

Estimation of N-terminal telopeptides of type I collagen in periodontal health, disease and after nonsurgical periodontal therapy in gingival crevicular fluid: A clinico - biochemical study

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ABSTRACT

Aim: This study explored gingival crevicular fluid (GCF) N-terminal telopeptides of type I collagen (NTx) levels in periodontal health, disease and after nonsurgical periodontal therapy along with its association with the clinical parameters.

Materials and Methods: Study comprised of three groups of 10 subjects each: Healthy (Group I), gingivitis (Group II), and periodontitis (Group III), while Group III patients after scaling and root planning (SRP) constituted Group IV. Gingival index (GI), probing pocket depth (PPD), clinical attachment loss (CAL), and radiological parameters were recorded. GCF samples were analyzed by competitive-enzyme-linked immunosorbent assay.

Results: Samples in Group III and Group IV tested positive for NTx whereas in Group I and Group II, NTx was not detected. Mean NTx levels were higher in Group III (6.79 ± 0.94 nanomole bone collagen equivalents per liter [nm BCE/L]) compared to Group IV (5.73 ± 0.95 nm BCE/L) which was statistically significant. Positive correlation was seen between the clinical parameters and the NTx levels in Group III and IV.

Conclusion: As NTx is specific bone turnover marker, it is detected only in periodontitis Group and the values decline after SRP. Failure to detect NTx in Group I and II, relates to the minimum or no resorption at the sample sites.

Key words: Collagen, gingival crevicular fluid, N-telopeptide, periodontitis

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Diagnostic data procured by a thoughtful history and a detailed examination are useful than anything that can be obtained from the diagnostic laboratory. Yet, there is a need for diagnostic information that is beyond traditional examination. Biochemical methods to measure specific markers are being currently used to provide information on bone resorption and formation, as these markers provide clinically useful evidence of the pathologic processes that reflect bone cell activity around the tooth.

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Osteoclasts are multinucleated cells with efficient and developed machinery to degrade organic bone matrix rich in collagen fibers. Hence, the direct indicators of bone resorption are the fragments of bone collagen produced by the osteoclastic activity.^[1]

Pyridinoline (Pyr) cross-links which stabilize the collagen chains in the extracellular matrix are nonreducible, these include Pyr and deoxypyridinoline (D-Pyr). Pyr is present in bone, cartilage matrix, and other connective tissues except skin. D-Pyr is found in bone and dentin. Although D-Pyr is not specific for the bone matrix, large amounts of D-Pyr have been found in type I collagen of bone. Amino- and carboxy-terminal fragments of collagen are also present in the organic phase of bone and are released into circulation as small peptides during the bone resorptive process.^[2-4] As they result from post-translational modification of collagen molecules, they cannot be reclaimed for collagen synthesis and are therefore highly specific to bone resorption.^[5]

Cross-linked N-terminal telopeptides of type I collagen (NTx) which is an amino-terminal telopeptide is exceptional

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because of its α -2(I) N-telopeptide and is released as a resolute end product of bone resorption.^[6] Detection of such a molecule in gingival crevicular fluid (GCF) might lead to the development of a marker directly related to tissue breakdown in periodontitis.^[7]

GCF being an ultrafiltrate of plasma provides advantages that are analogous to blood collection by the physician, it is noninvasive, site-specific about teeth, comparatively easy to perform, facilitates repeated sampling, and offers one of the most accessible entries in the body to assess the disease state.^[8]

In dental literature, there are few studies concerning the use of NTx as a biochemical resorption marker and the results are conflicting. Friedmann *et al.*^[9] have studied the levels of NTx in GCF and peri-implant crevicular fluid (PCF) and speculated that increased NTx levels may predict extensive bone destruction earlier than the GCF and PCF calprotectin levels. The levels of NTx along with other bone markers in chronic periodontitis patients were evaluated and it was stated that NTx may be useful as a resorption marker in periodontal bone destruction.^[7] Becerik *et al.*^[10] have estimated the GCF NTx levels in health and different periodontal diseases and it was concluded that fluctuating NTx levels might point out the abnormal bone turnover in periodontitis. However, studies have even failed to show NTx as a bone-specific marker of bone metabolism in cyclosporine – a induced gingival overgrowth.^[11] Till date, no studies have reported GCF NTx levels in healthy, gingivitis, chronic periodontitis patients, and after nonsurgical periodontal therapy of periodontitis patients. Hence, this study was designed to estimate NTx in GCF, assess its usefulness as a “bone-specific marker” and also correlate it with the clinical parameters.

MATERIALS AND METHODS

The study population comprised 30 subjects attending the outpatient Department of Periodontology, Government Dental College and Hospital, Bangalore from May 2007 to October 2008. Subjects were matched to eliminate age (25–50 years with a mean age $\pm$ standard deviation [SD] was 28.3 ± 2.627 , 28.9 ± 2.807 , and 32.3 ± 3.433 for Group I, II, and III, respectively) and sex as confounding factors. Ethical clearance for the study was obtained from the Ethical Committee of the Institution. The patients were explained regarding the study procedure and written informed consent was obtained from those who agreed to participate voluntarily in this study. The investigation was performed in accordance to the requirements of the “Declaration of Helsinki” as was adopted by the 18th World Medical assembly in 1964 and revised in Edinburgh (2000).

The exclusion criteria included - Pregnant, lactating and postmenopausal female subjects, patients on

anti-inflammatory drugs, bisphosphonates, alendronates, antibiotics, hormone replacement therapy, Vitamin D, and calcium supplements. Patients with systemic diseases and smokers were also excluded.

Patients were categorized into three groups based on probing pocket depth (PPD), clinical attachment loss (CAL), gingival index scores (GI) (Loe and Sillness 1963) and radiographic evidence of bone loss (assuming the physiologic distance between the cemento-enamel junction to alveolar crest to be 2 mm). After a full mouth periodontal probing, bone loss was recorded dichotomously using intraoral periapical radiographs (paralleling angle technique) to differentiate patients with chronic periodontitis from patients of other groups,^[12] without any delineation in the extent of alveolar bone loss. Paralleling angle technique was employed as it has a geometrical advantage over bisecting angle technique by reducing the distortion of the image.^[13]

- Group I: 10 subjects with clinically healthy periodontium (GI = 0, PPD $\leq$ 3 mm, and CAL = 0)
- Group II: 10 subjects with gingival inflammation (GI >1, PPD $\leq$ 3 mm, and CAL = 0)
- Group III: 10 subjects who showed clinical signs of gingival inflammation GI >1, PPD $\leq$ 5 mm, and radiographic bone loss with CAL $\geq$ 3 mm
- Group IV (after treatment): Subjects of Group III treated with scaling and root planning (SRP) (GCF samples were taken from same sites 6–8 weeks after treatment).

Site selection and gingival crevicular fluid sampling

The samples were collected a day later to the site selection in order to prevent contamination of GCF with blood as a result of probing. One site per subject was selected as a sampling site. In Group I subjects, sampling was predetermined to be from the mesiobuccal region of the maxillary right first molar, in the absence of which the left first molar was sampled. Sites with the highest clinical signs of inflammation (i.e., redness, bleeding on probing and edema) were selected in Group II subjects. In Group III subjects, sites with >3 mm of CAL as measured from the clinical cemento-enamel junction to the base of periodontal pocket using a Williams graduated periodontal probe were identified, and the site showing the highest CAL, along with the radiographical conformation of the bone loss, was assigned for sampling. On the subsequent day, after drying the area with a blast of air, supragingival plaque was removed without touching the marginal gingival, and GCF was collected using color-coded 1–5 μ L calibrated. Volumetric microcapillary pipettes (Sigma-Aldrich chemical co. Ltd., St. Louis, MO, USA). From each test site, a standardized volume of 1 μ L was collected using the calibration on the micropipette and by placing the tip of the pipette extracrevicularly (unstimulated). The GCF collected was immediately transferred to a plastic vial and stored at -70°C until the assay.

Competitive inhibition assay

NTx was quantitated using a commercially available competitive-inhibition enzyme-linked immunosorbent assay (ELISA) (Ostex, osteomark, Seattle, WA, USA) and expressed as nanomole bone collagen equivalents per liter (nm BCE/L). Sensitivity range of the ELISA kit to detect NTx is 3.2 nm BCE/L to 40 nm BCE/L.

Statistical analyses

All data were analyzed using a Statistical Software (SPSS version 10.5, SPSS, Chicago, IL, USA). A test for the validity of the normality assumption was carried out using Shapiro-Wilk test; if data were normal then parametric tests were carried out otherwise, nonparametric test was carried out for comparisons between the groups. Analysis of variance was carried out to find out if all four groups differed significantly. Further, pairwise comparisons using the Scheffé test were carried out to explore which pair or pairs differed with respect to GI. Nonparametric Kruskal-Wallis test was carried to find the difference between the four groups further Mann-Whitney test was used to compare the pair difference. The Wilcoxon signed ranks test was used to compare the pair difference between the groups with respect to GCF and CAL parameter. The Spearman rho correlation coefficient test was done to find any association between the clinical parameters and GCF levels. The level of statistical significance was set at $P < 0.05$.

Sample size determination and randomization

N-terminal telopeptides mean difference values (before and after treatment) were considered to calculate the power of the study. A sample of 10 achieved 87% power to detect the mean paired difference of 1.1 with an estimated SD of 0.9 and with a significance level of 0.05. Two-sided Wilcoxon test was carried out assuming that the actual distribution was normal.

RESULTS

The demographic data of gender and age in the study groups are summarized in Table 1. Table 2 presents the mean GI, PPD and CAL in Group I, Group II, Group III, and after nonsurgical treatment of Group III. There was a gradual increase in GI and PPD from Group I to Group III and these values declined after therapy. Pairwise comparison

Table 1: Demographic distribution of the study groups

	Group I (n=10)	Group II (n=10)	Group III (n=10)
Age			
Mean±SD	28.3±2.627	28.9±2.807	32.3±3.433
Minimum-maximum	26-34	26-34	27-38
Gender			
Male (%)	5 (50.0)	5 (50.0)	5 (50.0)
Female (%)	5 (50.0)	5 (50.0)	5 (50.0)

SD=Standard deviation

for GI and PPD was carried out between the groups and the differences between these values were found to be statistically significant [Table 3].

Laboratory findings

N-terminal telopeptides were detected in all the GCF samples of Group III and Group IV, but Group I and Group II failed to show any NTx. The mean NTx levels in Group III was 6.79 ± 0.94 nm BCE/L compared to Group IV, which was 5.73 ± 0.95 nm BCE/L. Significantly higher levels of NTx were present in Group III compared to Group IV and the difference were statistically significant [Table 4].

Correlation analysis

Spearman's rho correlation coefficient analysis between the GCF NTx levels and clinical parameters are shown in Table 5. GI, PPD, and CAL positively correlated with a reduction in GCF NTx levels from Group III to Group IV. In Group IV, there was a weak positive correlation with GI and CAL. There was a significant improvement in the clinical parameters after nonsurgical periodontal therapy in patients of Group III.

DISCUSSION

Parallel with better understanding of bone resorptive process and isolation of cellular components of the bone matrix, the number of new potential biochemical markers of bone formation and resorption are increasing. Traditional methods for taking X-rays or assessments of probing depth and clinical attachment level shows the previous periodontal tissue breakdown, however, these methods hardly confirm the disease activity and predict the disease outcome.^[14] Hence, the need for markers which predict sites at higher risk and monitor therapy has raised.

The diagnostic tests utilize GCF that can be harvested from the sulcus or periodontal pocket. A number of components evaluated in GCF to date, lack specificity to alveolar bone destruction, and essentially constitute soft-tissue inflammatory events. Hence, the actual detection of connective tissue derived molecules may lead to a more accurate assessment of tissue breakdown. Among the collagen markers, Pyr cross-linked carboxyterminal telopeptide of type I collagen (ICTP) is the most studied marker. ICTP is 12–20 kD fragment of bone type I collagen released during bone resorption. However, studies have found measurable levels of ICTP in gingiva postulating that a smaller proportion of ICTP can have its source from soft tissue breakdown.^[15]

Osteocalcin is a 5.4 kD calcium binding protein of bone and has been involved in the most catabolic and anabolic stages of bone turnover however like ICTP, osteocalcin is also released during soft tissue loss making it less specific to bone resorption.^[16]

Table 2: Comparison of mean clinical parameters between the groups

One-way ANOVA test to compare mean PPD between the groups						
Clinical parameter	Group I	Group II	Group III	Group IV	Test value	P
GI						
Mean±SD	0.31±0.098	1.68±0.179	2.53±0.264	1.41±0.157	246.149	0.001*
Minimum-maximum	0.17-0.45	1.34-1.94	1.98-2.82	1.18-1.60		
Kruskal-Wallis test to compare mean PPD between the groups						
PPD						
Mean±SD	1.30±0.483	2.20±0.632	6.10±1.101	3.60±0.699	34.211	0.001*
Median	1.00	2.00	6.00	3.50		
Minimum-maximum	1-2	1-3	5-8	3-5		
Wilcoxon signed ranks test to compare mean CAL between the groups						
CAL						
Mean±SD	N/A	N/A	3.40±0.516	1.60±0.516	-2.842	0.004*
Median	N/A	N/A	3.00	2.00		
Minimum-maximum	N/A	N/A	3-4	1-2		

*Level of statistical Significance at $P<0.05$. N/A=Not applicable, GI=Gingival index, PPD=Probing pocket depth, CAL=Clinical attachment loss, SD=Standard deviation

Table 3: Pairwise comparison between the groups

Clinical parameters	Groups	P
Scheffe test for GI	I and II	0.001*
	I and III	0.001*
	I and IV	0.001*
	II and III	0.001*
	II and IV	0.011*
	III and IV	0.001*
Mann-Whitney test for PPD	I and II	0.005*
	I and III	0.001*
	I and IV	0.001*
	II and III	0.001*
	II and IV	0.001*
	III and IV	0.001*

*Level of statistical significance at $P<0.05$. GI=Gingival index, PPD=Probing pocket depth

C-terminal cross-linked telopeptide (CTX) of type I collagen are released at acidic pH and are released into circulation from bone matrix, but CTX has a much shorter half-life (1 h) than NTx (11 h) making it easily degradable.^[17] Cross-linked NTx of type I collagen are degradation products of type I collagen and are not a part of soft tissues around the teeth.^[14] Hence, they are accepted as reliable markers for subtle changes during bone resorption reflecting true osteoclastic activity. However, skin and other soft tissues have histidine cross-links and do not have Pyr cross-links.^[18] In this regard, the present study was designed to detect NTx in GCF of healthy, gingivitis, and periodontitis affected subjects. Periodontitis affected subjects were treated with SRP and further evaluated for NTx levels. In addition, the purpose of this study was also to evaluate whether the GCF NTx could be related to clinically identified disease.

N-terminal telopeptides were detected from the GCF collected in precalibrated pipettes. The extra crevicular method was employed to ensure atraumatic, to obtain an undiluted sample of native GCF and also to avoid nonspecific attachment of analyte to filter papers resulting in false reduction of detectable NTx which can underestimate the correlation of NTx levels to disease severity. A standard volume of 1 µL of GCF was collected from the selected sites to avoid discrepancies regarding the sample volume during

analysis. However, 1 µL of sample collection in healthy and gingivitis affected sites was more time consuming due to lack of clinical inflammation and the reduced flow of GCF.

In our study, NTx was not detected in Group I and Group II whereas in Group III and Group IV, it was detected in all the samples and the difference was statistically significant. Detection of NTx in Group III could be related to the greater amount of bone resorption at the diseased sites or the large pocket volume. Decreased values of NTx in Group IV compared to Group III could be speculated to the reduced inflammatory process which in turn, could have arrested the resorptive process at the affected site after SRP. Failure to detect any NTx in Group I and II can be explained by the absence of resorptive process at the sampled site or the levels of NTx which could be much lesser than the sensitive range of the kit (3.2 nm BCE/L to 40 nm BCE/L). Our study has shown the presence of NTx only in Group III and Group IV delineating it from Group II and Group I where there is no resorptive process of bone, being very specific to the transition from gingivitis to periodontitis. Further, the levels of NTx in GCF were found to be highest in periodontitis affected sites which decreased after SRP. GCF NTx may reflect events and conditions which are beyond the reach of the clinical parameters and it is possible that high NTx levels are related to active periodontal destruction. Spearman rho correlation test showed a positive correlation with the clinical parameters and the NTx levels. Till date, no studies have detected NTx in periodontal health, disease and after nonsurgical therapy, however, recently a study by Becerik *et al.*^[10] have measured GCF NTx levels in health and different periodontal diseases and stated that fluctuating NTx levels might point out an abnormal bone turnover in periodontitis, but this study does not state the effect of nonsurgical therapy on NTx levels. Wilson *et al.*^[7] have even measured GCF NTx levels along with other markers in periodontitis affected subjects and concluded that NTx may be useful as a bone resorption marker. Friedmann *et al.*^[9] have even measured NTx in PCF and speculated that its detection may reflect an up-regulated bone turnover

Table 4: Wilcoxon signed ranks test to compare of GCF NTx levels between group III and group IV

Group	n	Mean	SD	Median	Minimum	Maximum	Mean difference	SE	Z	P
Group III	10	6.790	0.9433	6.450	5.4	4.0	1.060	0.302	-2.812	0.005*
Group IV	10	5.73	0.9522	6.000	4.0	7.0				

*Level of statistical significance at $P < 0.05$. GCF=Gingival crevicular fluid, NTx=N-terminal telopeptides, SE=Standard error, SD=Standard deviation

Table 5: Spearman's rho correlation coefficient to correlate GCF NTx levels with clinical parameters

Group	GCF versus PPD		GCF versus GI		GCF versus CAL	
	R	P	r	P	r	P
Group III	0.406 [†]	0.244	0.653 [†]	0.040	0.572 [†]	0.084
Group IV	0.070*	0.705	0.361 [†]	0.305	0.120*	0.692

*r value between 0 and 0.3-weak positive correlation, [†]r value between 0.3 and 0.7-moderate positive correlation. GCF=Gingival crevicular fluid, PPD=Probing pocket depth, GI=Gingival index, CAL=Clinical attachment loss, NTx=N-terminal telopeptides

around implants. However, a study by Isik *et al.* have even failed to detect NTx in GCF during orthodontic intrusive movement. It was concluded that remodeling associated with orthodontic tooth movement may not generate NTx or may remain in tissues without its release into circulation.^[19]

A study conducted by Gursoy *et al.* failed to detect salivary NTx in periodontitis subjects. High thermal denaturation of NTx at a physiologic temperature in comparison with that of ICTP or CTX explained the fragments inability to be detected in the saliva sample.^[20]

There is limited data on the use of GCF NTx in periodontal diseases, but the use of urinary and serum NTx in the field of medicine is enormous. It has been extensively used in monitoring the effectiveness of bisphosphonate therapy, diagnosing multiple myeloma, breast cancers, and many other systemic conditions.^[21-23] Such a marker with high specificity to bone resorption should be utilized in periodontal diagnosis and treatment, as there is a definite need for a sophisticated and a precise predictor. Moreover, a marker that characterizes the transition between gingivitis and periodontitis could be a major discovery in identifying cases of gingivitis that are at risk of progressing to periodontitis and cross-linked NTx could be one such marker.

Our study showed that cross-linked NTx can be successfully estimated in GCF however, longitudinal studies with large sample size are needed to validate NTx as "specific marker of bone resorption." Further, research and new techniques might aid in the development of a very sensitive and a specific chair side NTx test kit in periodontal practice. These chair side kits might open new avenues by reducing the time required for elaborate laboratory investigations and in singling out individuals who are more susceptible to periodontal diseases. In many instances, these tests are valuable in establishing end point of the therapy before placing the patients on maintenance therapy. However, these tools are intended to augment clinician's professional expertise and not replace it.

CONCLUSIONS

The following conclusions can be drawn from the present study:

- Cross-linked NTx can be successfully estimated in GCF of chronic periodontitis subjects
- Cross-linked NTx could mark the transition from gingivitis to periodontitis
- Clinical parameters and the GCF NTx levels can be positively correlated
- Cross-linked NTx could be used as a bone-specific "resorption marker" in periodontal diagnosis.

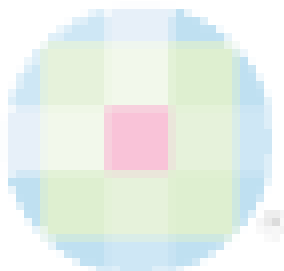
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