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Novel rinse assay for the quantification of oral neutrophils and the monitoring of chronic periodontal disease

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Background and Objectives: The aim of this study was to develop a single, rapid, noninvasive oral rinse assay to enable the accurate quantification of oral neutrophils. Products released by neutrophils are partly responsible for the destruction observed in periodontitis. Quantification of oral neutrophil levels is important for understanding their role in periodontal diseases. Previous studies have relied on time-consuming serial rinses and cumbersome counting techniques for the collection and quantification of oral neutrophils.

Material and Methods: Patients with chronic periodontal disease provided rinse samples before and after phase I periodontal treatment. Cells in the rinse samples were stained with acridine orange, and neutrophil counts were carried out using a fluorescence microscope and a hemocytometer.

Results: This assay allowed us to detect a significant difference in pretreatment oral neutrophil counts between periodontal disease and healthy control groups ($p < 0.001$). Patients who responded favorably to phase I therapy demonstrated a 43% reduction in oral neutrophil counts compared with their pretreatment levels ($p = 0.019$). Patients who did not respond to phase I periodontal treatment showed no significant difference in oral neutrophil levels ($p = 0.39$).

Conclusion: Oral neutrophil levels, as determined by a rapid oral rinse, reflect the severity of periodontal disease and treatment response. A single, rapid, oral rinse assay is an effective means of collecting and quantifying oral neutrophil levels and may serve as an excellent research tool for further study of the role of neutrophils in periodontal diseases.

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Periodontitis is an inflammatory disease that results in the destruction of the tissues of tooth support and, without treatment, ultimately leads to tooth loss. Neutrophils provide crucial defense functions against invading micro-organisms and are a core element of innate immunity (1). However, although their primary role is protective, neutrophils release toxic products that are thought to be partly responsible for the destruction seen in periodontal disease (2,3). The human mouth has a constant bacterial presence that is kept under control, in part, by a continual influx of neutrophils from the surrounding periodontal tissues. Yet, despite the importance of neutrophils in the pathogenesis of periodontitis, a simple and rapid method for the

quantification of neutrophil levels in the oral environment has not been described.

Researchers began studying the numbers of leukocytes found in saliva more than 70 years ago. The majority of neutrophils in the oral cavity enter via the gingival crevice (4–6). Early studies revealed that saliva from patients with periodontitis contained a greater number of leukocytes than saliva from healthy patients (7,8). The rate at which neutrophils migrate through the gingival sulcus into the oral cavity [i.e. the orogranulocytic migratory rate (OMR)] is increased in the presence of gingival inflammation (9,10). The OMR also correlates with increased pocket depth and the gingival index. It is not sensitive to the presence of plaque, debris, or calculus (11,12). The idea of using neutrophil quantification to assess periodontal disease status and the effectiveness of therapy was first proposed by Raeste *et al.*, in 1978 (13). That study described an oral rinse assay for neutrophil counts, which was used in 81 patients with periodontal disease. The assay used a series of 12 sequential rinses to assess oral neutrophil levels. The study concluded that 'this test gives information about the severity of the inflammation'. A citation search (ISI Web of Science) reveals that this work has not been cited by any studies looking at the further use of this test as an assay for inflammation. One reason for abandoning this approach may be the impracticality of using 12 successive patient rinses and labor-intensive counting methods to determine oral neutrophil levels. However, the availability of oral neutrophils, and their involvement in periodontal disease, supports the need for a simple method of oral neutrophil quantification. Therefore, the objectives of this study were to (i) develop and validate a single, noninvasive oral rinse assay to enable the expedient quantification of oral neutrophils, (ii) utilize the assay to study the relationship between periodontal disease severity and oral neutrophil levels and (iii) use oral neutrophil levels to monitor periodontal disease status and effectiveness of therapy.

Materials and methods

Reagents

Hank's Balanced Salt Solution (HBSS), plus 2 mM calcium and 0.4 mM magnesium, was used for all rinses (University of Toronto Media Preparation services Toronto, Ontario, Canada). Acridine orange [3,6-bis(dimethylamino) acridium chloride hemi(zinc chloride salt)] (AO; Sigma Chemical, Burlington, Ontario, Canada), 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.

Study population and clinical protocol

Patients in this study were adults referred to the Periodontal Post-Graduate Clinic at the Faculty of Dentistry, University of Toronto, for the diagnosis and treatment of moderate-to-severe periodontal diseases. The study protocol was explained to each potential subject, and written, informed consent was obtained prior to the commencement of treatment. This study was approved by the Scientific and Ethics Review Boards at the University of Toronto. Patients who were diagnosed with moderate or severe adult periodontitis were enrolled (28 women and 25 men; mean age: 48 $\pm$ 1.7 years). Disease severity was categorized by the number of pockets recorded that were > 5 mm deep (moderate periodontitis: 10 or fewer pockets > 5 mm deep; severe periodontitis: more than 10 pockets > 5 mm deep) (14). All examiners were calibrated prior to the start of the study, and the same examiners completed pretreatment and post-treatment probings on a given patient. A medical history was taken and updated at each appointment to ensure that all patients were in good health. Patients with systemic diseases that could affect immune function/neutrophil response, such as diabetes, human immunodeficiency virus (HIV), and hematological disorders, were eliminated from the study. Furthermore, patients with oral pathological conditions other than periodontal disease (such as chronic apthous ulcers, tonsillitis, or rampant caries), which might contribute to elevated oral neutrophil levels, were not enrolled. One patient was dismissed from the study after being diagnosed with leukemia. The 14 control subjects (seven men and seven women; mean age: 33.7 $\pm$ 2.7 years) had no clinical signs of periodontal disease or any systemic health conditions that might influence oral neutrophil levels. Although the control group was, in general, younger than the disease group, an age-matched disease subgroup was used to verify that age was not a factor in oral neutrophil levels (data not shown).

All patients received a complete extra- and intraoral examination to help rule out oral infections, other than chronic periodontitis, that might contribute to an elevated oral neutrophil count. A specific periodontal examination was completed at the initial and re-evaluation appointments, and the following clinical parameters were obtained: probing depth (six points per tooth – University of Michigan 'O' probe), bleeding on probing, tooth mobility, furcation involvement, and recession. Each patient also received a full mouth series of radiographs; bone loss was graded as mild (≤ 2 mm of bone loss), moderate (up to 50% bone loss), or severe ($\geq$50% bone loss) (15).

Patients received the standard periodontal nonsurgical protocol used in the Graduate Periodontal Clinic at the Faculty of Dentistry, University of Toronto. Phase I therapy consisted of supra- and subgingival scaling and root planing for 2–4 h, and oral hygiene instruction. All patients received treatment from either a qualified hygienist or a periodontal resident in the Graduate Periodontal Clinic at the Faculty of Dentistry.

Collection of oral neutrophils

The protocol used to collect and count oral neutrophils was a modification of that of Wright *et al.* (16). Briefly, HBSS was measured into two 15-ml aliquots. Patients were asked to rinse with each aliquot for 30 s and then to expectorate both samples into one

50-ml Falcon Tube (Becton Dickinson, Franklin Lakes, NJ, USA). Two millilitres of 37% formaldehyde was added immediately and the sample stored at 4°C until required. All samples were analyzed on the day of collection. Patients were not allowed food or drink for a minimum of 30 min prior to providing oral rinse samples to avoid clearance of neutrophils prior to donations. In order to avoid contaminating the sample with blood, all collections were performed before any intraoral procedures were carried out.

Collection frequency

Oral neutrophils were collected, before commencing any treatment in the Graduate Periodontal Clinic, to provide a baseline measurement. A second oral rinse sample was collected 4–6 wks following phase I therapy during the re-evaluation examination. As a means of validating the oral rinse assay and the baseline oral neutrophil levels prior to treatment, some patients were asked to provide an additional rinse sample on a different day before commencing phase I treatment.

Recovery and counting protocol for oral neutrophils

All cells in the sample were collected by centrifugation (18 *g* at room temperature; Hettich Rotina 35R, Rare Scientific, Edmonton, Canada). After decanting and discarding the supernatant, cell pellets were resuspended by pipette in 500 µl of HBSS. 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 min. To establish uniform suspensions following incubation, the samples were again resuspended by pipette. To facilitate accurate counting, the sample was diluted by a factor of 10 prior to counting. Neutrophils were counted visually by fluorescence microscopy (Leitz Orthoplan Microscope, Wetzlar, Germany). 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 16 grids from both sides of the hemocytometer were counted for each sample.

Statistical analysis

The Student's *t*-test for paired comparisons was used to determine the significance of the difference between initial and re-evaluation rinse samples for diseased patients. The Student's *t*-test, single factor analysis of variance (ANOVA), and Tukey's studentized range test were used to determine the significance of the difference between control and diseased subjects. Linear and multiple regression analysis were used to determine the significance of the relationship between oral neutrophil count and other measures/modifiers of disease activity (probing depth, bleeding on probing, radiographic bone loss, smoking status, age, gender, and number of teeth). Total probing depth was expressed as a sum of all pocket depths that were > 5 mm for each patient. A p-value of < 0.05 delineated statistical significance. A standard sample size calculation determined that a minimum of 29 patients must complete phase I therapy for the difference between initial and re-evaluation oral neutrophil counts to be statistically significant when $\alpha = 0.05$, $\beta = 0.1$, $\delta = 1 \times 10^6$, and $\sigma = 1.8 \times 10^6$ (13,17).

Results

Rinse reproducibility

The reproducibility of a 30-s saline rinse to collect oral neutrophils was assessed by comparing the initial oral rinse neutrophil counts with a second rinse obtained at least 1 wk after the initial rinse but before starting any periodontal therapy ('before hygiene'). A Student's *t*-test for paired comparisons showed no statistically significant difference between the initial and 'before hygiene' neutrophil counts, verifying a stable baseline oral neutrophil level prior to treatment ($p = 0.69$, $n = 12$; Fig. 1). Furthermore, the control group's initial and re-evaluation neutrophil counts can also be used as a measure of this oral rinse assay's reproducibility. As the control subjects have no signs of periodontal disease, their oral neutrophil levels should remain constant. 'Re-evaluation' samples were obtained for six control subjects. There was no statistically significant difference between initial and re-evaluation levels (Student's *t*-test for paired comparisons, $p = 0.84$; Fig. 2). Immediate chemical fixation with paraformaldehyde of rinse samples preserved the cellular contents of the rinses, allowing accurate counting to be carried out several hours after collection (data not shown).

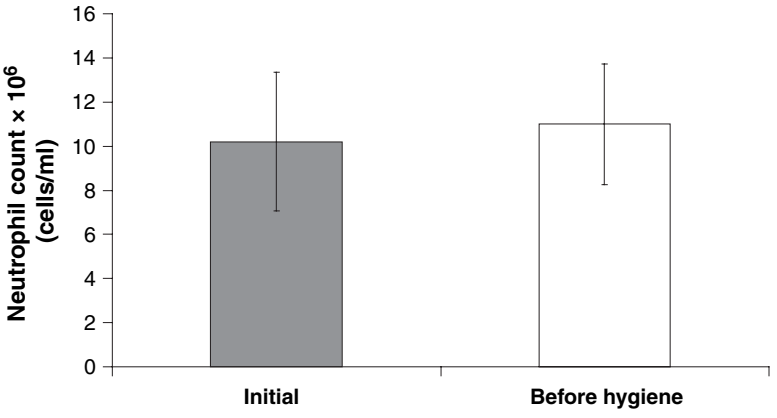

Fig. 1. Comparison of the mean initial neutrophil count with the 'before hygiene' neutrophil count in patients with periodontal disease. There was no statistically significant difference between 'initial' and 'before hygiene' counts (Student's paired *t*-test, $p = 0.69$, $n = 12$; mean ± standard deviation).

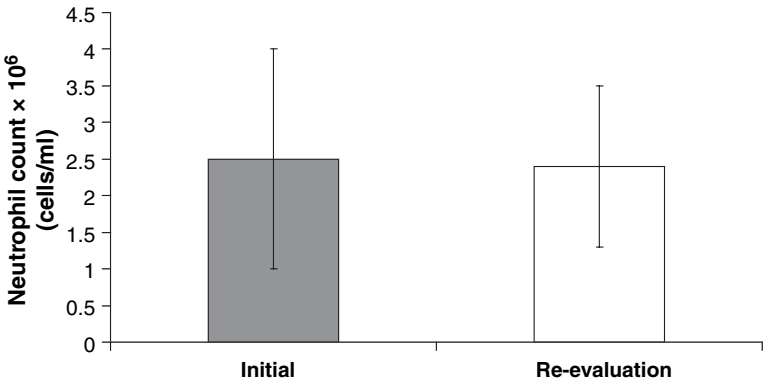

Fig. 2. Comparison of initial and re-evaluation oral neutrophil counts for control subjects. There was no statistically significant difference between rinse samples (Student's paired *t*-test, $p > 0.8$, $n = 6$; mean ± standard deviation).

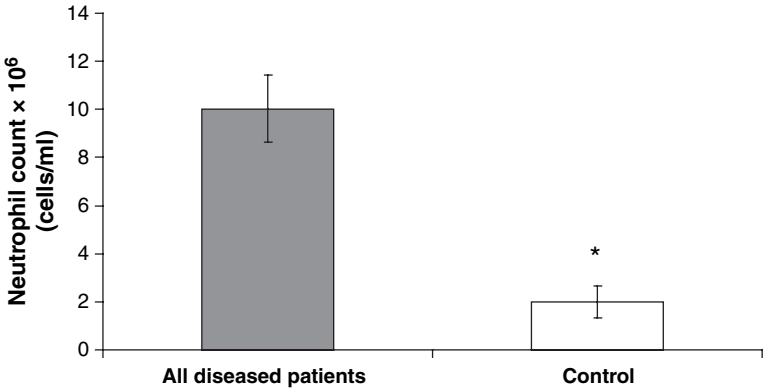

Fig. 3. Comparison of initial mean oral neutrophil count for all patients with periodontal disease ($n = 53$) and controls ($n = 14$). There was a statistically significant difference between patients with periodontal disease and controls (Student's *t*-test; $*p < 0.001$; mean ± standard deviation).

periodontitis (> 10 pockets of > 5 mm deep) (14). Patients with severe periodontitis ($n = 32$) exhibited a sixfold higher level of oral neutrophils compared with controls, and the group of patients with moderate periodontitis ($n = 21$) exhibited more than twice the level of oral neutrophils compared with controls (ANOVA; $p < 0.001$; the results of a Tukey studentized range test indicated that the significant differences lie between the groups of patients with severe periodontitis and controls, and the groups of patients with severe and moderate periodontitis, but not between the groups of patients with moderate and control periodontitis; Fig. 4). The sensitivity and specificity of this oral rinse assay, as a measure of periodontal disease presence, was also calculated at various cut-off points (Fig. 5). A neutrophil count of $> 3 \times 10^6$ yielded a sensitivity of 0.83 and a specificity of 0.79 for moderate-to-severe disease. The positive predictive value and negative predictive value were 0.94 and 0.55, respectively.

We assessed the relationship between initial oral neutrophil counts (diseased and control subjects) and total initial probing depth (> 5 mm; Fig. 6). Regression analysis confirmed that the positive relationship between initial oral neutrophil count and pocket depth was statistically significant ($p = 4.7 \times 10^{-9}$,

Relationship between periodontal disease severity and oral neutrophil levels

To determine whether patients with periodontal disease had elevated oral neutrophil levels, initial mean oral neutrophil counts for all diseased subjects ($n = 53$) were compared with the mean initial oral count from the periodontally healthy controls ($n = 14$). Diseased patients exhibited more than a fivefold greater initial mean oral neutrophil count than the controls (Student's *t*-test; $p < 0.001$; Fig. 3).

The diseased patients were then divided into two groups based on disease severity: moderate periodontitis (≤ 10 pockets of > 5 mm); and severe

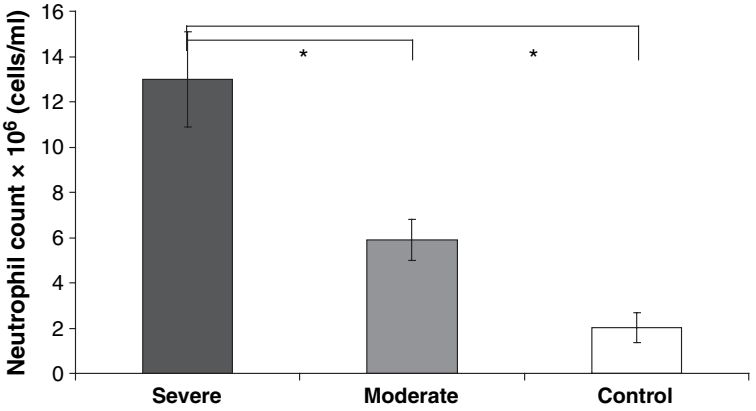

Fig. 4. Comparison of initial neutrophil counts for patients with periodontal disease and healthy controls. Patients were segregated based on the severity of periodontal disease. Significant differences between groups were determined by analysis of variance (ANOVA; $*p < 0.001$). A Tukey studentized range test established that differences lie between the severe/control and severe/moderate groups; $n = 32, 21, 14$, respectively; mean ± standard deviation).

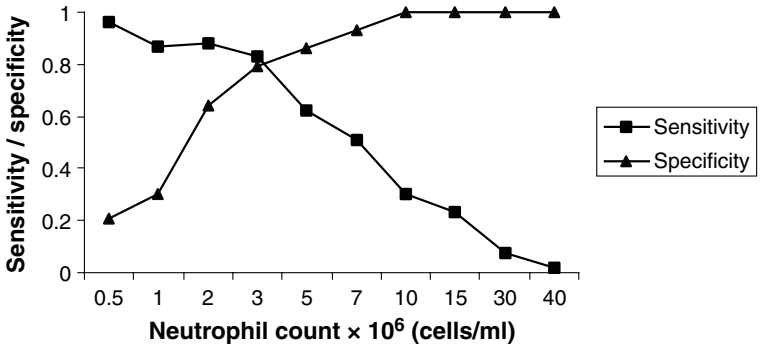

Fig. 5. Sensitivity and specificity values for increasing oral neutrophil level cut-off values. A neutrophil count of $>3 \times 10^6$ yielded a sensitivity of 0.83 and a specificity of 0.79.

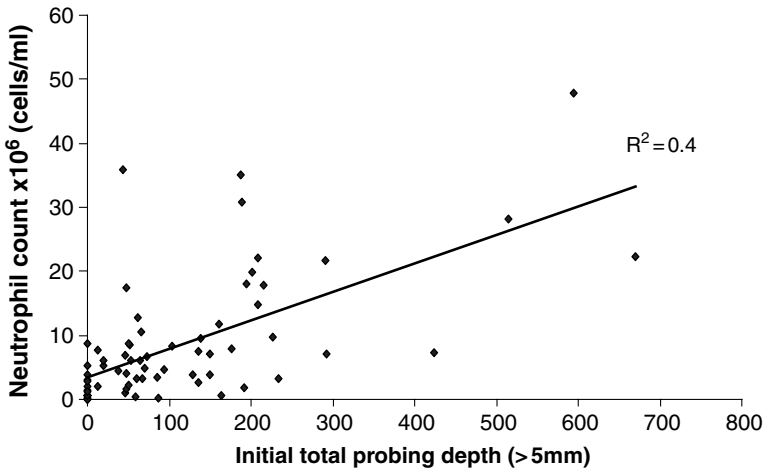

Fig. 6. Comparison of initial oral neutrophil count and initial total probing depth (> 5 mm). A statistically significant linear positive relationship was found to exist between pocket depth and neutrophil count (regression: $p < 0.006$, $n = 67$).

$R^2 = 0.4$). No statistically significant relationship was found between initial oral neutrophil counts and the percentage of sites with bleeding on probing ($p = 0.34$, $R^2 = 0$).

A multiple regression analysis was performed to examine the influence of age, gender, smoking status, radiographic bone loss, bleeding on probing, number of teeth, and depth index on oral neutrophil levels. The only statistically significant explanatory variable among those tested was the depth index ($F = 24.56$, $p < 0.0001$). Thus, of all the parameters collected in the study, total probing depth (> 5 mm) was the only factor to significantly influence the initial oral neutrophil count. It should be noted that in this study there was no statistical difference in initial mean neutrophil count between smokers and nonsmokers (Student's *t*-test, $p = 0.6$; $n = 16$ smokers, 38 nonsmokers). Perhaps a larger sample size of smokers is required to detect the influence of smoking on neutrophil levels.

Oral neutrophil response to Phase I therapy

No statistically significant reduction in oral neutrophil counts was observed if all treated patients were considered (Student's *t*-test for paired comparisons; $p = 0.16$, $n = 35$). However, patients who demonstrated a reduction in total probing depth following treatment (i.e. 'improvers', defined as having a reduction of at least 33% in total probing depth) showed a concomitant 43% reduction in oral neutrophil count ($p = 0.019$, Student's *t*-test for paired comparisons, $n = 17$). Conversely, patients who experienced less than a 33% reduction in total probing depth following phase I therapy ('nonimprovers') demonstrated no significant change in oral neutrophil levels (Student's *t*-test for paired comparisons, $p = 0.39$, $n = 18$; Fig. 7). The average reduction in total probing depth for all patients following therapy was 33%. It should also be noted that there was no statistical difference in the severity of initial disease (based on initial total probing depth) between

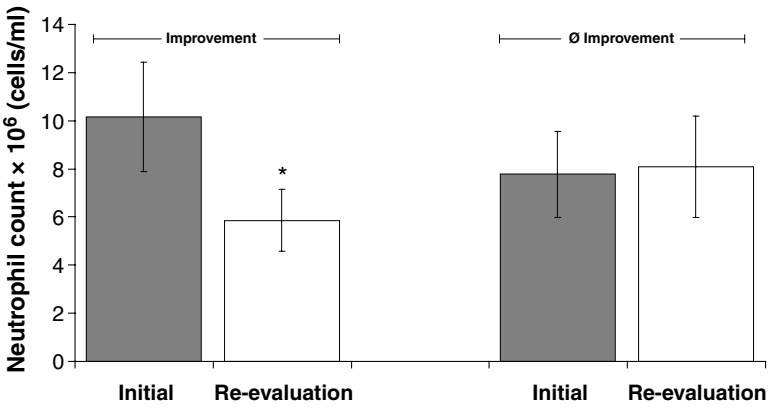

Fig. 7. Comparison of initial and re-evaluation oral neutrophil levels for patients with periodontal disease who completed phase I sanative therapy ($n = 35$). Improvement was defined as a minimum of 33% reduction in total probing depth (> 5 mm) following sanative treatment. 'Improvers' demonstrated a 43% reduction in mean oral neutrophil levels (*$p < 0.02$, Student's paired *t*-test, $n = 17$; mean ± standard deviation). Patents who did improve had no significant reduction in oral neutrophil count ($p > 0.4$, Student's paired *t*-test, $n = 18$). Note that the mean change in total probing depth for all patients following phase I therapy was 33%.

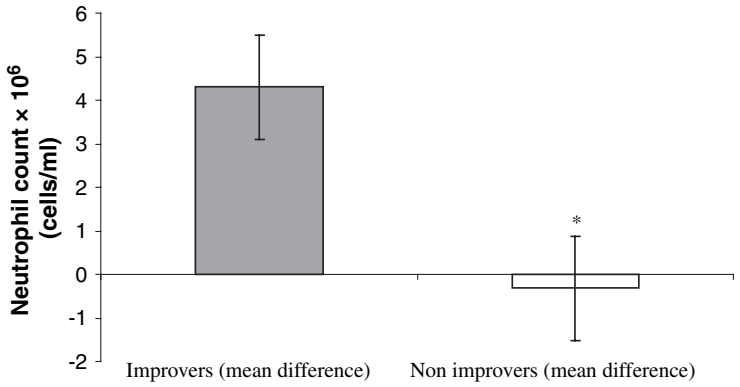

Fig. 8. Comparison of the mean difference of neutrophil levels in patients with periodontal disease who completed phase I sanative therapy ($n = 35$). Improvement was defined as a minimum of 33% reduction in total probing depth (> 5 mm) following sanative treatment. 'Improvers' ($n = 17$) demonstrated a greater mean difference in neutrophil levels following treatment when compared with nonimprovers ($n = 18$) (*$p < 0.03$, Student's paired t-test; mean ± standard deviation).

'improvers' and 'nonimprovers' ($p = 0.296$, Student's t-test, $n = 17$, 18, respectively).

Figure 8 compares the mean change in neutrophil levels following phase I therapy. 'Improvers' (defined as having a reduction of at least 33% in total probing depth) demonstrated a statistically significant greater mean change in neutrophil levels when compared with nonimprovers ($p < 0.03$, Student's paired t-test; $n = 17$ improvers, 18 nonimprovers). Interestingly, patients who failed to show clinical improvement following phase I treatment demonstrated no change in their post-treatment mean neutrophil levels.

Discussion

The data obtained from this study demonstrates that it is possible to collect and quantify oral neutrophils using a single, rapid, noninvasive oral rinse assay. Compared with previous investigations, this rinse protocol is a practical, rapid and reproducible means of collecting and quantifying oral neutrophils from a clinical setting (9–13,18). We tested the reproducibility of this rinse protocol by performing duplicate rinses on subjects and controls on separate days before treatment. We validated the reproducibility of the simplified collection and counting protocol by demonstrating that there was no significant difference in oral neutrophil levels in samples taken from patients on separate days before treatment. The oral neutrophil levels in follow-up samples from control subjects also remained constant over the course of the study. It is important to note that although the control subjects were free from periodontal disease, oral neutrophils were still detected by our oral rinse quantification protocol. This is expected as there is always a bacterial presence in the sulcus, and the constant presence of neutrophils and other mediators of the immune response are important in the maintenance of gingival health (18–20).

This study also sought to determine whether this oral rinse protocol was a potential research tool for evaluating the relationship between oral neutrophil levels and periodontal disease severity. A critical local host defense event, which occurs during periodontal infections, is the efflux of neutrophils into the gingival crevice (21). The primary function of neutrophils is the phagocytosis and killing of invading micro-organisms (22); however, growing evidence points towards the destruction seen in periodontitis being mediated by hyperactive neutrophils (2,3). Given that neutrophils and their products are largely responsible for the destruction of periodontal tissues, a simple test aimed at quantifying oral neutrophils may prove useful for the detection and monitoring of active periodontal tissue breakdown and further our understanding of the role of neutrophils in oral inflammatory diseases.

In this study, we utilized our oral rinse assay to determine the relationship between oral neutrophil levels and the severity of chronic periodontal disease. The positive relationship observed is likely because increased probing depths lead to increased ulcerated epithelium through which greater numbers of neutrophils migrate in response to the presence of subgingival bacteria. Earlier studies, which utilized time-consuming serial rinses and counting techniques, found similar correlations (12,18). It has been previously shown that the rate at which oral neutrophils migrate into the oral cavity increases in the presence of inflammation (10) and that oral neutrophil counts positively correlate with both increasing pocket depth and the gingival index (11,12). Interestingly, Raeste & Aura (13) found that the leukocyte count in the first rinse of the OMR series also reflects the severity of periodontal disease. Although the definition of disease used in their study is unclear and inconsistent with the criteria described above, meaningful comparisons can still be made. Raeste & Aura's first rinse values exhibited a threefold higher leukocyte count in patients with chronic periodontitis than in healthy controls (13). A 25-fold higher leukocyte count was noted in patients with acute periodontitis than in controls. Using our single rinse assay, patients with severe periodontitis demonstrated sixfold more neutrophils than healthy controls. Moderately diseased patients had approximately twice the number of oral neutrophils when compared with controls. Even though direct comparisons cannot be made owing to the differences in study design, Raeste & Aura's results (13) corroborate our finding that a single rinse assay to quantify oral neutrophils does reflect periodontal disease status. It is important to note that the level of inflammation, as measured by the percentage of sites with bleeding on probing, did not correlate with oral neutrophil levels, as determined by our rinse protocol. Studies have shown that the presence of bleeding on probing is a poor measure of active disease (23). Given that this oral rinse assay has a

high sensitivity and positive predictive value (0.83 and 0.94, respectively), it may prove to be an effective and efficient method for quantifying the degree of inflammation and possibly the likelihood of future periodontal breakdown.

Monitoring the effectiveness of various periodontal therapies is important from a research standpoint and there is currently no absolute gold standard to compare the effectiveness of various approaches. Following active periodontal therapy, clinicians often have difficulty determining whether their efforts, utilizing traditional measures, have truly arrested disease. As neutrophils play a significant role in the pathogenesis of some periodontal diseases, a simple test that quantifies the neutrophil response to treatment may be useful. Early studies on oral neutrophil counts used serial rinses to accomplish this goal (13). The single oral rinse assay, used in this study, was able to detect a significant reduction in oral neutrophil counts in patients who responded to phase I therapy. It is important to note that the patients who did not demonstrate a clinical improvement following phase I therapy also failed to show a reduction in oral neutrophil levels. Perhaps these patients would benefit from more aggressive treatment, such as antibiotic therapy, surgery, or modulation of the host response (24,25). It is likely that the persistent presence of neutrophils reflects ongoing inflammation and the destruction of periodontal tissues mediated by neutrophils and their products. For this reason, the oral rinse protocol described here may therefore prove to be a very useful tool in monitoring the progression and/or remission of periodontal disease.

In summary, this study demonstrates that it is possible to collect and quantify oral neutrophils using a single, rapid, noninvasive oral rinse assay. The ability to perform rapid, accurate quantification of oral neutrophils is an important tool in the study of oral diseases, such as periodontitis. This assay was used to demonstrate a positive correlation between oral neutrophil levels and periodontal disease severity, suggesting that it may prove to be a very useful research tool in monitoring the etiology, progression and/or remission of some periodontal diseases. Future work will focus on determining whether this assay can be used to monitor periodontal health in patients with neutrophil-related disorders. Longitudinal monitoring of patients will allow us to determine if neutrophil levels can be used to monitor susceptibility to periodontal diseases in this unique population of patients.

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