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Research Article

GINGIVAL CREVICULAR FLUID LEVELS OF N-TELOPEPTIDE OF TYPE I COLLAGEN BEFORE AND AFTER NON- SURGICAL PERIODONTAL THERAPY IN GENERALIZED CHRONIC PERIODONTITIS SUBJECTS

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ABSTRACT

Aim: To estimate the levels of N-telopeptide of type I collagen (NTx) in gingival crevicular fluid (GCF) in periodontally healthy, gingivitis, generalized chronic periodontitis subjects and after nonsurgical periodontal therapy for periodontitis patients along with its association with the clinical parameters.

Materials and Methods: A total of 72 subjects were selected from the out-patient department of Periodontology, Meenakshi Ammal Dental College and Hospital. The subjects were categorized as: (Group I) Healthy, (Group II) gingivitis, and (Group IIIA) generalized chronic periodontitis, Group IIIA subjects after scaling and root planing, constituted Group IIIB. GCF samples were analyzed by competitive- Enzyme Linked Immunosorbent Assay (ELISA) for NTx.

Results: There was a statistically significant reduction in PPD and gain in CAL in Group IIIB when compared to Group IIIA. Mean NTx levels were higher in group IIIA (23.42±11.41 nm BCE/L) compared to group IIIB (11.74±5.43 nm BCE/L) which was statistically significant. Positive correlation was seen between the clinical parameters and the NTx levels in Group IIIB.

Conclusion: Cross linked NTx can be used as a bone specific "resorption marker" in periodontal diagnosis.

INTRODUCTION

Biochemical bone turnover marker measurements cannot independently establish a specific bone disease diagnosis. They do reflect metabolic abnormalities such as accelerated bone turnover and thus allow dynamic assessment of bone remodelling and monitoring of treatment response in metabolic bone diseases. (Baim S *et al* 2009). Informative biomarkers can further serve as early sentinelsof disease, and this has been considered as the most promising alternative to classic environmental epidemiology. (Khashu H *et al* 2012).

The discovery of Cross Linked N-telopeptides of type I Collagen (NTx) has provided specific biochemical marker of human bone resorption which can be analyzed by immunoassay. The N-telopeptides of type I collagen molecule is specific to bone due to the unique amino acid sequences and orientation of the cross linked alpha-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. Detection of such a molecule in gingival crevicular fluid (GCF) might lead to the development of a marker directly related to bone resorption in periodontitis. (Wilson AN *et al* 2003).

A number of studies have reported the acceptability of Cross-linked N-terminal telopeptide (NTx) of type I collagen as a reliable marker for subtle changes in bone turnover. Gertz *et al* (1998) stated that NTx are not further degradable and represent the end products of bone type I collagen degradation, thus reflecting the true osteoclastic activity. Friedmann *et al* (2006) 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 calprotectin levels. Becerik *et al* (2011) 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.

One foreseeable benefit of an oral fluid–based periodontal diagnosis would be identification of highly susceptible individuals prior to overt disease. Timely detection and diagnosis of disease may considerably affect the clinical management of periodontal patients by offering earlier, less invasive and more cost-effective treatment therapies. (Kinney JS *et al* 2007). Thus, the aim of this study was to estimate the GCF levels of N- Telopeptide of type I collagen before and after non-surgical periodontal therapy in generalized chronic periodontitis subjects.

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MATERIALS AND METHODS

The study population comprised of 72 subjects attending the Outpatient Department of Periodontology, Meenakshi Ammal Dental College and Hospital, Chennai, India, from March 2015-June 2016. Subjects were in the age group of 25-50 years. 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 inclusion criteria included:(i)Systemically healthy subjects, (ii) Subjects with age group between 25-50 years, (iii) Presence of at least 20 teeth, (iv) Subjects with only gingivitis and (v) Subjects with generalized chronic periodontitis.

The exclusion criteria included: (i) Subjects with aggressive periodontitis, (ii) Bone disorders, (iii) Pregnant, lactating and postmenopausal subjects, (iv) Subjects on antibiotics, (v) Subjects taking drugs affecting bone metabolism like Bisphosphonates, Alendronates, Hormone Replacement Therapy, anti-inflammatory drugs, Vitamin D and Calcium Supplements, (vi) Smokers, (vii) Subjects with a history of periodontal treatment within six months.

Patients were categorized into three groups based on Probing Pocket Depth (PPD), Clinical Attachment Loss (CAL), Gingival Index Scores (GI) (Loe & Sillness 1963) and radiographic evidence of bone loss (assuming the physiologic distance between the cemento-enamel junction to alveolar crest to be 2mm). After a full mouth periodontal probing, bone loss was recorded dichotomously using intra-oral periapical radiographs to differentiate chronic periodontitis subjects from patients of other groups, without any delineation of the extent of alveolar bone loss.

Group I (Healthy): This group consisted of 24 subjects with clinically healthy periodontium (GI < 1, PPD ≤ 3mm).

Group II (Gingivitis): This group consisted of 24 subjects who showed clinical signs of gingival inflammation but there was no evidence of attachment loss. The intraoral periapical radiographs did not show any bone loss. (GI >1, PPD≤ 3mm).

Group IIIA (Generalized Chronic Periodontitis): This group consisted of 24 subjects who showed clinical signs of gingival inflammation and attachment loss with radiographic evidence of bone loss. (GI > 1, PPD ≥ 5mm and radiographic bone loss with CAL ≥ 5mm)

Group IIIB (After Treatment Group): This group consisted of subjects from Group IIIA, who were treated with non-surgical periodontal therapy (SRP).

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 mesio-buccal 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 IIIA subjects, sites with ≥ 5mm of CAL were identified, and the site showing the highest clinical attachment loss, along with the radiographic 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 gingiva, and GCF was collected using color-coded 1-5µL calibrated, volumetric microcapillary pipettes (Sigma-Aldrich chemical co. Ltd, StLouis, MO, USA). From each test site, a standardized volume of 1 µL 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 -70C until the assay.

Non-Surgical Periodontal Therapy

After collection of GCF at baseline, subjects with generalized chronic periodontitis were treated with scaling and root planing. After 8 weeks, the clinical parameters were recorded and GCF was collected from the same site and assigned as Group IIIB. All the GCF samples collected were immediately transferred to plastic vial and stored at -70ºC until the assay.

Competitive Inhibition Assay

NTx was quantitated using a commercially available competitive-inhibition enzyme-linked immunosorbent assay (Fine Test, Wuhan Fine Biological Technology Co.,Ltd, China) and expressed as nanomole Bone Collagen Equivalents per liter (nm BCE/L). Sensitivity range of the ELISA kit to detect NTx was 3.125 nm BCE/Lto 200 nm BCE/L.

Statistical Analysis

Statistical analysis was done using Statistical Package for Social Sciences (SPSS) version 20.1 (Chicago, USA Inc). Mean and Standard Deviation was estimated for both the groups. The Pearson's correlation test was used to evaluate relationship between variables. In the present study, $p \leq 0.05$ was considered as the level of significance.

RESULTS

The age and gender distribution in all the three groups is shown in Table 1.

Table 1 Age and gender distribution in group I, group II and group IIIA.

Variables		Group I (N = 24)	Group II (N = 24)	Group IIIA (N = 24)	*P value*
Gender	Male	13 (54.2%)	11 (48.8%)	12 (50%)	-
	Female	11 (48.8%)	13(54.2%)	12 (50%)	
Age (years)		27.54 ± 2.3	33.08 ± 5.0	39.87± 4.4	0.000**

Comparison of mean gingival index, probing pocket depth and CAL between the groups showed statistical significance. Mean NTx levels of group II and group IIIA subjects were higher when compared to group I. On comparison of group II and group IIIA, mean NTx levels showed statistical significance (Table 2).

Mean gingival index, probing pocket depth, CAL and NTx levels reduced following non-surgical periodontal therapy in group IIIB and the reduction was statistically significant (Table 3).

Table 2 Inter-group comparison of gingival index (GI), probing pocket depth (PPD), clinical attachment level (CAL) and GCF NTx levels in group I, II and IIIA.

Variables	GROUP I	GROUP II	GROUP IIIA	Comparison between Group I and Group II (p value)	Comparison between Group II and Group IIIA (p value)	Comparison between Group I and Group IIIA (p value)
Gingival Index	0.72 ± 0.20	1.46 ± 0.27	1.71 ± 0.28	0.000**	0.003**	0.000**
Probing Pocket Depth (mm)	1.75 ± 0.19	2.28 ± 0.27	6.07 ± 1.02	0.013*	0.000**	0.000**
Clinical Attachment Level (mm)	1.75 ± 0.19	2.28 ± 0.27	6.37± 1.16	0.031*	0.000**	0.000**
NTx (nm BCE/L)	10.85 ± 4.17	15.85 ± 5.97	23.42 ± 16.71	0.076 (NS)	0.004**	0.000**

Table 3 Inter-group comparison of gingival index (GI), probing pocket depth (PPD), clinical attachment level (CAL) and GCF NTx levels in group IIIA and group IIIB.

Variables	GROUP IIIA	GROUP IIIB	p value
Gingival Index	1.71±0.28	0.78 ± 0.17	0.000**
Probing Pocket Depth (mm)	6.07±1.02	4.27 ± 0.76	0.000**
Clinical Attachment Level (mm)	6.37±1.16	4.42± 0.94	0.000**
NTx (nm BCE/L)	23.42±11.41	11.74± 5.43	0.000**

The correlation between periodontal parameters and NTx levels in group IIIA suggests that there was no significant correlation in the GI and NTx levels (p value 0.599), the PPD and NTx levels also showed no significant correlation (*p* value 0.762). Similarly, there was no significant correlation between CAL and NTx levels in group IIIA (*p* value 0.579). In group IIIB the correlation between periodontal parameters and NTx levels suggested that there was a significant correlation in the GI and NTx levels (p value 0.000), the correlation was significant in the PPD and NTx levels (*p* value 0.000). Similarly, there was a significant correlation in the CAL and NTx levels of group IIIB (*p* value 0.000) (Table 4).

Table 4 Correlation between gingival index, probing pocket depth and clinical attachment level with NTx levels in Group IIIA and Group IIIB.

Periodontal Parameters	Pearson Correlation	NTx (nm BCE/L)	
		Group IIIA	Group IIIB
Gingival Index	R- value	0.112	0.810
	***p*-value**	0.599(NS)	0.000**
Probing Pocket Depth (mm)	R- value	-0.065	0.699
	***p*-value**	0.762(NS)	0.000**
Clinical Attachment Level (mm)	R- value	-0.119	0.718
	***p*-value**	0.579(NS)	0.000**

DISCUSSION

Advances in bone cell biology over the past decade have resulted in several new biochemical markers for the measurement of bone homeostasis. As reviewed by Loos and Tjoa (2005), more than 90 different components in GCF have been evaluated for periodontal diagnosis. Of the numerous constituents in GCF, however, the vast majority constitute soft tissue inflammatory events, while only a few are regarded as specific biomarkers of alveolar bone destruction. Bone-related biomarkers from oral fluids associated with periodontal diseases include alkaline phosphatase (ALP), cathepsin B, collagenase-2 (MMP-8), gelatinase (MMP-9), collagenase-3 (MMP-13), calprotectin, osteocalcin, pyridinoline cross-links (ICTP), osteonectin and osteopontin.

Two of the well-known mediators of bone loss studied in GCF include pyridinoline cross-linked carboxy terminal telopeptide of type I collagen and osteocalcin. However both these markers are also released during soft tissue loss making it less specific to bone resorption. (Nakashima K *et al* 1994). C-terminal cross linked telopeptide of type I collagen (CTx) has a much shorter half-life (1 hr) when compared to NTx (11 hr) which makes it easily degradable. (Cremer S *et al*2008). This Cross-linked N-terminal telopeptides of type I collagen (NTx) which is an amino-terminal telopeptide is a recent addition to this group of bone related proteins identified in GCF. It is exceptional because of its α-2(I) N-telopeptide and is released as a resolute end product of bone resorption. (Alfaqeeh SA *et al* 2011). 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. (Ishihara Y*et al* 2006). Hence, it is accepted as reliable marker for subtle changes during bone resorption reflecting true osteoclastic activity.

The present study was designed to detect NTx levels in GCF of healthy, gingivitis and periodontitis affected subjects. The periodontitis subjects were treated with SRP and evaluated again after 8 weeks for NTx levels. There are various methods to collect GCF; in this study it was collected using extracrevicular method to ensure atraumatism and to obtain an undiluted sample of native GCF whose volume can be accurately assessed. (Griffiths GS *et al* 2003).

In our study, GCF levels of NTx were assessed using a sandwich enzyme-linked immunosorbent assay technology and all the samples showed the presence of NTx. This assay has high sensitivity and excellent specificity for detection of NTx. Alveolar bone is always in a state of flux, but in health there is a balance between the rate of bone formation and rate of bone destruction. So, healthy and gingivitis group showed lower levels of NTx when compared to generalized chronic periodontitis group. During periodontal disease, the balance is tipped towards destruction and active bone resorption takes place. So in group IIIA, the GCF levels of NTx were significantly higher compared to Group I and Group II. A similar study done by Aruna G *et al* (2015) failed to detect NTx levels in healthy and gingivitis subjects because the levels of NTx could have been much lesser than the sensitive range of the elisa kit used in the study.

Following baseline measurements, periodontitis affected subjects underwent nonsurgical therapy (SRP), to form Group IIIB. After 8 weeks, clinical measurements and GCF collection were repeated. The mean GI, PPD, CAL and NTx values at 8

weeks reduced significantly when compared to the baseline values, the levels of NTx was found to be highest in periodontitis subjects which decreased significantly after SRP. Becerik *et al* (2001) and Wilson *et al* (2003) have measured GCF NTx levels in periodontitis affected subjects and concluded that NTx may be useful as a bone resorption marker. A recent study by Aruna G *et al* (2016) measured the plasma levels of NTx in healthy and periodontitis subjects, they concluded that NTx levels differ substantially with respect to periodontal health, disease and after treatment of chronic periodontitis subjects.

In inflamed periodontal tissues, along with the breakdown of periodontal structures, resorption of alveolar bone also occurs. The factors involved in bone destruction are bacterial and host-mediated. Bacterial plaque products induce the differentiation of bone progenitor cells into osteoclasts and stimulate gingival cells to release mediators that have the same effect. They can also act directly on osteoblasts or their progenitors, inhibiting bone formation and reducing their numbers. Many host factors released by inflammatory cells like prostaglandin E2, interleukin (IL) - 1α, IL-1β and tumor necrosis factor (TNF) – α are capable of inducing bone resorption and play a role in progression of periodontal disease.

We have also correlated the GCF levels of NTx with the progression of the periodontal disease. The NTx levels were correlated with clinical parameters assessed at baseline and at 8 weeks. There was no significant correlation between GI, PPD, CAL and NTx levels at baseline in group IIIA. This might be because of exaggerated clinical measurements due to the presence of tissue inflammation. So they did not correlate with the amount of bone resorption, which is reflected as low GCF levels of NTx. In group IIIB there was a significant correlation between GI, PPD, CAL and NTx levels. This indicates that the reduction of gingival index, probing pocket depth and gain in clinical attachment level at 8 weeks is associated with reduction in GCF levels of NTx.

Nonsurgical periodontal therapy is a critical aspect of periodontal treatment. Gingival inflammation is usually substantially reduced or eliminated within 3 to 4 weeks after removal of local factors. Gradual reductions in inflammatory cell population, crevicular fluid flow, and repair of connective tissue result in decreased clinical signs of inflammation and alveolar bone loss. This is clinically manifested as reduced GI score, improvement in probing pocket depth, gain in clinical attachment level and reduction in the GCF levels of NTx.

The results of our study show that GCF NTx levels are reduced in group IIIB which could be attributed to the beneficial effect of scaling and root planing. Within the limitations of this study, it is fair to assume that the estimation of NTx levels in GCF has the potential to serve as a diagnostic marker for periodontal disease and its response to nonsurgical periodontal therapy.

CONCLUSION

To conclude, non-surgical periodontal therapy leads to reduction in both clinical parameters and GCF NTx levels. The use of this biomarker NTx as a diagnostic tool for bone resorption can be established only after the validation of the findings by multiple studies and larger sample size.

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