Original Article

Quantification and Comparison of Salivary Neutrophils in Periodontal Health and Disease

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Abstract

Background: Neutrophils continuously migrate into the oral cavity from various sources like gingival crevicular fluid and saliva both in health and in inflammation. The migration of the neutrophils into the various tissues and into the oral cavity occurs when the host microbial interplay tips the balance favoring the initiation of the inflammatory and immune reactions which depending on the amount of the microbial load results in the development of acute and chronic infections in the susceptible host. **Aim:** The present study was designed to quantify and compare the oral salivary neutrophil levels in patients with gingivitis and chronic and aggressive periodontitis as well as in healthy controls, before and after scaling and root planing (SRP) and to compare the difference within the selected study groups. **Materials and Methods:** Forty subjects were classified into four groups, that is, healthy controls, gingivitis, and chronic and aggressive periodontitis. Oral rinse samples were collected using Hank's balanced salt solution from each patient before and after phase I periodontal therapy. Cells in the rinse samples were stained with Acridine orange, and neutrophil counts were carried out using a fluorescence microscope and a hemocytometer. **Results:** Baseline oral salivary neutrophil levels were maximum in the chronic periodontitis group followed by the aggressive group and then the gingivitis group. Oral salivary neutrophil levels also positively correlated to probing pocket depth, plaque index, calculus index, and gingival index in all four study groups. Maximum reduction in the oral salivary neutrophil levels after phase I periodontal therapy was seen in the gingivitis group. **Conclusion:** From our study, we conclude that the oral salivary neutrophil levels decreased significantly after SRP. Estimation of changes in the oral salivary neutrophil levels has the potential to aid in monitoring treatment outcomes. Thus, it suggests that it could be used as a simple, noninvasive laboratory technique to monitor the periodontal status and disease progression.

Keywords: *Neutrophil, oral rinse, orogranulocytic migratory rate, periodontal disease, saliva*

Introduction

In absolute health, the pristine gingiva shows no inflammatory infiltrate. But, the clinically healthy gingiva features an inflammatory infiltration, predominantly neutrophils, and as the inflammatory process progresses, lymphocyte infiltration may occur as a response to microorganisms.[1]

The initial challenge posed by the microorganisms in the oral cavity is efficiently dealt with by the

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defense mechanisms. These mechanisms involve the gingival epithelium which acts as a physical barrier, as well as gingival crevicular fluid and saliva which contains various proteins, antimicrobial molecules, and polymorphonuclear leukocytes (PMNs) which can lyse the bacteria directly or together with antibodies.[2]

It is a well-known fact that neutrophils continuously migrate into the oral cavity from various sources like gingival crevicular fluid and saliva both in health and in inflammation.[1]

Gingivitis occurs as an initial inflammatory response to periodontal pathogens. This may further progress to periodontitis or remain the same depending upon the microbial activity and the hosts' response. The gingival changes can be characterized by the enhanced migration of the neutrophils into the gingival tissues as well into the oral cavity as an inflammatory response.[3]

The progression of periodontitis from gingivitis is marked with the increase in the number of PMNs. The chronicity of periodontitis further leads to the shift in the cells to lymphocytes and plasma cells as a response to the tissue-invasive microorganisms.[3,4]

The neutrophils which form the first line of defense, in fact, act against the microorganisms to a certain extent, beyond which, the neutrophils cause damage to the host tissues as seen in aggressive periodontitis. The defective functioning of neutrophils is thought to be one of the main reasons for severe loss of support to the teeth in aggressive periodontitis. But it is now believed that it is due to the hyper-responsiveness of the neutrophils rather than their defective functioning.[5,6]

When host-microbial interactions tip the balance in favor of the initiation of inflammatory and immune responses, neutrophil migration into various tissues and the oral cavity occurs. Depending on the microbial load, this leads to the development of acute and chronic infections in the susceptible host.[7]

Inflammatory chemical mediators such as Interleukin 8 (IL-8) and Intercellular Adhesion Molecule 1 act as chemoattractants and regulate the neutrophil migration into the gingival sulcus through the junctional epithelium, thereby influencing neutrophil migratory rate into the oral cavity.[8]

The rate of migration of the leukocytes into the oral cavity has been estimated by Klinkhamer and others[9,10] in health and diseased conditions. The variations in the levels of leukocytes in saliva have been positively correlated to periodontal health, gingivitis, and periodontitis, thereby signifying the role of orogranulocytic migratory rate in the assessment of disease severity and as well the importance of quantification of oral salivary neutrophil levels in understanding periodontal disease progression.[9-11]

In light of the above facts, the present study is designed to quantify and compare the oral salivary neutrophil levels in periodontal health and disease.

Materials and Methods

Forty patients attending the Department of Periodontics, College of Dentistry were included in the study. All participants were otherwise systemically healthy (no added systemic illness, as per medical history assessment/interview) and had not undertaken periodontal therapy within the last 6 months of the precise study assessment. Both male and female, aged between 18 and 45 years, having a minimum number of 24 teeth were selected and classified based on clinical parameters such as plaque index, calculus index, gingival index, bleeding index, probing pocket depth, clinical attachment loss, and bone loss into four groups viz., healthy controls, gingivitis, chronic periodontitis, and aggressive periodontitis.

Medically compromised patients and those on medications (e.g., corticosteroids, chemotherapeutic agents, anticancer drugs, and immune modulators), smokers, pregnant, and lactating women were excluded from the study, and any oral desquamative conditions or diseases. The recruited patients were divided into four groups of 10 each as assigned below:

Group 1 (healthy control) consisted of 10 patients with no signs of any form of gingival or periodontal disease. The gingival was characterized clinically by its pink color, firm consistency, and scalloped margins. The interdental papillae were firm, did not bleed on gentle probing, and filled the space below the contact areas. The gingival exhibited a stippled appearance and there was a knife-edge margin between the tooth and soft tissues.

Group 2 (gingivitis) consisted of 10 patients who demonstrated gingival inflammation but with no demonstrable loss of attachment. Clinical findings included erythema, edema, bleeding, sensitivity, tenderness, and an increase in the size of the gingiva in ≥20 teeth. Radiographic analysis and/or probing attachment levels did not indicate loss of supporting structures in any of the teeth.

Group 3 (chronic periodontitis) consisted of 10 patients who have chronic periodontitis manifested by ≥5 mm deep probing pocket depths in at least four teeth at six different sites with radiographic evidence of bone loss.

Group 4 (aggressive periodontitis) consisted of 10 patients who were diagnosed with aggressive periodontitis with American Academy of Periodontology 1999 classification criteria, that is, except for the presence of periodontitis, patients were otherwise systemically healthy, showing severe attachment loss and bone destruction, with a limited amount of microbial deposits inconsistent with disease severity.

Periodontal status evaluation

Clinical parameters assessed for the study were plaque index,[12] calculus index,[2] gingival index,[13] bleeding index,[14] probing pocket depth, and clinical attachment level.

All the measurements were made 1 day before the collection of the oral rinse at baseline and 6-8 weeks following the phase I therapy which includes patient education, motivation, oral hygiene instructions, and SRP.

All the patients received a complete extra-oral and intra-oral examination to help rule out oral infections, other than periodontal diseases, that might contribute to an elevated oral leukocyte count.

Procedure for the collection of oral rinse

Patients were prohibited from eating or drinking for at least 30 minutes prior to giving oral rinse samples to prevent clearance of neutrophils from the oral cavity, thereby avoiding the bias in the total neutrophils count. The estimations of neutrophils were made between noon and 2 p.m., to avoid the diurnal variation, of PMNs, in the orogranulocytic migratory rate.

Hank orogranulocytic migratory rate avoids the diurnal variation, ophils from the oral cavity, thereby avoiding the bias in the total and then to expectorate both samples directly into two 15 mL collecting tubes. Two millilitres of 37% formaldehyde were added immediately to the samples and the samples were stored at 4°C until required. All samples were analysed on the day of collection. To avoid contaminating the sample with blood, all collections were performed before any intraoral procedures were carried out.

Each selected patient, after collection of oral rinses, was subjected to thorough scaling and root planing and was recalled every 2 weeks for evaluation and reinforcement of oral hygiene. After 6 weeks from the time of completion of Scaling and Root Planing (SRP), a second oral rinse sample was collected post SRP and recording of all clinical parameters plaque index, calculus index, gingival index, bleeding index, probing pocket depth, and clinical attachment level of the study were recorded. The entire study was completed within 1 year.

Hank's Balanced Salt Solution, plus 2 mM calcium and 0.4 mM magnesium, was used for all rinses. Acridine orange [3,6-bis(dimethylamino) acridinium chloride hemi (zinc chloride salt)] (AO), a fluorescence lysosomal marker that gives a characteristic fluorescent staining pattern in neutrophils and helps differentiate them from other cells, was added to the rinse samples after rinse collection. Both the reagents were supplied by Sigma Aldrich Company.

Recovery and counting protocol for oral neutrophils

All cells in the sample were collected by centrifugation (18 g at room temperature). After decanting and discarding the supernatant, cell pellets were resuspended by pipette in 500 µL of Hank's Balanced Salt Solution. From this suspension, 250 µL of cells were stained with 4 µg of AO. The sample was incubated in the dark at room temperature for 15 minutes. To establish uniform suspensions following incubation, the samples were again resuspended by a pipette.

To facilitate accurate counting, the sample was diluted by a factor of 10 prior to counting. Neutrophils were counted visually by fluorescence microscopy using a hemocytometer. The cells observed were either neutrophils or epithelial cells. Neutrophils were easily identified by their size and characteristic multilobulated nucleus (which fluoresced) and were distinct from the larger epithelial cells. A total of 64 grids from both sides of the hemocytometer were counted for each sample.

Statistical methods

All statistical analyses were performed using IBM SPSS Statistics version 25.0 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY, USA: IBM Corp). Analysis of variance (ANOVA) was used to find the significance of study parameters between three or more groups of patients. Student's *t*-test (two-tailed, dependent) was used to find the significance of study parameters on the continuous scale within each group.

Results

A total of 40 subjects, 10 subjects in Group I (healthy control), 10 subjects in Group II (gingivitis), 10 subjects in Group III (chronic periodontitis), and 10 subjects in Group IV (aggressive periodontitis) were included in the study.

The age of patients who were included in the four groups ranged between 18 and 45 years with a mean age of 23.10 ± 2.99 years in group I, 23.00 ± 2.49 years in group II, 37.80 ± 4.71 years in group III, and 27.70 ± 3.40 years in group IV.

Table 1: Comparison of salivary neutrophils among participants

Salivary neutrophils	Group I	Group II	Group III	Group IV	*P*
Pre-SRP	77.2±22.91	149.70±28.75	267.50±30.85	194.20±24.99	<0.001**
Post-SRP	-	90.60±23.10	177.00±25.59	131.00±21.83	<0.001**
% Change	-	39.5%	33.8%	32.5%	-
P	-	<0.001**	<0.001**	<0.001**	-

**Statistically significant $P \leq 0.001$

Table 2: Comparison of Plaque Index between the groups

	Group I	Group II	Group III	Group IV	*P*
Pre-SRP	0.36±0.11	0.78±0.21	1.71±0.27	1.15±0.39	<0.001**
Post-SRP	-	0.43±0.11	0.57±0.23	0.58±0.18	0.127
% Change	-	44.9%	66.7%	49.6%	-
P	-	<0.001**	<0.001**	<0.001**	-

**Statistically significant $P \leq 0.001$

Table 3: Comparison of Calculus Index between the groups

Calculus index	Group I	Group II	Group III	Group IV	*P*
Pre-SRP	0.46±0.12	0.83±0.40	1.84±0.27	1.04±0.37	<0.001**
Post-SRP	-	0.01±0.04	0.33±0.16	0.19±0.17	<0.001**
% Change	-	98.8%	82.1%	81.7%	-
P	-	<0.001**	<0.001**	<0.001**	-

**Statistically significant $P \leq 0.001$

Table 4: Comparison of Gingival Index between the groups

Gingival index	Group I	Group II	Group III	Group IV	*P*
Pre-SRP	0.30±0.12	0.77±0.29	1.78±0.28	1.19±0.44	<0.001**
Post-SRP	-	0.34±0.09	0.71±0.16	0.49±0.15	<0.001**
% Change	-	55.8%	60.1%	58.8%	-
P	-	<0.001**	<0.001**	<0.001**	-

**Statistically significant $P \leq 0.001$

Group I consisted of two males and eight females, group II of eight males and two females, group III of six males and four females, and group IV of eight males and two females.

When compared among all four groups, the mean count of salivary neutrophils at baseline was maximum in group III with 267.50 ± 30.85, followed by group IV with 194.20 ± 24.99, group II with 149.70 ± 28.75, and group I with 77.2 ± 22.91. The mean count of salivary neutrophils was minimum in group II with 90.60 ± 23.10 as compared to 131.00 ± 21.83 in group IV and 177.00 ± 25.59 in group III at 6 weeks post-SRP. The percentage of change was found to be maximum in group II with 39.5% This was when followed by group III with 33.8% in group III and 32.5% in group IV. The mean change in the count of salivary neutrophils from

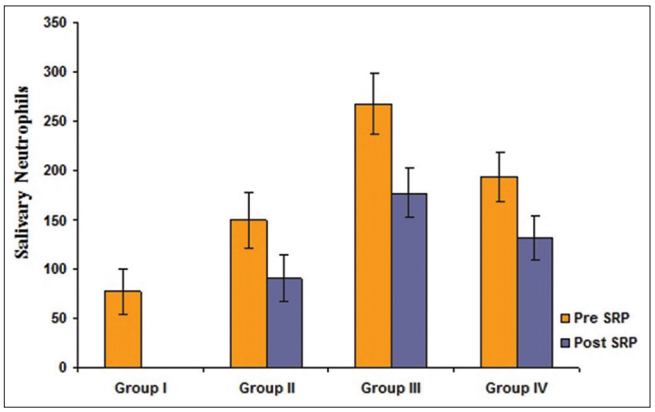

Figure 1: Comparison of salivary neutrophils levels, before and after SRP

baseline to post SRP is statistically significant $P \leq .001$ [Table 1 and Figure 1].

The mean plaque index in group III at baseline was 1.71 ± 0.27, decreased to 0.57 ± 0.23 after SRP, that is, at 6 weeks post SRP with a percentage change of 66.7%, followed by group IV with a percentage change of 49.6%, and group II with 44.9%. The minimum mean plaque index was found in group II with 0.43 ± 0.11 as compared to 0.57 ± 0.23 in group III, and 0.58 ± 0.18 in group IV. The decrease post-SRP is statistically significant [Table 2].

The mean calculus index was the highest with respect to group III with 1.84 ± 0.27, followed by group IV, II, and I at baseline. The least mean calculus index was seen in group II with 0.01 ± 0.04 followed by group IV with 0.19 ± 0.17, and group III with 0.33 ± 0.16 at 6 weeks post-SRP. But the maximum percentage change was seen in group II with 98.8% followed by group III and group IV post-SRP, respectively, and is statistically significant [Table 3].

The mean gingival index was the highest in group III with a mean of 1.78 ± 0.28 at baseline and a greater decrease in percentage was noticed in this group with a percentage change of 60.1%, as compared to the other groups at 6 weeks post-SRP. This was followed by group IV, which had shown a percentage change of 58.8% and group II with 55.8% and it is statistically significant [Tables 4 and 5].

The mean probing pocket depth in group III was the maximum with 3.87 ± 0.55 at baseline as compared to

Table 5: Comparison of Bleeding on probing between the groups

Bleeding on probing	Group I	Group II	Group III	Group IV	*P*
Pre-SRP	54.83±8.58	75.64±11.29	98.26±3.83	88.26±14.75	<0.001**
Post-SRP	-	51.31±10.27	65.09±14.74	64.81±15.73	0.079
% Change	-	24.33%	33.14%	23.45%	-
P	-	0.005**	0.005**	0.005**	-

**Statistically significant $P \leq 0.001$

Table 6: Comparison of Probing pocket depth between the groups

Probing pocket depth	Group I	Group II	Group III	Group IV	*P*
Pre-SRP	1.87±0.16	2.12±0.19	3.87±0.55	2.96±0.75	<0.001**
Post-SRP	-	1.86±0.08	2.81±0.35	2.52±0.69	<0.001**
% Change	-	12.26%	27.39%	14.86%	-
P	-				

**Statistically significant $P \leq 0.001$

2.96 ± 0.75 in group IV, 2.12 ± 0.19 in group II, and 1.87 ± 0.16 in group I. The mean probing pocket depth was 1.86 ± 0.08 at 6 weeks post-SRP in group II, which was the minimum Probing pocket depth (PPD) when compared to 2.52 ± 0.69 in group IV and 2.81 ± 0.35 in group III. The maximum percentage change was found in group III, that is, 27.39% as compared to 14.86% in group IV and 12.26% in group II, respectively. The difference in probing pocket depth post-SRP is statistically strongly significant [Table 6].

Discussion

The periodontal diseases are assessed at various levels of their clinical presentation with an array of conventional techniques involving clinical parameters and radiographs. Although the amount of loss of supporting tissues and the severity of the disease can be estimated, these conventional techniques cannot always measure the disease activity as periodontal diseases show a discontinuous pattern of exacerbation and remission. Additional investigations involving microbiological, genetic assays, and analysis of the host response using various biological markers from blood, saliva, and Gingival crevicular fluid (GCF) have been used in diagnosing, monitoring the disease activity, and in identifying the susceptible patients.[15]

PMNs play a major role in host defense against infections by phagocytizing the microorganisms. Systemic disturbances in the number or function of peripheral blood PMNs commonly result in significant oral complications[16] Gingival or crevicular PMNs have been comprehensively investigated, and their role in periodontal health and disease is well established.[17-19] It is already shown that in diseased conditions, there is an obvious increase in the number of neutrophils. The potential use of salivary neutrophils in periodontal disease assessment has been identified by many authors they have shown a relationship between the orogranulocytic migratory rate and periodontal disease.[4,9,11,20] Based on their findings, the present study was designed to quantify and compare the salivary neutrophils in health, gingivitis, chronic periodontitis, and aggressive periodontitis cases before and after SRP. The assessment of disease activity was done by comparing the oral salivary neutrophil levels in the study groups along with clinical parameters viz, plaque index, calculus index, gingival index, bleeding index, and probing pocket depths.

Group I had the lowest gingival index at baseline, which can be explained by the low plaque index scores. The gingival index for group III was maximum at baseline (1.78 ± 0.28). After SRP, at 6 weeks' maximum change in the gingival index was seen in group III followed by group IV and II with 60.1%, 58.8%, and 55.8%, respectively, and with minimum change in group II as compared to the healthy controls group. This can be explained by the fact that the gingival inflammatory changes observed were due to plaque and once plaque was eliminated by effective SRP, gingival inflammation and gingival index scores obviously reduced.[4,10,20]

Reduction of bleeding index scores after SRP was maximum for group III, that is, 33.14% followed by group II and IV as compared to group I. The intergroup difference in bleeding index scores is minimal at 6 weeks post-SRP and were comparable to group I at baseline. These results were in correlation with those shown by Raeste AM *et al.* 1978, in their studies.[9]

The probing pocket depth reduction was maximum in group III, that is, chronic periodontitis, with 27.39% followed by groups IV and II. The probing pocket depths were maximum in group III both at baseline and post-SRP, suggesting that only nonsurgical approach is inadequate and further periodontal therapy is required.

The probing pocket depth reduction in gingivitis group was comparable to that of the healthy controls, as it is a reversible condition.[4,20]

This overall improvement of the periodontal conditions and the reduction in probing pocket depths is in close agreement with findings from studies by Rosling *et al*. (1976a, b) and Isidor (1981). This observation is also in accordance with data presented by Badersten *et al*. (1984) in their studies.[21-24]

At baseline, the healthy control group had shown minimal oral salivary neutrophil levels compared to other groups. The presence of oral salivary neutrophils in this group which is free from periodontal disease can be attributed to the persistent bacterial presence in the gingival sulcus. This is reflected by the constant appearance of neutrophils and other mediators of the immune response in the gingival sulcus to maintain the gingival health.[1] Baseline oral salivary neutrophil levels were maximum for chronic periodontitis, that is, group III (267.50 ± 30.85), followed by aggressive periodontitis (Group IV) and gingivitis (Group II) with 194.20 ± 24.99 and 149.70 ± 28.75, respectively. This result is almost similar to the studies done by Raeste and Aura in 1979 and Bender *et al*. in 2006.[4,20] These high OMR counts indicate that the degree of inflammation is more in severe cases of gingivitis and periodontitis. In our study, the chronic periodontitis group had both increased levels of plaque and gingival index as compared to gingivitis and aggressive periodontitis groups. The oral salivary neutrophil levels in the aggressive periodontitis group are relatively low compared to the chronic periodontitis group. This is due to the relatively lower scores of PI, CI, GI, BI, and PPD as compared to the chronic periodontitis group. Also, the oral salivary neutrophil levels at baseline reflect the severity of periodontal disease.

In accordance with the study done by Raeste and Aura, and Bender, our study too showed a positive and statistically significant correlation between oral salivary neutrophil levels and pocket depth, that is, patients with deeper pockets showed increased levels of oral salivary neutrophils, thus indicating increased disease activity.[4,20]

After SRP, changes in levels of oral salivary neutrophils in all three groups was statistically significant, but the maximum change was seen in the gingivitis group, that is, 39.5%, followed by chronic periodontitis (33.8%) and then aggressive periodontitis (32.5%). This indicates that disease activity was reduced to a maximum after SRP in the gingivitis group than in the other two groups, as gingivitis is a reversible condition and does not involve deeper periodontal tissues, unlike chronic and aggressive periodontitis groups. The local factors and microbial load contributing to inflammation could be more efficiently removed in the gingivitis group, compared with chronic periodontitis and aggressive periodontitis groups. However, in aggressive periodontitis cases, host immune defects and the presence of tissue-invasive microorganisms could not be altered by SRP alone.[5,25]

In our study, the gingival index is positively correlated to the oral salivary neutrophil levels both at baseline and 6 weeks after SRP in all three groups. This is in accordance with the results shown by Bender *et al*. in their study.[20] This is due to the SRP which is successful in decreasing the disease activity in these groups owing to its association with plaque-induced inflammation and reduction of the bacterial load.[20]

A relatively minimal change in PPD is seen in the aggressive periodontitis group after SRP as compared to other groups. This could thus be explained due to the presence of tissue-invasive pathogens as shown by Carranza and Newman, which cannot be eliminated by SRP alone. This reinforces the rationale for further periodontal therapy. Similarly, the decrease in oral salivary neutrophil levels in the chronic periodontitis group after SRP was less as compared to the gingivitis group.

It is important to note that the patients who did not demonstrate a clinical improvement following SRP at the end of 6 weeks also failed to show a significant reduction in oral salivary neutrophil levels. This can be explained by the persistent presence of the neutrophils which reflects the ongoing inflammation and destruction of the periodontal tissues mediated by neutrophils and their products. But, however, the quantification and comparison of the oral salivary neutrophil levels in health and disease have its own limitations. Although elevated levels of neutrophils indicate inflammation in the oral cavity, it does not specifically demarcate the two forms of periodontitis, that is, chronic and aggressive due to the tissue-invasive microorganisms and the involvement of the deeper sites.

Conclusion

Oral salivary neutrophil levels are increased in gingivitis, chronic periodontitis, and aggressive periodontitis before SRP and its levels decreased significantly after SRP. Estimation of changes in the oral salivary neutrophil levels has the potential to aid in monitoring treatment outcomes. Thus, it suggests that it could be used as a simple, noninvasive laboratory technique to monitor the periodontal status and disease progression. Nevertheless, we could not differentiate chronic periodontitis and aggressive periodontitis based only on oral salivary

neutrophil levels due to the small sample size. Our study warrants the need for longitudinal studies to concur, whether the baseline changes in oral salivary neutrophil levels and after SRP can be used accurately to assess disease severity; its progression determine which patients are at risk and assess the effectiveness of the treatment.

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Conflicts of interest

There are no conflicts of interest.

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