to the severe periodontitis group.

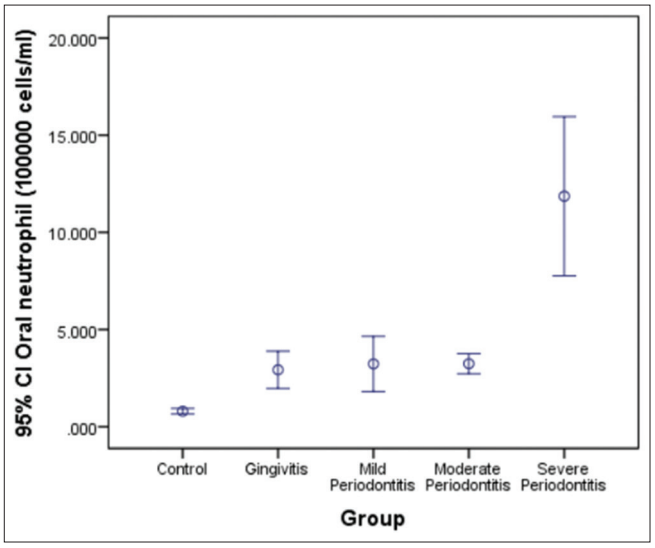
J.S. Bender *et al.* in 2006 had evaluated oral neutrophil counts utilising a similar oral rinse assay to determine the relationship between oral neutrophil levels and the severity of chronic periodontal disease. The progressive relationship observed is probably because of increased probing depths, leading to increased ulcerated epithelium through which greater numbers of neutrophils migrate in response to the presence of subgingival bacteria.[8]

Table 4: Descriptive statistics of number of oral neutrophils and one-way ANOVA of number of oral neutrophils

Diagnosis	*n*	Mean	Std. Deviation	Std. Error	Comparison	Sum of Squares	df	Mean Square	*F*	Sig.
Control	25	0.797	0.349	0.070	Between Groups	1836.975	4	459.244	19.554	0.000
Gingivitis	25	2.929	2.313	0.463	Within Groups	2818.344	120	23.486		
Mild Periodontitis	25	3.235	3.438	0.688						
Moderate Periodontitis	25	3.244	1.254	0.251						
Severe Periodontitis	25	11.859	9.928	1.986						
Total	125	5.847	6.141	0.549		4655.319	124			

Table 5: Pair wise comparisons of number of Oral Neutrophil

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.
Control	Gingivitis	-2.132	1.371	0.529
	Mild Periodontitis	-2.438	1.371	0.391
	Moderate Periodontitis	-2.448	1.371	0.387
	Severe Periodontitis	-11.062	1.371	0.000
Gingivitis	Control	2.132	1.371	0.529
	Mild Periodontitis	-0.305	1.439	1.000
	Moderate Periodontitis	-0.315	1.439	0.999
	Severe Periodontitis	-8.930	1.439	0.000
Mild Periodontitis	Control	2.438	1.371	0.391
	Gingivitis	0.305	1.439	1.000
	Moderate Periodontitis	-0.010	1.439	1.000
	Severe Periodontitis	-8.625	1.439	0.000
Moderate Periodontitis	Control	2.448	1.371	0.387
	Gingivitis	0.315	1.439	0.999
	Mild Periodontitis	0.010	1.439	1.000
	Severe Periodontitis	-8.61.5	1.439	0.000
Severe Periodontitis	Control	11.062	1.371	0.000
	Gingivitis	8.930	1.439	0.000
	Mild Periodontitis	8.625	1.439	0.000
	Moderate Periodontitis	8.615	1.439	0.000’



Graph 2: Error bar for number of oral neutrophils

The present study showed an increasing count from healthy to severe periodontitis group. Our results are in accordance with the investigation by M. Landzberg *et al.* in 2014.[3] They also obtained an increase in oral neutrophil count from healthy group to severe periodontitis group.

In the study by Landzberg *et al.*,[3] there was a marked difference in the orogranulocyte counts between groups of mild periodontitis and moderate periodontitis. This is contrary to the results that we obtained as they did not show much difference between these groups. The possible explanation for this difference may be that there was not much difference in the total number of periodontal pockets among the participants between these two groups in our study and there would have been considerable variations in the Landzberg *et al.* research.[3]

This study reiterates the relevance in quantifying oral neutrophils using a single, rapid, non-invasive oral rinse assay. The facility to perform rapid, accurate quantification of oral neutrophils may be an important diagnostic tool in the study of oral diseases, such as periodontitis. Longitudinal studies with larger samples sizes will allow us to determine if oral neutrophil levels can be used to monitor susceptibility to periodontal diseases. The oral neutrophil levels will be an indicator for the presence of an active periodontal infection. This may guide the clinician to make a more comprehensive periodontal examination, which may help in early diagnosis of a condition and intervention. This tool can be used as a routine diagnostic tool, and early diagnosis of as yet identified oral conditions may be possible. This may pave way for early referral and management of oral diseases.

CONCLUSION

A positive correlation between the oral neutrophil counts and severity of the periodontal disease was noted in the study. Oral neutrophil counts increased across the groups of healthy, gingivitis, mild periodontitis, moderate periodontitis, and severe periodontitis in an ascending order, which correlated with the disease severity.

It can be concluded that the assessment of the oral neutrophil count utilising a standardised oral rinse is an easy and non-invasive method of determining the oral inflammatory load. Further long-term studies with a large sample size assessing the progression and outcome to therapy in periodontal disease patients are needed.

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Conflicts of interest

There are no conflicts of interest.

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