Dental Research Journal

Original Article

Plasma levels of N-telopeptide of Type I collagen in periodontal health, disease and after treatment

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Received: May 2015
Accepted: August 2015

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ABSTRACT

Background: To determine plasma concentrations of bone resorption marker cross-linked N-terminal telopeptide (NTx) of Type I collagen in periodontal health, disease and after nonsurgical periodontal therapy in chronic periodontitis group. In addition, to know the association between plasma NTx levels and the different clinical parameters.

Materials and Methods: Thirty subjects were divided on the basis of their periodontal status and were categorized as Group I: Healthy, Group II: Gingivitis, and Group III: Chronic periodontitis. Group III subjects were treated with scaling and root planing, 6-8 weeks later blood samples were analyzed, and they constituted Group IV. NTx levels in plasma were analyzed by competitive - enzyme-linked immunosorbent assay. All data were analyzed using statistical software (SPSS) (α = 0.05).

Results: All the samples tested positive for the presence of NTx. The mean NTx concentration was highest in Group III (18.77 nanomole Bone Collagen Equivalent [nm BCE]) and the lowest in Group IV (16.02 nm BCE). The values of Group I and Group II fell between the highest and the lowest values (16.23 nm BCE and 16.70 nm BCE, respectively). The difference in mean NTx levels in Group III and Group IV were statistically significant. NTx levels in all the groups positively correlated with the clinical parameters. All data were analyzed using statistical software (SPSS) (α = 0.05).

Conclusion: Within the limits of this study, it may be suggested that plasma NTx levels may provide distinguishing data between periodontally healthy diseased sites and after nonsurgical therapy of diseased sites.

Key Words: Collagen, periodontitis, plasma, resorption

INTRODUCTION

Periodontitis is an inflammatory condition initiated by chronic microbial load affecting the tooth-supporting tissues. Despite studies focusing on microbial biofilm as the causative factor for periodontitis, there is a shift toward osteoimmunology which explains the interaction between host immune responses and cytokines in the development of periodontal diseases.[1]

Bone remodeling being a multifaceted process requires several cross-talk mechanisms, and pathological activation of one system is bound to affect the other. During inflammation, the balance between formation and resorption is skewed toward osteoclastic resorption leading to the release of bone breakdown products into local tissues and also

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www.ncbi.nlm.nih.gov/pmc/journals/1480

How to cite this article: Aruna G. Plasma levels of N-telopeptide of Type I collagen in periodontal health, disease and after treatment. Dent Res J 2016;13:18-23

to the systemic circulation. Collagen cross-links are generally reliable markers of bone resorption because they are stable in plasma and urine. As they result from posttranslational modification of collagen molecules they cannot be reclaimed for collagen synthesis, therefore, are highly specific to bone resorption.[2] In addition, calcium kinetic studies of the bone formation and resorption have also shown that the cross-links of collagen correlate highly with resorption.[3]

Cross-linked N-terminal telopeptide (NTx) of Type I collagen is an amino-terminal telopeptide which is exceptional because of its α-2(I) N-telopeptide. It is released as a resolute end product of bone resorption and is not a part of soft tissues around the teeth.[4,5] Skin and other soft tissues have histidine cross-links and do not have pyridinoline crosslinks.[6]

Studies assessing the role of NTx in gingival crevicular fluid (GCF), serum and peri-implant crevicular fluid (PCF) as a diagnostic marker of periodontal disease activity have reported conflicting results so far. Friedmann *et al*.[7] have studied the levels of NTx in GCF and PCF and speculated that increased NTx levels may predict extensive bone destruction earlier than 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.[8] Becerik *et al*.[9] 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.[10]

To date changes associated with plasma NTx levels in healthy, gingivitis, chronic periodontitis (CGP), and after nonsurgical periodontal therapy of CGP have not been clarified. Our hypothesis states that alterations in plasma levels of NTx may be one of the systemic manifestations of periodontal bone resorption. Thus, the aim of this study was to investigate whether periodontally healthy, gingivitis and CGP subjects exhibit different plasma levels of NTx, to know the levels of NTx in CGP subjects after nonsurgical periodontal therapy and to correlate the levels with the clinical parameters.

MATERIALS AND METHODS

The subjects enrolled in this study were fully informed about the protocol of this study and written informed consent was obtained according to the Helsinki Declaration. The study was approved by the Ethical Committee of the Institution. A total of 30 subjects were recruited from February 2007 to January 2008. Subjects were matched to eliminate age (25-50 years) and sex as confounding factors (Table 1). Exclusion criteria included a history of periodontal therapy, use of antibiotics, anti-inflammatory drugs within the previous 3 months, pregnancy, or lactation, systemic diseases and smokers. Patients on bisphosphonates, alendronates, hormone replacement therapy, Vitamin D, and calcium supplements were also excluded.

Patients were categorized into three groups based on probing pocket depth (PPD), clinical attachment loss (CAL), gingival index (GI) scores (Loe and Sillness 1986) and radiographic evidence of bone loss (assuming the physiologic distance from 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 CGP from patients of other groups, without any delineation in the extent of alveolar bone loss.

- Group 1: 10 subjects with clinically healthy periodontium (GI = 0, PPD ≤3 mm, and CAL = 0).
- Group 2: 10 subjects with gingival inflammation (GI >1, PPD ≤3 mm, and CAL = 0).
- Group 3: 10 subjects who showed clinical signs of gingival inflammation GI >1, PPD ≤5 mm and radiographic bone loss with CAL ≥3 mm.
- Group 4(after treatment): Subjects of Group 3 treated with scaling and root planing (SRP) (plasma samples taken 6-8 weeks after treatment).

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.

Plasma samples collection

The skin over the antecubital fossa was disinfected, and 2 ml of blood was collected by venipuncture using a 20-gauge needle with 2 ml syringes. Vacutainer previously coated with 3.2% sodium citrate was used and was centrifuged at 1000 rpm for 10 min (1000 g, 4°C) to separate the plasma component. The plasma was extracted within 30 min and stored at-70°C until the time of the assay procedure.[11]

The assay procedure was done according to the kit-manufacturers' instructions. Competitive inhibition assay procedure is often used to measure small analytes because it requires the binding of 1 antibody rather than 2 as used in standard enzyme linked immunosorbent assay (ELISA) formats. Here monoclonal antibody (MoAb) is coated onto 96-well microtiter plate. When the sample is added, the MoAb captures the free analyte out of the sample. In the next step, a known amount of analyte labeled with biotin is added. The labeled analyte will also attempt to bind to the MoAb absorbed onto the plates; however, the labeled analyte is inhibited from binding to the MoAb by the presence of previously bound analyte from sample. This means that the labeled analyte will not be bound by the MoAb on the plate, if the MoAb has already bound unlabeled analyte from sample. The amount of unlabeled analyte in the sample is inversely proportional to the signal generated by the labeled analyte. NTx was quantitated using a commercially available competitive-inhibition ELISA (Ostex, Osteomark, Seattle, WA, USA) and expressed as nanomole Bone Collagen Equivalents (nm BCEs). Sensitivity range of the ELISA kit to detect NTx is 3.2 nm BCE to 40 nm BCE.

Statistical analyses

All data were analyzed using statistical software (SPSS version 10.5, SPSS, Chicago, IL, USA). A test for the validity of the normality assumption was carried out using Shapro–Wilk test; if data were normal then parametric tests were carried out otherwise the nonparametric test was carried out to compare between the groups. Analysis of variance was carried out to find out if all four groups differed significantly (Table 2). Further, pairwise comparisons using the Scheffe 's test were carried out to explore which pair or pairs differed with respect to gingival parameters (Table 3). Nonparametric Kruskal–Wallis test was carried out to find out the difference among four groups further Mann-Whitney test was used to compare the pair difference. Wilcoxon signed ranks test was used to compare the difference among the groups with respect to CAL and Paired *t* test was done to compare NTx levels in Group III and Group IV. The Spearman rho correlation coefficient test was used to find any association between the clinical parameters and GCF concentration. The level of statistical significance was set at $P < 0.05$. NTx mean difference values (before and after treatment) were considered to calculate the power of the study. A sample of 20 achieved 87% power to detect the mean paired difference of 1.1 with an estimated standard deviation 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

All the samples tested positive for the presence of NTx. The mean NTx concentration was highest in

Table 2: Comparison of mean clinical parameters between the groups

Clinical parameter	Group I	Group II	Group III	Group IV	Test value	*P*
One-way ANOVA test to compare mean GI between the groups						
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; CAL: Clinical attachment loss; PPD: Probing pocket depth; ANOVA: Analysis of variance.

Group III (18.77 nm BCE) and the lowest in Group IV (16.02 nm BCE). The values of Group I and Group II fell between the highest and the lowest values (16.23 nm BCE and 16.70 nm BCE, respectively). Statistical significance was seen between mean NTx levels of Groups I, II and III but not between Groups I, II, and IV [Tables 4-6] however, the difference between Groups III and IV was statistically significant [Table 7]. There was also a positive correlation between the clinical parameters and the mean NTx levels [Tables 8 and 9].

DISCUSSION

The traditional method of assessing probing depth, gingival bleeding, and plaque score along with clinical attachment level and X-rays has been extensively used by the clinicians. However, these measurements neither give information on disease activity nor the susceptibility of patients towards disease progression. Hence, this drawback has directed the clinicians to explore various markers in biofluids such as plasma, saliva, urine, and GCF. Few of the extensively studied markers are the interleukins, tumor necrosis factor, matrix metalloproteinases, etc., but, these markers are general inflammatory signals and are not specific to bone destruction.[12] Furthermore, it has even been speculated that higher levels of these markers in systemic circulation could be a result of spillover from the local tissues.

Biochemical monitoring of bone metabolism depends upon measurement of enzymes and proteins released during bone formation and degradation of products produced during bone resorption. However, the diagnosis of active phases of periodontal disease and the identification of patients at risk for disease presents a major challenge to the clinicians. In this, regard various biochemical markers are available that allow a specific and sensitive assessment of bone formation and bone resorption.[13]

However, these bone markers exhibit substantial short-term and long-term fluctuations related to diet, time of day, the phase of the menstrual cycle, season of the year, exercise, and anything else that alters bone remodeling. These biological factors, in addition to assay imprecision, produce significant intra- and inter-individual variability in markers.[14] The most important biologic factors are diurnal and day-to-day variability in bone forming and bone-resorbing activities. Bone turnover marker levels are highest in the early morning and lowest in the afternoon and evening. Levels of urinary markers can vary 20-30% from the highest to the lowest value of the day. Plasma markers change to a smaller degree except for carboxy terminal telopeptides (CTx), which can vary by more than 60% during the day.[15] The plasma markers of bone formation appear to vary less from day to day.[16]

In our exploratory study, the plasma NTx was assessed using a competitive inhibition ELISA and all the samples showed the presence of NTx. The reference values for NTx in men and women are 14.8 and 12.6, respectively (as per the Ostemark® NTx serum kit) and in our study the highest plasma NTx levels were seen in the periodontitis group and the lowest in after treatment group. The healthy and gingivitis levels fell between the highest and lowest values. One possible explanation for this finding is the active phase of bone

Table 3: Pairwise comparison between the groups

Clinical parameters	Groups	*P*
Scheffe's 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.

Table 4: Results of ANOVA comparing the mean NTx levels in plasma between Groups I, II and III

Serum	Sum of squares	df	Mean squares	*F*	Significance
Between groups	36.525	2	18.262	7.038	0.003*
Within groups	70.062	27	2.595		
Total	106.587	29			

*The mean difference is significant at 0.05 level; NTx: N-terminal telopeptide; ANOVA: Analysis of variance.

Table 5: Results of ANOVA comparing the mean NTx levels in plasma between Groups I, II, and IV

Serum	Sum of squares	df	Mean squares	*F*	Significance
Between groups	2.425	2	1.212	0.322	0.727
Within groups	101.537	27	3.761		
Total	103.962	29			

NTx: N-terminal telopeptide; ANOVA: Analysis of variance.

Table 6: Multiple comparison using Bonferroni test for Group I, II, and III NTx levels in plasma

Group (I)	Group (J)	Mean difference (I-J)	SE	Significant	95% CI	
					Lower bound	Upper bound
Group I	Group II	−0.47000	0.72040	1.000	−2.3088	1.3688
	Group III	−2.54000*	0.72040	0.005	−4.3788	−0.7012
Group II	Group I	0.47000	0.72040	1.000	−1.3688	2.3088
	Group III	−2.07000*	0.72040	0.023	−3.9088	−0.2312
Group III	Group I	2.54000*	0.72040	0.005	0.7012	4.3788
	Group II	2.07000*	0.72040	0.023	0.2312	3.9088

*The mean difference is significant at 0.05 level; NTx: N-terminal telopeptide; SE: Standard error; CI: Confidence interval.

Table 7: Paired *t*-test to compare NTx levels in plasma in Group III and Group IV

Group	Mean	SD	Mean difference	*t*	*P*
Group III	18.77	1.51	2.750	2.876	0.018*
Group IV	16.02	2.40			

*The mean difference is significant at 0.05 level; SD: Standard deviation; NTx: N-terminal telopeptide.

Table 8: "Wilcoxon signed ranks test" to compare CAL in Group III and Group IV

Group	Mean	SD	Minimum	Maximum	*Z*	*P*
Group III	3.4	0.52	3	4	−2.842	0.004*
Group IV	1.6	0.52	1	2		

*The mean difference is significant at 0.05 level; SD: Standard deviation; CAL: Clinical attachment loss.

Table 9: Spearman rank correlation test comparing plasma NTx with GI, PPD, and CAL

Group	Plasma with GI	Plasma and CAL	Plasma and PPD
Group I	#	#	0.634*
Group II	#	#	0.567*
Group III	0.647*	0.398*	0.546*
Group IV	0.545*	0.218*	0.765*

*If the "*r*" value is between 0 and 0.5, there is a weak positive correlation; if the "*r*" value is between 0.5 and 1, there is a strongly positive correlation; and if *r* is 1, there is 100% positive correlation between the two sets of data compared. #Since CAL is 0 and NTx level is below the detection limit of the kit in Group I and Group II (GCF) these correlations are N/A. N/A: Not applicable; GCF: Gingival crevicular fluid; NTx: N-terminal telopeptide; GI: Gingival index; CAL: Clinical attachment loss; PPD: Probing pocket depth.

resorption in periodontitis group leading to release of collagen breakdown fragments into the circulation and further reduction in the resorptive phases after SRP. Unlike the periodontitis group and after treatment group, healthy and gingivitis group showed no significant difference most likely, due to the absence of alveolar bone destruction or the levels below the sensitivity range of the assay kit which could not contribute necessarily to the systemic circulation.

Similarly, study by Wilson *et al*. detected NTx in the serum samples and it was stated that serum represents combined bone turnover activity of both trabecular and cortical bone, and the bone turnover rate of trabecular bone is greater than the cortical bone.[8]

Studies in dental literature have highlighted the use of NTx in other samples such as GCF, PCF, saliva, and the results are not confirming. Friedmann *et al*. have studied the levels of NTx in GCF and PCF and speculated that increased NTx levels may predict extensive bone destruction earlier than calprotectin.[7] A study conducted by Gursoy *et al*.[17] failed to detect salivary NTx in periodontitis subjects concluding that high thermal denaturation of NTx at physiologic temperature in comparison with ICTP and CTx explained the inability of NTx to be detected in the saliva sample. Moreover, study by Isik *et al*.[18] have even failed to detect NTx in GCF during orthodontic intrusive movement speculating that remodeling associated with orthodontic tooth movement may not generate NTx or may remain in tissues without its release into circulation.

Our previous study evaluated NTx levels in GCF, NTx was detected only in periodontitis and after treatment group, however, inability of NTx to be detected in healthy and gingivitis was attributed to absence of resorptive process at the sampled site. [19]

It has been proposed that patients with periodontitis may have elevated circulating levels of some inflammatory markers. Monocytes, macrophages, and other cells respond to the dental plaque microorganisms by secreting a number of chemokines and inflammatory cytokines. The elevation in cytokine expression by cells within the gingival connective tissue in chronic periodontitis lesions can theoretically spill over into the circulation where it can induce or perpetuate systemic effects.[20] Furthermore, the plasma provides information about the inflammatory stimulus and/or response generated in circulation toward the periodontal pathogens.[21]

Although several authors have highlighted the use of these markers in systemic conditions,[22,23] no studies

have hypothesized their causative role on a systemic level *per se* Further research aiming the process of resorption and explaining collagen breakdown products as mere products of resorption or their ability to perpetuating a disease process are required.

CONCLUSION

1. Plasma NTx levels can differ substantially with respect to periodontal health, disease and after treatment of chronic periodontitis subjects.
2. NTx levels in plasma can be positively correlated with the clinical parameters.
3. The use of biochemical markers in medical practices are controversial, as interpreting the values for individual patients are complex related to the intricacies inherent in bone metabolism.
4. Lack of standardization has led to unacceptable levels and variation.

ACKNOWLEDGMENTS

I would like to acknowledge Dr. Suman, Dr. Vidyapriya Dharshini. Praveena M. N. and Lalitha Ganganna for helping me write the manuscript.

Financial support and sponsorship
Nil.

Conflicts of interest
None declared.

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