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ORIGINAL ARTICLE

Methodological evaluation of gingival crevicular fluid volume and neutrophil elastase levels: sequential sampling, length of sampling time and two different sampling methods

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ABSTRACT

Objectives: The mechanisms underlying the formation and composition of gingival crevicular fluid (GCF) and its flow into and from periodontal pockets are not understood very well. The aim of this study was to evaluate the length of sampling time and sequential sampling of GCF neutrophil elastase (NE) enzyme levels by using intracrevicular and orifice methods.

Material and methods: Twenty adults (mean age of 41.8 years, ranged 31–60 years, 18 males and 2 females) with chronic periodontitis were enrolled and all completed the 3-d study. GCF was collected by both intracrevicular and intrasulcular methods, 720 samples of GCF were collected. In first, second and third day, the length of sampling time in seconds (s) and order were '5- 10-30-s'; '10- 30- 5-s' and '30- 5- 10-s,' respectively. GCF elastase levels were determined by hydrolysis of neutrophil specific substrate N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide.

Results: NE activity (μU) and NE activity/volume (μU/μl) were significantly different for order of sampling ($p < .05$), but not for the length of sampling time ($p > .05$).

Conclusions: Within the limits of this study, the choice of sampling technique in GCF-profile studies seems to be a critical decision as it has the potential to affect the GCF volume and NE activity.

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Introduction

Gingival crevicular fluid (GCF) is accepted as a physiological fluid and/or inflammatory exudate. Analysis of the constituents of GCF sample enables to investigate the processes in periodontal health and periodontal disease depending on the sampling site-specific nature of the samples. These characteristics of GCF also provide exploration of the samples as a diagnostic and prognostic indicator [1].

GCF sampling is usually made by two methods; either by placing a calibrated filter paper strip [2–4] to the opening of the gingival crevice, so-called orifice method or periodontal pocket, so called intracrevicular method. GCF fluid was allowed to accumulate for a relatively short period of time, usually 30 s (s). The sampling of the GCF with an absorbent paper strip is an objective and non-invasive procedure which is also a highly sensitive sample collection method [2–4]. Intracrevicular GCF collection technique is generally preferred, however, insertion depths of these strips vary depending on the sampling methods [2,5–12]. The fluid flow is affected by mechanical irritation, therefore, the strip insertion depth, repetition of insertions (sequential samplings) and/or duration of insertions (sampling time) may alter the amount of GCF volume and/or GCF composition-constituents [3,4,9,11–18]. To establish a standard methodology of GCF sampling, numerous studies were conducted and the need of a standardized method was emphasized [2–4,6,19–23]. The choice of sampling time is concerning in collecting GCF as variable collection durations were reported [24–27]. With regard to reporting data of the GCF, it is acceptable to either measure volume (e.g. with a Periotron) and then calculate mediator concentration according to the original GCF volume, or to report total mediator content. Most authors nowadays prefer to report total mediator content [28].

Neutrophil elastase (NE) is a serine protease stored in azurophilic granules which degrades the periodontal tissue components, when released from granulocytes due to bacterial phagocytosis or cell lysis [29–31]. The proteolytic activity of this enzyme contributes to the body's defence against infectious agents by promoting the destruction of pathogenic bacteria [32]. High levels of unregulated NE have also been associated with the inflammatory state found in a wide range of acute and chronic diseases [33], with excess NE causing extracellular matrix degradation, cellular receptor cleavage and healthy tissue disruption [34,35]. NE is, therefore, a potential diagnostic marker for a number of disease states and methods for the detection of this enzyme with high sensitivity are of clinical importance [36,37]. Depending

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on the characteristics of the enzyme, the elevated levels of NE activity might be expected during periodontal tissue breakdown [38] which is known to be associated with future attachment loss [39]. Building on these findings, quantification of GCF constituents may be considered as a tool for understanding of the development and progression of periodontal diseases and may serve as an objective measure of periodontal disease status. The analysis of NE activity is effective in differentiating the healthy state from gingivitis and aids in treatment planning [11]. Furthermore, as emphasized in a recent meta-analysis published by Arias-Bujanda et al. [40], NE was the second most researched and had the second best performance values (estimated sensitivity and specificity median = 75% and 81%) of GCF biomarkers. While harvesting GCF, collecting fluid without affecting the biological flow of fluid is crucial in the methodological step. Therefore, the liquid collected from the sulcus is very limited and the GCF flow can be easily affected by mechanical irritation [4,39]. There is no consensus on preferred sampling time, however, it is recommended to be less than 5 min [11]. Further, while some authors use the first GCF sample for laboratory analysis [11,41], some others avoid the first one as they reflect the situation within crevice [42,43]. There are studies in literature assessed the impact of different methods and argued which standard method was valuable to determine the GCF profile and the biological properties of GCF [4,44]. However, few systematic studies examined the differences in amount or composition of GCF in correlation with sampling time or sequential sampling. Therefore, there is a need for a study exploring the features of GCF in comparison with variable sampling methods. The aims of this study were to evaluate the effect of the length of sampling time (5-s 10-s and 30-s) and the sequential sampling (first, second and third day) on the NE activity and GCF volume by using intracrevicular and orifice methods.

Material and methods

Study population

The study population comprised 20 patients (18 males and 2 females). Based on the new classification system for periodontal diseases [45], periodontal status was classified as follows: localized or generalized periodontitis of periodontal Stage II or Stage III with grade B or grade C, with age of >35 years (mean age: 41.8 years, ranged 31–60 years) with probing depths (PDs) >5 mm in at least two sites, radiological detectable alveolar bone loss in all quadrants.

Exclusion criteria were presence of systemic disease; use of antibiotics and/or anti-inflammatory drugs within the past 6 months; history of any oral hygiene prophylaxis and scaling and root planning within the past 6 months. This study was approved by the Research Ethics Committee of Selcuk University Faculty of Dentistry. Written informed consent was obtained from all subjects prior to participation. The study was registered at ClinicalTrials.gov (NCT02676362).

Clinical periodontal evaluation

The plaque index (PI)[15], gingival index (GI)[16], sulcus bleeding index (SBI)[9], clinical attachment level (CAL) and PD [with Williams Periodontal Probe (Hu Friedy, Chicago, IL)] were recorded by the same examiner (NAK).

Gingival crevicular fluid collection and processing

Two sites presenting ≥5 mm of PD, alveolar bone loss as assessed on panoramic radiographs and GI of 2 (with preference of two maxillary non-molar teeth) were selected for GCF sampling procedure for each individual. GCF was harvested from mesiofacial or distofacial angle of two different maxillary anterior incisors or premolars to eliminate the risk of contamination with saliva. Samples were collected in the morning 2–3 h after the breakfast. Prior to GCF sampling, plaque scores were determined, then the sample site was isolated with cotton rolls and lightly scaled to remove supragingival plaque, and gently air dried. In the first set, paper strips (Periopaper strips, IDE, Amityville, NY) were used according to the orifice method described by Rüdin et al. [10]. Paper strip was inserted 1 mm into the pocket and left in place for 5s. The volume of GCF (μl) on strips was determined with Periotron 8000 (Harco, Winnipeg, Canada) previously calibrated with serum [46]. GCF was harvested at the same interproximal site with the length of sampling times of 5, 10 and 30 s with 60 s intervals between repeat samples. Each site was sampled three times in the first set and each of the paper strips was placed into eppendorf tube containing 150 μl of phosphate-buffered saline (PBS) and stored at −30 °C until assayed. Ten min following the first set, the same procedure was performed using intracrevicular placement of paper strips. The strips were inserted into the crevice until mild resistance was felt and then left in place. The same procedures were repeated for three consecutive days with different order in terms of sampling time. In a few cases, bleeding occurred while performing repeated sampling, we recalled patients following day for repeat sampling procedure. On the first, second and third day, the length of sampling time in seconds were '5 s, 10 s, 30 s'; '10 s, 30 s, 5 s' and '30 s, 5 s, 10 s', respectively (Table 1). Total actual sampling time for orifice and intracrevicular methods were 340 s; for orifice method 165 s; intracrevicular method 165 s with 10 m interval in between two methods. A total of 720 samples of GCF were collected by the same examiner (NOA).

Table 1. The length of sampling time, sequence position of each day by using the orifice and intracrevicular methods.

		The length of sample time		
Days	Sampling methods	First	Second	Third
First day	Orifice (Set I)	5 s	10 s	30 s
	Intracrevicular (Set II)	5 s	10 s	30 s
Second day	Orifice (Set I)	10 s	30 s	5 s
	Intracrevicular (Set II)	10 s	30 s	5 s
Third day	Orifice (Set I)	30 s	5 s	10 s
	Intracrevicular (Set II)	30 s	5 s	10 s

Neutrophil elastase activity in GCF

NE level in GCF was determined by measurement of p-nitroanilide resulting from hydrolysis of neutrophil elastase-specific peptide (N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (Sigma Chem. Co., St Louis) using ELISA plates. Hydrolysis of this substrate was accompanied by a colour change that can be quantified using an immunoassay plate reader which measures functionally active NE levels. Standard solutions of NE (range 2–1000 μU/μl) were prepared with ELISA diluent buffer.

At the time of analysis, samples were vortexed and incubated at 1 h at 22 °C. On each microtiter plate, 25 μl of elution media (physiologic saline ± 0.1% Tween 20) and 200 μl of substrate was added into two wells in the first column as the substrate blank. Duplicate 25 μl samples of each standard and 200 μl of substrate was placed into standard wells. A total of 25 μl of sample and 200 μl of substrate was added to sample wells. A 25 ml aliquot of GCF was incubated with 200 ml of 1.25 mM synthetic substrate specific for NE, N-methoxysuccinyl-ala-ala-pro-val-p-nitroanilide (Sigma Chem. Co., St Louis) in a 96-well ELISA plate for 2.5 h at 37 °C. At the end of the incubation, the optic density was read at 405 nm. This method measures functionally active NE, which includes free NE and NE bound to a2-macroglobulin (A2MG). Elastase-a1-antitrypsin (A1AT) inhibitory complex was not recorded by this assay since it was functionally inactive [47]. Active NE levels (free elastase and elastase bound to A2MG) were calculated from the standard curve. NE activity on the strips was expressed in terms of μU (pmols of substrate hydrolysed per minute) and μU/μl [48,49].

Statistical analysis

For normalization of biochemical values, natural logarithmic transformations were undertaken. The effects of the orifice and intracrevicular methods, the length of sampling time and sequential sampling measurements on crevicular fluid NE were analysed by two-way analysis of variance (ANOVA) and the level of significance was set at $p < .05$ and Bonferroni correction applied for multiple *post hoc* comparisons with adjusted p level of 0.016. Statistical analyses were performed using SPSS version 15.0 (SPSS 15.0, SPSS, Chicago, IL).

Results

A total of 40 teeth in 20 consecutive patients with chronic periodontitis were included in this study. The mean and standard deviation (SD) for PI and GI were 2.45 ± 0.6 and 2.05 ± 0.2, respectively. All sampling sites were SBI+ (2.05 ± 0.8). The mean ± SD for PD and CAL were 5.8 ± 0.9 mm and 5.7 ± 1.5 mm, respectively.

Study design and sampling protocols were presented in Table 1. A total of 720 GCF samplings were obtained from 20 periodontitis patients with two interproximal sites of non-molar teeth during 5, 10 and 30 s with 1-min intervals and varying sample order (first, second and third) by using orifice and intracrevicular methods (Table 1).

Table 2 represents data for median values and interquartile range of GCF volume, NE activity (μU and μU/μl) for different sample times (5, 10 and 30 s). The 5 s sample of the GCF volume was significantly lower than 30 s sample ($p < .05$). There were no statistically significant differences in terms of NE activity (μU and μU/μl) between different sampling time periods ($p > .05$).

The NE activity (μU and μU/μl) in the first samples was higher than in second and third samples (Table 3, $p < .05$). There was a statistically significant difference in GCF volume for the first and third samples ($p < .05$). However, no significant differences were found between first and second samples ($p > .05$), also third and second samples ($p > .05$).

Data for median values and interquartile range of GCF volume and NE activity (μU and μU/μl) for each of two sets (both orifice and intracrevicular methods) were presented in Table 4. The values of NE activity (μU and μU/μl) with orifice

Table 2. Median values (interquartile range) of NE activity and GCF volume for the sampling time.

	Sampling time		
	5 s ($n = 240$)	10 s ($n = 240$)	30 s ($n = 240$)
NE Activity/volume (μU/μl), median (IQR)	771.25 (2118.34)	556.36 (2008.10)	957.29 (2546.29)
NE activity/time (μU/s) median (IQR)	112.92 (458.48)	133.93 (427.68)	175.94 (678.68)
GCF volume (μl) median (IQR)	0.29[a] (0.43)	0.31 (0.48)	0.40 (0.56)

Differences between 5 and 30 s; n = number of samples.
[a]$p < .016$ was considered statistically significant when compared 30 s.

Table 3. Median values (interquartile range) of NE activity and GCF volume for sequential sampling.

	Sequential sampling		
	First ($n = 240$)	Second ($n = 240$)	Third ($n = 240$)
NE activity/volume (μU/μl)	1882.24[a,b] (2674.78)	637.76[a] (1973.81)	111.0230 (1087.70)
NE activity/time (μU/s)	436.30[a,b] (907.34)	124.30[a] (483.45)	25.12 (183.93)
GCF volume (μl)	0.30[b] (0.38)	0.33 (0.56)	0.34 (0.53)

Differences between first, second and third samplings.
[a]$p < .016$ was considered statistically significant when compared to second sampling.
[b]$p < .016$ was considered statistically significant when compared to third sampling.

method were significantly lower than with intracrevicular method ($p < .05$).

Distribution of data for the NE activity (μU and μU/μl) and GCF volume for both sampling methods at different sampling durations were presented in Table 5. The highest NE activity (μU) was achieved with orifice technique on the third day, in 30 s and at the first measurement. The lowest NE activity (μU/μl) was obtained with intracrevicular technique on the first day, in 30 s and at the third measurement. While highest NE activity (μU/μl) was achieved at the first measurement in 30 s with orifice technique on the third day, lowest NE activity (μU/μl) was obtained at the first measurement in 30 s with intracrevicular technique on the third day. The least amount of GCF volume was obtained on the second day in 5 s and at the third measurement with orifice technique. The highest amount of GCF volume was revealed in 30 s with intracrevicular technique on the first day and at the third measurement.

Discussion

A better understanding of the GCF flow frame and its molecular composition is essential when gingival fluid tests serve as a precursor for objective diagnostic disease activity. Since periodontitis is largely site-specific, it is important to distinguish areas that will not respond to treatment and areas at high risk for future deformation. This study analysed the GCF flow and NE activity in untreated periodontitis. To the best of our knowledge, this is the first study elucidating the sequential gingival fluid collection procedures for NE levels by using orifice and intracrevicular methods. The key finding of the study presented here is that NE activity and GCF volume were affected by the selected sequential sampling in both methods. NE activity in the first samples was higher than in the second and third samples. When considering the sampling time, 5, 10 and 30 s samples had similar amounts of NE in GCF.

MMP-8 and NE are the only two enzymes that have each been studied in terms of their predictive value for periodontal disease progression and their ability to predict the outcome of treatment [40,50–53]. NE was examined in terms of its ability to predict disease progression to provide an indicator of bone loss in several studies. In one study, sensitivity/specificity values were found to be 84%/66%, respectively [52] whereas they were estimated as 77/61% in another study [4]. Therefore, apart from the several limitations to the use of biomarkers for diagnosing/predicting disease outcome (such as appropriate marker selection, reliable analysis methods), selection of sampling methods and sampling time can affect amounts of biomarkers.

Length of sampling time

This study comparatively analysed two different sampling techniques to determine methodologies and potential impacts on GCF volume and sampling time variations. The results of this study showed that 5 s samples contained less GCF volume than 30 s samples ($p < .05$). An earlier study [2] has reported that, considering only first samples, the 5 and 10 s samples had significantly more GCF volume than 20 and 30 s samples. The reason for this contradictory outcome may be that they have worked in patients who received periodontitis treatment and were in the maintenance phase. The inflammatory status of periodontal tissues was suggested as an important factor affecting GCF volume [17,44,54].

Only few studies were designed to assess relationship between sampling time and GCF enzyme activity [2,3,52]. In the study of Jin et al. [54], two well-matched groups of sites

Table 4. Median Values (interquartile range) of NE activity and gingival crevicular fluid (GCF) for intracrevicular method and orifice method.

	Sampling methods	
	Intracrevicular method ($n = 360$)	Orifice method ($n = 360$)
NE activity/volume (μU/μl)	378.49[a] (1688.94)	1094.31 (2470.78)
NE activity/time (μU/s)	150.56[a] (776.88)	136.55 (384.76)
GCF volume (μl)	0.57[a] (0.48)	0.18 (0.23)

Differences between orifice method and intracrevicular method.
[a]$p < .05$ was considered statistically significant when compared orifice method.

Table 5. Median Values (interquartile range) of GCF parameters in different time periods arranged by sampling sequence divided by orifice and intracrevicular methods.

Day	Sampling methods	The length of sampling time	Sequential sampling (1-m intervals)	*n*	NE activity (μU/μl)	NE activity (μU/s)	GCF volume
First day	Orifice	5 s	First	40	395 (2428.37)	56.92 (254)	0.14 (0.15)
		10 s	Second	40	118.70 (1527.51)	13.95 (252)	0.27 (0.28)
		30 s	Third	40	175.15 (928.48)	12.83 (140)	0.25 (0.35)
	Intracrevicular	5 s	First	40	1086.57 (1822.90)	428.09 (978.09)	0.53 (0.38)
		10 s	Second	40	150.49 (814.02)	53.57(363.78)	0.69 (0.49)
		30 s	Third	40	7.67 (69.19)	5.57 (39.65)	0.84 (0.52)
Second day	Orifice	5 s	Third	40	1116.19 (2418.77)	68.08 (251.08)	0.12 (0.27)
		10 s	First	40	2222.09 (38.58)	256.02 (497.20)	0.14 (0.23)
		30 s	Second	40	967.22 (3005.38)	121.67 (390)	0.19 (1.09)
	Intracrevicular	5 s	Third	40	32.69 (940)	15.62 (302.79)	0.50 (0.37)
		10 s	First	40	2137.29 (2300.53)	713.41 (1918.55)	0.46 (0.43)
		30 s	Second	40	978.14 (2058.85)	318.98 (1193.23)	0.65 (0.56)
Third day	Orifice	5 s	Second	40	1949.57 (2260.82)	212.20 (429.67)	0.13 (0.19)
		10 s	Third	40	972.77 (1329.62)	154.41 (261.04)	0.14 (0.15)
		30 s	First	40	2577.56 (2631.08)	438.23 (750.79)	0.16 (0.20)
	Intracrevicular	5 s	Second	40	504.55 (1375.83)	110.29 (617.83)	0.42 (0.58)
		10 s	Third	40	21.94 (174.33)	10.03 (33.13)	0.58 (0.57)
		30 s	First	40	2376.19 (1729.03)	851.35 (1702.70)	0.48 (0.38)

(static GCF–flow GCF at 1 min and static GCF–flow GCF at 5 min), effect of time on GCF flow and NE activity were investigated. Their study results showed that the sampling time was very important to test the static GCF variable. In this study, we collected only static GCF and found that the values of NE activity (μU and μU/μl) were not affected by the length of sampling time ($p>.05$). The result of this study showed that the 5 s sampling time was sufficient to evaluate NE activity. Thus, shorter sampling time may help reduce the risk of contamination during sampling as well as increasing patient comfort.

In our study, NE activity in GCF was not statistically different at 5, 10 and 30 s of sampling time ($p>.05$). In a past study, with sampling times of 5 and, 30 s, aspartate aminotransferase activity, enzyme and fluid flow rate decreased while the volume of GCF was found at longer sampling time [2]. The reason for this contradictory result may be due to the feature of GCF that contains many enzymes of different molecular size and concentration; and accordingly the kinetics of accumulation on paper strips varies from one another. Our findings suggested that as the NE activity in GCF was not affected by the sampling time, it can be concluded that a sampling time of more than 5 s is not required to evaluate NE activity in GCF.

Jin et al. [54] showed that static GCF volume was not consistently correlated with the change of GCF flow in subjects with gingivitis and periodontitis, but only in the healthy subjects. In terms of NE activity, the subjects with periodontitis had the highest levels in both static GCF and flow GCF, as compared to subjects with gingivitis and periodontitis. Building on this, it would be useful to examine the relationship between the NE and disease onset, transition from gingivitis to periodontitis, or whether a site is undergoing active destruction.

Sequential sampling

Effect of sequential sampling on GCF volume and GCF NE activity was another concern of this study. We observed that GCF volume in the first sample was lower than the third sample ($p<.05$). This higher volume in the third sample may be attributed to the additional sampling time, which could have led to the mechanical effect of the previous two sampling attempts thus increasing the volume, regardless of the technique. The effect of sequential sampling on GCF volume and composition has been evaluated in several past studies with different time intervals [2–4,11,17,18,41]. Lamster et al. [3] reported minimal variation in sequential sampling protocol including 0, 4, 8, 30 and 60 s. Ozkavaf et al. [4] found that a volumetric increase with both sampling techniques were generally in accordance with the time-limited stability of GCF. Compatible with other studies [3,4,11,17], we propose that GCF volume is influenced by increased vascular permeability due to the intensity of inevitable mechanical irritation.

As for the analysis of repeated sampling results (1 min interval), more enzyme activity was reported in the first samples regardless of sampling time in previous studies [2]. In our study, the values of NE activity (μU and μU/μl) in the first samples were higher than in second and third samples. Higher enzymatic activity of the first samples is mainly due to the reservoir activity in the sulcus/pocket [2,3,11]. Our results were generally consistent with most previous studies showing a higher enzyme activity in the first GCF samples.

The quantity of GCF NE [2,11] has been shown to be affected by sequential sampling performed with various time intervals. For the analysis performed on the last two of three sequential samplings, GCF flow rate and NE activity was appeared to increase with the severity of gingival inflammation. Jin et al. [54] studied GCF parameters between the two matched sites for subsequent collection of flow GCF samples either 1 or 5 min after static GCF sampling in all subjects. These investigators were assigned to determine to what extent the time variable accounts for the difference in GCF flow and NE activity. Their study results showed that a rapid replenishment of GCF flow was observed in all subjects with gingivitis and periodontitis and depletion of NE activity in GCF at 1 min was found in all subjects. This was the reason that we waited 1 m between repetitive samples. We showed that NE activity was significantly decreased for sequential sampling ($p<.05$). This may be due the fact that NE activity was diluted by the increased GCF volume primarily dependent on vascular permeability [55]. Besides a decrease in neutrophil proteins including NE has been reported in each repeating sample due to the binding of neutrophils to the periopaper and the reduction in their numbers [1].

Sampling method (orifice and intracrevicular methods)

We preferred GCF collection with paper-based methods since they can detect higher levels of the NE activity than washing method [56]. The effect of sampling method on GCF volume and composition was another concern of this study. In this study although symmetrical sampling sites had a similar clinical status, intracrevicular technique provided higher amounts of GCF as well as the higher flow rate compared to orifice method ($p<.05$). This is in accordance with an early study of Ozkavaf et al. [4] who reported greater amounts of GCF with the intracrevicular technique than with the orifice technique. The main reason for this result was the intensity of the unavoidable mechanical irritation during strip placement, which was placed deeply into the base of the pocket, thus increasing vascular permeability.

In this study, we found that NE activity (μU/μl) in the intracrevicular was lower than in the orifice method ($p<.05$). Also, NE activity (μU) with intracrevicular method was higher than with the orifice method ($p<.05$). The reason for these results may be mechanical and/or chemical irritation of gingival tissue results in an increased permeability of the vessels of the dento-gingival plexus [57]. The higher flow rate of GCF in the intracrevicular method compared to orifice method could be another explanation to this result.

In this study, we analysed different sampling techniques with sequential sampling and with variable sampling time comparatively to explore the potential effects of these techniques on GCF volume as well as NE activity. To the best of

our knowledge, this the first study in literature exploring these relationships as a whole. Our study results showed that the 5 s sampling time was sufficient for analysis of NE activity in GCF. Therefore, we suggest 5 s sampling time for either GCF sampling method, thus reducing the risk of contamination of saliva as a benefit by means of less chair-side time for sampling.

We acknowledge some limitations of the study. GCF sampling procedure was only performed from the sites presenting $\geq$5 mm PD, alveolar bone loss and GI of 2 in patients with periodontitis. Future studies with addition of patients with gingivitis would allow more accurate analysis for the determination of the sampling time and the sampling method. It would be of useful to investigate NE activity for the transition from the gingivitis to periodontitis stage. Also, small sample size was another limitation of this study.

Conclusion

Intracrevicular method provides higher NE activity and GCF volume compared to orifice method and first samples contain higher NE activity compared to repeat samples. As a result, in each repeated GCF sample, NE activity decreases rapidly, thus the first sample reflects the actual disease state more than second and third samples. Also, shorter sampling times such as 5 s are sufficient to analyse the NE activity in GCF. Therefore, we recommend avoiding repeated sampling procedures and long period of sampling time during GCF sample collection. This may help reduce the risk of the contamination of saliva.

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Disclosure statement

The authors declare no conflicts of interests and have nothing to disclose in this study.

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