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Estimation of Oral Neutrophil Counts Following Periodontal Therapy in Patients with Chronic Periodontal Disease Utilizing a Standardized Oral Rinse - Interventional Study

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Abstract

Background: Orogranulocytic Migratory Rate (OMR) is defined as the amount of neutrophils move into the oral cavity, through the gingival sulcus. When inflammatory changes occur in the oral cavity, there is an increase in the level of orogranulocytic migratory rate. The oral neutrophil count may be an indicator of the periodontal health status and also it correlates with the severity of periodontal disease. The aim of the study was to assess the relationship of oral neutrophil counts with severity of chronic periodontal diseases and to estimate their levels in response to phase 1 periodontal therapy. M**aterials and Methods:** A total of 66 subjects were selected from the Departments of Periodontics, Annoor Dental College and Hospital, Kerala, India. The subjects were divided into three groups of 22 subjects in each group with two visits. The groups were diagnosed on the basis of gingival index and the number of periodontal pockets. Oral rinse samples of subjects were taken before and 4 weeks after periodontal treatment ((phase 1). Acridine orange was used to stain the cells in the rinse samples. A fluorescence microscope and hemocytometer was used to count neutrophils. **Results:** There was significant difference in mean value of oral neutrophils before and after treatment for control, moderate periodontitis and severe periodontitis groups. **Conclusion:** The oral rinse assay was capable to identify a major decrease in oral neutrophil counts in patients who reacted to periodontal treatment (phase 1 therapy). This method is safe, easy, reliable and noninvasive method of quantification of oral neutrophils.

Keywords: Acridine Orange, Fluorescence Microscope, Hemo Cytometer, Oral Neutrophils, Periodontitis

1. Introduction

'Periodontitis can be defined as an inflammatory disease that results in the destruction of the tissues of tooth support, without treatment lead to tooth loss'. The inflammatory disease is originated by oral bacterial biofilms that develop in the soft tissue pockets between the gingival and the roots of the teeth. Neutrophils are a key host cells in defense against invading microorganisms, and play role in both acute and chronic inflammation[1]. It plays a pivotal role in the resolution of microbial infections. Neutrophils are continuously entering the mouth through the gingival sulcus and their role in the oral cavity is to control the presence of oral bacteria[2]. Poly-Morphonuclear Neutrophils (PMNs) are the most abundant leukocytes in humans, accounting for 50–70% of all circulating white[3] blood cells. Neutrophils also release products which are toxic to the host and play a large role in the breakdown of periodontal tissues seen in periodontitis[4]. The normal range of circulating PMN levels is increased in infectious diseases known as neutrophilia (mainly acute bacterial infections). As a result, neutrophil counts are used to recognize patients with infections[5]. Within the human mouth, space between tooth surface and free gingiva (gingival crevice) appear to be the only tissues that deal the path for polymorphonuclear granulocytes. It migrate into the mucous secretion, that covers all free surfaces of the oral cavity. In the mucus, polymorphonuclear granulocytes is related to the rate of the movement of polymorphonuclear leukocytes

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through the walls of capillaries and in to the tissues named leukopedesis. Increase in the rate of leukopedesis leads to the formation of a cellular infiltrate and also to an increase in the rate of granulocytic migration; in the presence of an inflammatory reaction in the periodontal tissues. In the oral mucus the living polymorphonuclear granulocytes have been considered as "Oro-Granulo-Cytes" (OGC's). It is estimated that 30,000 PMNs transit through periodontal tissues every minute and that their presence in the gingival crevicular fluid is physiologic[6]. In the collected mucus, the number of OGC's can be determined by counting the cells. The aim of our study was to assess the relationship of oral neutrophil counts with severity of chronic periodontal diseases and to estimate their levels in response to phase 1 periodontal therapy. The purposes of this study was to confirm the efficacy of a non-invasive, single standardized oral rinse to allow the convenient quantification of oral neutrophils. To study the association between oral neutrophil levels and periodontal disease severity and to evaluate the efficacy of using oral neutrophil counts to monitor effectiveness of therapy and periodontal disease status. The hypothesis of our study was to decrease in the oral neutrophil count in patients with chronic periodontitis after phase 1 therapy.

2. Materials and Methods

The present interventional study was conducted, after institutional ethical committee approval (Ref no – 016-A/09), on 66 patients drawn from department of Periodontics, Annoor Dental College and Hospital, Kerala, India. The oro-granulocyte evaluation was done in the Department of Oral Pathology and Microbiology, Annoor Dental College, Kerala, India during February to October 2018 and duration of study was 9 months.

2.1 Sampling Criteria

The patients attending to the Department of Periodontology were first informed about the study and after obtaining the written consent. They were tested to wash out with 10 milliliter of 0.9 percent saline for 30 seconds and then to expectorate in a paper cup. Following this, periodontal examination was carried out to determine whether the subject would be included in our study. The selected subject's rinse samples were utilized for neutrophil evaluation and the others discarded.

2.2 Sampling Method

Test group patients with chronic periodontitis which can be sub divided into two group's as[7]:

1. Moderate periodontitis – (ten or less pockets >5mm deep)
2. Severe periodontitis - (more than ten pockets >5mm deep)

Control group: Healthy patients with no clinical signs of periodontal disease.

The inclusion criteria of the study firstly included both male and female patients and secondly included systemically healthy individuals. The exclusion criteria of the study included the patients with systemic diseases that could affect immune function for example HIV, diabetes and haematological disorders. Secondly, patients with oral pathological conditions such as rampant caries, chronic apthous ulcers, tonsillitis, etc other than periodontal disease.

2.3 Sample Size

The sample size was calculated using statistical package G*Power (3.1.5). The sample size for this study with three groups and two visits each group under 5% error and 80% power was calculated. Thus the sample size was obtained as 22 per group; therefore the total sample size considered for this study with 3 groups was 66. There was no bias and drop of study noted in our study.

2.4 Study Method

2.4.1 Collection of Oral Neutrophils

The selected samples expectorate was taken in to a 15ml calibrated test tube, which was centrifuged at 2500 RPM for 5 minutes and the supernatant was removed using a 5-mL pipette. The pellet (group of cells) which was remaining at the bottom of the calibrated test tube after removing the supernatant was suspended in 5 mL of Hank's Balanced Salt Solution (HBSS). One milliliter of this suspension was removed from the test tube. 4 Microliter of acridine orange (a fluorescent nucleic acid marker) was added to one millilitre of this solution to stain the oral Neutrophils. The stain can accelerate the solution. It allows neutrophils to be distinguished from other cells using fluorescence microscope. A ten microliter aliquot of this suspension was then measured on a hemocytometer and the oral polymorphonuclear neutrophils were counted visually using fluorescence

microscope. Neutrophils were counted and quantified based on the standard protocol for haemocytometer use. Neutrophils were counted in sixteen grids. From each corner of the hemocytometer for each sample and were easily distinguished from other cells and debris by the characteristic fluorescent staining of their multi lobulated nuclei. C-MOS Camera was used to capture the image of the cells. IS- Capture image software was used to transfer it to a laptop. A study done by Landzberg *et al.*[3] stated that cell concentration per ml is equal to total cell count in 4 squares x 2500 x dilution factor.

2.4.2 Collection Frequency

To provide a baseline measurement, the selected samples expectorate was taken, before starting any treatment. After 4 weeks following phase 1 therapy during the re-evaluation examination, a second oral rinse sample was collected.

3. Statistical Analysis

This study deals with testing whether there is any significant difference in mean values of oral neutrophils, gingival index and number of pockets before and after treatment for control, moderate periodontitis and severe periodontitis groups. Paired t-test was used for the analysis and error bars were drawn to visually understand the difference. In all the analysis significance level was taken to be 0.05, and the tests are two-tailed. Statistical Analysis was carried out using statistical package, SPSS (version 22.0.0.0).

4. Results

Table 1 represents the difference of age among the groups and as well as the association of gender with groups. The mean value for age was found to be highest for severe groups followed by moderate and control groups. Table 2 depicts one way ANOVA test. It shows a significant difference in mean value of age among the three groups. No significant association of gender with the study groups was found in this test. Table 3 pair-wise comparison was conducted and observed that mean value of age was different for control and moderate, control and severe groups. There was no difference in moderate and severe groups. Graph 1 an error bar was drawn to compare the mean value of each group and represents the variability of the data. The point rounded in the Graph 1 shows the mean values and the length of the bar represent the confidence interval (95%). These graphs also help us to compare mean values of 2 groups visually. If they are significantly different, they will not overlap. Graphs 2 show the bar chart of gender groups. Table 4 shows that difference in oral neutrophils before and after treatment and observed that there are zero oral neutrophils for control group after the treatment, for other groups there was a reduction in the mean number of oral neutrophils and also paired t – test observed that there was significant difference in mean value of oral neutrophils before and after treatment for control, moderate and severe groups. Graph 3 represents the error bars. It can be observed that the bars are not overlapping with the value zero. Table 5 shows difference in gingival index before and after treatment and it can be observed that there was a reduction in the mean value of gingival index after the treatment and also paired t test observed that there was significant difference in mean value of gingival index before and after treatment for control, moderate and severe groups. Graph 4 the error bar observed that the bars are not overlapping with the value zero. Table 6 shows that difference in number of pockets before and after treatment. The analysis was not done for control group as there were no pockets in that groups and also paired t test observed that there was significant difference in mean value of number of pockets before and after treatment for moderate and severe groups. In Graph 5, the error bars observed that the bars are not overlapping with the value zero Table 7 shows that comparison of oral neutrophil, gingival index and number of pockets before and after treatment and also paired t test suggests that there was significant difference among all the pairs.

Table 1. Descriptive statistics of age among the groups and frequency distribution of subjects by gender

	N	M	SD	SE	Female	Male	Total
Control	22	26.818	10.098	2.153	13	9	22
Moderate	22	44.000	11.468	2.445	11	11	22
Severe	22	47.136	11.465	2.444	14	8	22
Total	66	39.318	14.103	1.736	38	28	66

N - number, M - mean , SD – standard deviation, SE – standard error.

Table 2. Depicts the mean value of age and gender among the three groups

	SS	df	MS	F	Sig.	Value	df	Asymp. Sig. (2-sided)
Between Groups	5264.455	2	2632.227	21.638	.000	0.868	2	.648
Within Groups	7663.864	63	121.649					
Total	**12928.318**	**65**						

MS - Mean Square, SS - Sum of Squares.

Table 3. Pair-wise comparison of mean value of age among three groups.

(I) Group	(J) Group	MD (I-J)	SE	Sig.
Control	Moderate	-17.182	3.326	0.000
	Severe	-20.318	3.326	0.000
Moderate	Control	17.182	3.326	0.000
	Severe	-3.136	3.326	0.349
Severe	Control	20.318	3.326	0.000
	Moderate	3.136	3.326	0.349

MD - Mean Difference, SE - Standard Error.

Table 4. Depicts oral neutrophil counts among three groups before and after treatment

Group	ON	M	N	SD	SEM	t	df	Sig. (2-tailed)
Control	Before	7.909	22	6.171	1.316			
	After Before –After	4.545 3.364	22	4.896 2.036	1.044 0.434	7.747	21	0.000
Moderate	Before	173.591	22	214.488	45.729			
	After Before –After	33.000 140.591	22	49.715 172.546	10.599 36.787	3.822	21	0.000
Severe	Before	530.182	22	389.486	83.039			
	After Before –After	190.500 339.682	22	230.376 192.823	49.116 41.110	8.263	21	0.000

ON – Oral Neutrophil, M - Mean, N – Number, SD – Standard Deviation, SEM - Standard Error Mean.

Table 5. Depicts gingival index among three groups before and after treatment

Group	GI	M	N	SD	SEM	t	df	Sig. (2-tailed)
Control	Before	1.315	22	0.627	0.134			
	After Before –After	0.566 0.750	22	0.287 0.463	0.061 0.099	7.587	21	0.000
Moderate	Before	1.438	22	0.454	0.097			
	After Before –After	0.901 0.537	22	0.310 0.319	0.066 0.068	7.903	21	0.000
Severe	Before	1.743	22	0.327	0.070			
	After Before –After	1.214 0.529	22	0.320 0.387	0.068 0.082	6.409	21	0.000

GI – Gingival Index, M - Mean, SD - Standard Deviation, SEM – Standard Error Mean.

Table 6. Depicts number of pockets among two groups

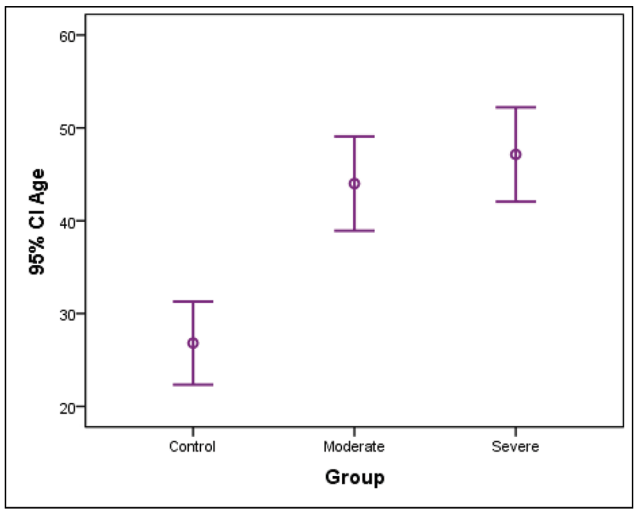
Group	GI	M	N	SD	SEM	t	df	Sig. (2-tailed)
Moderate	Before	15.773	22	13.582	2.896			
	After Before –After	4.273 11.500	22	6.017 8.279	1.283 1.765	6.515	21	0.000
Severe	Before	56.864	22	27.634	5.892			
	After Before –After	36.864 20.000	22	30.784 10.151	6.563 2.164	9.241	21	0.000

GI - Gingival Index, M - Mean, N - Number, SD - Standard Deviation, SEM - Standard Error Mean.

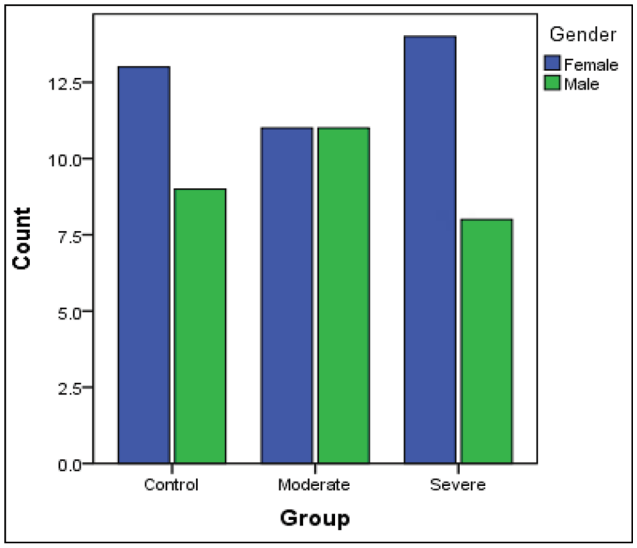
Table 7. Depicts the comparison of gingival index, number of pockets, oral neutrophils count

	Mean	N	SD	SEM	t	df	Sig. (2-tailed)
ON [Before]	237.227	66.000	334.818	41.213			
GI [Before]	1.499	66.000	0.511	0.063			
ON [Before] – GI [Before]	235.729		334.658	41.194	5.722	65	0.000
ON [Before] PP [Before]	237.227 24.212	66.000 66.000	334.818 29.827	41.213 3.672			
ON [Before] – PP [Before]	213.015		310.216	38.185	5.579	65	0.000
ON [After]	76.015	66.000	157.303	19.363			
GI [After] ON [After] – GI [After]	0.893 75.122	66.000	0.402 157.126	0.050 19.341	3.884	65	0.000
ON [After]	76.015	66.000	157.303	19.363			
PP [After] ON [After] – PP [After]	13.712 62.303	66.000	24.353 137.134	2.998 16.880	3.691	65	0.000

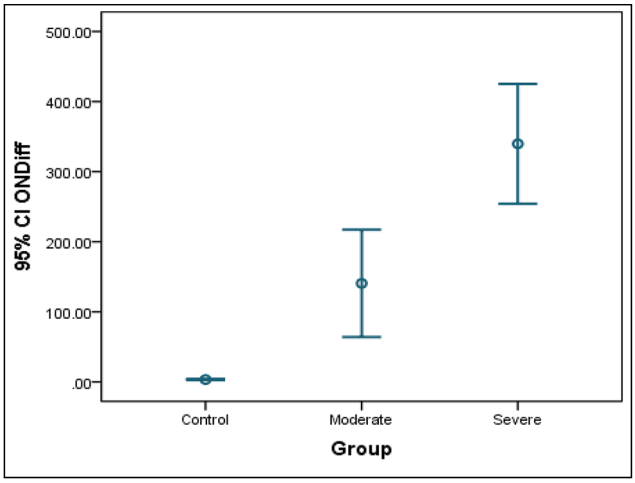
ON - Oral Neutrophil Count, GI - Gingival Index, PP - Periodontal Pocket, N - Number, SD - Standard Deviation, SEM - Standard Error Mean.



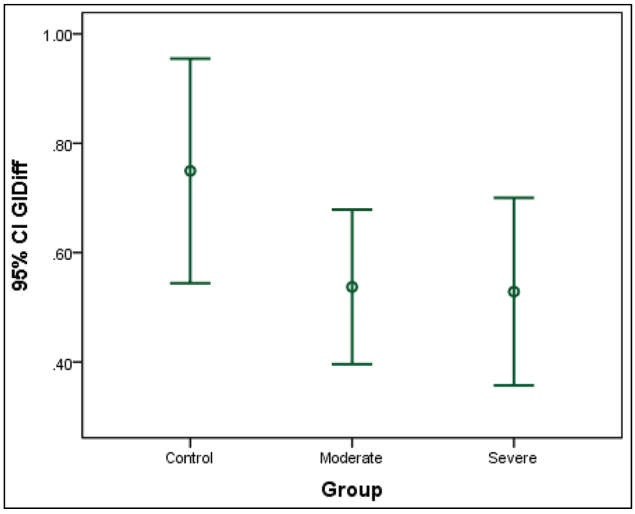
Graph 1. Error bar was plotted with the mean value of age against the three groups.



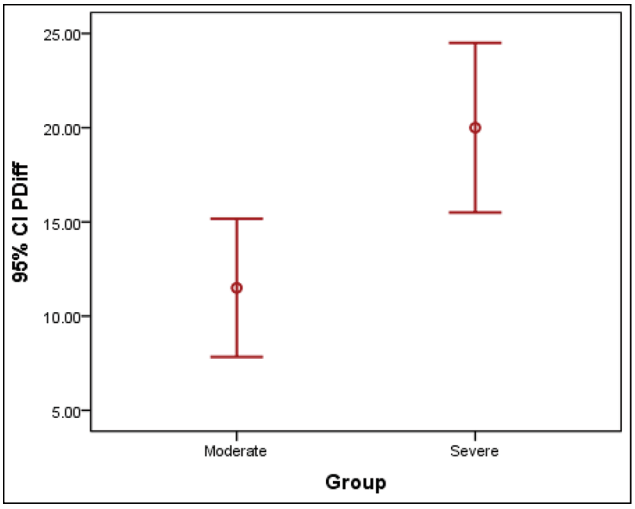
Graph 2. Bar chart shows gender among the three groups.



Graph 3. Error bar shows oral neutrophil counts among three groups.



Graph 4. Error bar depicts gingival index among three groups.



Graph 5. Error bar depicts no: of pockets among two groups.

5. Discussion

Neutrophils are a vital component of the periodontal host response. Neutrophils denote the majority of the leukocytes move to the gingival crevice specially space between the tooth surface and the free gingival to the associated biofilm[8,9]. From the gingival plexus of blood vessels neutrophils exit and enter the crevice (via the gingival junctional epithelium which under inflammatory conditions)[10]. To prevent bacterial invasion into the underlying tissues of periodontal pocket, neutrophils form a defense wall against the tooth associated biofilm[11]. The function of the neutrophil is related to its ability to engulf and destroy microorganisms. 'OMR is the rate at which neutrophils migrate through the gingival sulcus in to the oral cavity.' OMR is greater in the occurrence of gingival inflammation. The first attempts to correlate OMR with gingival health were made by Klinkhamer (1968)[11]. He put forward the technique of rinsing the mouth with hypertonic saline and examining the collected samples. The saline controlled the shrinkage of the leukocytes and making them healthier and did not damage the gingival tissues. He described an Oral Migratory Rate Index (OMRI) as an index of gingival health taking into account the number of teeth present. Skougaard *et al.* (1969)[12] found an affirmative correlation between OMR and the Gingival Index (GI). In our study on comparing the oral neutrophil count and gingival index before and after treatment in control, moderate periodontitis and severe periodontitis groups (Table 7), showed that there was major difference in mean value between oral neutrophil count and gingival index among three groups.

In our study, Table 4 shown that there was significant difference in mean value of oral neutrophils before and after treatment for control, moderate periodontitis and severe periodontitis groups. Our results are in accordance to the study by Bender *et al.* (2006)[13]. He proved that a major change was noticed in pretreatment oral neutrophil counts between healthy control groups and periodontal diseases. Patients who reacted positively to periodontal treatment confirmed a forty-three percent decrease in

oral neutrophil counts compared with their pretreatment levels and patients who did not react to periodontal treatment revealed no major change in oral neutrophil levels.

A study done by Wool Weaver *et al.* (1972)[14] proposed that increased periodontal pocket depth provide a greater surface area for migration of leukocytes. And therefore the oral migratory rate would increase. But the relationship between oral migratory rate and pocket depth may be for the reason that patients with increased pocket depth generally have increased gingival inflammation. In our study, Table 6 compared the number of periodontal pockets before and after treatment in moderate periodontitis and severe periodontitis groups, showed that there was significant difference in mean value of number of pockets among two groups. The statistical analysis was not done for control group as there were no periodontal pockets in this group.

Huda *et al.* (2015)[15] suggested an association between periodontal disease and adverse birth outcome. 63 pregnant females were recruited and 15 seconds saline rinses were collected to measure oral inflammatory load as represented by counts of oral neutrophil levels. To determine the extent of the correlation between the periodontal pockets and clinical attachment loss to the oral inflammatory load periodontal examinations were performed. Oral neutrophil counts found in patients with periodontitis had statistically showed that there was a significant increase when compared with those without periodontitis. In our study we evaluated the periodontal pockets, oral neutrophil count, and gingival index. There was statistically significant reduction in periodontal pockets, oral neutrophil count and gingival index in moderate periodontitis and severe periodontitis groups. The statistical analysis was not done in control group as there were no pockets in this group.

Rijkschroeff (2017)[16] reported that edentulous mouth might be associated with a loss of oPMNs since these cells enter the oral cavity through the gingival crevices. The aim was to examine the numbers of oPMNs in rinse samples. Samples were obtained from 21 edentulous and 20 dentate subjects. When compared with dentate subjects the edentulous subjects with lower oPMN counts and higher percentages of apoptotic oPMNs. In our study Edentulous subjects wearing full dentures are excluded and partial dentures or dentate subjects were taken and the numbers of oPMNs in rinse samples obtained from periodontitis groups were higher compared to control group.

Nicu (2018)[17] aimed to describe the oPMNs numbers and function, in patients with untreated periodontitis and compare those to cells from subjects without periodontitis. Venous blood and oral rinse samples were obtained from 19 patients with untreated periodontitis and 16 control subjects for PMN isolation. Periodontitis patients presented with over 4 times higher oPMN counts compared to controls subjects. In our study only oral rinse samples were obtained from periodontitis and control group. There was statistically significant reduction in oral neutrophil count in moderate periodontitis and severe periodontitis groups. The statistical analysis was not done in control group as there were no pockets in this group.

Padhye and Padhye (2019)[18] done a study to quantify and compare the impact of smoking status on oPMN and Malon-Di-Aldehyde (MDA) in individuals with Chronic Periodontitis (CP) before and after scaling and root planning. 66 patients divided into 4 groups, group A-periodontally healthy, B-Current smokers with CP, C-former smokers with CP, D-nonsmokers with CP. Greater periodontal destruction is seen in current smokers than former and nonsmokers with CP. MDA can be measured as a consistent biomarker for oxidative stress and directly related to oPMN levels. In our study, we assess the relationship of oral neutrophil counts with severity of chronic periodontal diseases and to estimate their levels in response to phase 1 periodontal therapy. The study concluded that oral rinse assay was capable to detect a major decrease in oral neutrophil counts in patients who responded to periodontal treatment.

Naiff *et al.* (2020)[19] aimed that in severe periodontitis patients, whether periodontal treatment improves the phagocytic function of blood monocytes. In the study 55 participants was enrolled and divided in to two groups, control includes healthy subjects without periodontal disease and second group includes patients with periodontitis. Phase 1 therapy (scaling and root planning) was performed until the clinical resolution of inflammation and periodontal pockets. Study concluded that phase 1 therapy in patients with severe periodontitis offers a state of homeostasis due to the reestablishment of phagocytic function. Our study established that oral rinse assay was capable to distinguish a major decrease in oral neutrophil counts in patients who responded to periodontal treatment.

The limitations of our study may be the sample size assessed which was comparatively small for each groups. Larger sample sizes are necessary to confirm the exact correlation between oral neutrophil counts and

periodontal diseases. An additional limitation was that oral neutrophil count levels were not compared with that of the circulating neutrophil levels which is a conventional and time tested method used by medical practitioners. In the future**,** research is required to evaluate the potential relationship between oral PMN counts derived from the saliva and systemic measures of inflammation. It is the inflammatory and host responses, and not the clinical signs of periodontitis that are currently used to assess gingival health status, which are associated with systemic conditions, and could be useful in identification of patients susceptible to the inflammatory disorder.

6. Conclusion

Our study demonstrates that it is possible to collect and quantify oral neutrophils using a rapid, single, noninvasive, economical oral rinse test and also confirmed that the level of oral polymorphonuclear neutrophils is a good sign of oral inflammatory load and periodontal diseases status. The study concluded that oral rinse assay was capable to detect a major decrease in oral neutrophil counts in patients who responded to phase 1 therapy. Clinical parameters such as gingival index and number of pockets used in this study had significant reduction after phase 1 therapy. In our study oral PMNs were examined in a group of orally and systemically healthy dentate individuals. Limited knowledge exists concerning PMN functionality in the partial dentate oral cavity. Long term studies with a larger sample size evaluating the progression and outcome to therapy in periodontal disease patients are required to confirm the results of this study. Future will focus on determining whether this assay can be used to screen periodontal health in patients with neutrophil-related conditions.

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