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GINGIVAL CREVICULAR FLUID OSTEOPONTIN LEVELS IN HEALTHY AND PERIODONTALLY DISEASED GROUPS BEFORE AND AFTER NON-SURGICAL TREATMENT - A COMPARATIVE BIOCHEMICAL STUDY

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ABSTRACT

Aims and objectives: The aim of the study was to estimate the levels of osteopontin (OPN) in gingival crevicular fluid (GCF) of subjects with clinically healthy periodontium and subjects with gingivitis and chronic periodontitis and also to investigate the effect of treatment (scaling and root planing) on osteopontin levels in GCF in chronic periodontitis subjects.

Materials and methods: A total of 45 subjects in the age group of 20-45 years were selected. The subjects were divided into three groups as Group I (Healthy), Group II (Gingivitis) and Group III (Chronic periodontitis). Group IV consisted of 15 subjects of group-III, who were treated with non-surgical approach. Samples of GCF were collected at the initial visit in Group I, Group II and Group III subjects. Scaling and root planing was performed for Group III subjects. After 8 weeks, GCF was collected from the same site of the subjects in Group III. The concentration of osteopontin was determined using a human osteopontin enzyme immunometric assay (EIA) kit. **Results**: The highest mean OPN concentration in GCF (12.733 µg/ml) was observed in Group III and the lowest mean OPN concentration (1.946 µg/ml) was observed in Group I. After treatment, the mean concentration of OPN in GCF decreased from12.733 µg/ml to 5.690 µg/ml (Group IV). OPN levels in GCF increased proportionally with the progression of periodontal disease and reduced significantly following treatment.

Conclusion: The results observed in this study indicate that osteopontin could serve as a potential biomarker of periodontal disease progression.

Keywords: GCF, osteopontin, EIA kit

INTRODUCTION

Periodontitis is a chronic inflammatory response to the subgingival bacteria, producing irreversible periodontal tissue destruction and tooth loss. The progression of periodontitis is chronic, with cyclic periods of exacerbation and remission, and may remain unnoticed with minimal symptoms in the early stages.

The host immune and inflammatory response to the microbial challenge is a critical determinant of susceptibility to develop the destructive disease, under the influence of multiple behavioral, environmental and genetic factors. Hence, although disease progression is episodic in nature on a tooth site level, the risk of developing periodontal disease is principally patient-based rather than site-based.

Periodontitis is diagnosed clinically by loss of attachment between the tooth and the supporting tissues (clinical attachment loss), by deepening of the pocket between the root of the tooth and the supporting tissues (pocket depth), and/or by radiographic evidence of bone loss. The clinical measurements used in the diagnosis of periodontitis are of limited usefulness, as they more often indicate previous periodontitis rather than current disease activity.

Human gingival crevicular fluid obtained from patients with periodontal disease contains periodontopathic bacteria, inflammatory cells, degradation products of periodontal tissues and several other components which are involved in inflammatory and immune reactions. These components include inflammatory cytokines such as IL-1β, TNF-α, prostaglandins and several enzymes. Some of these components serve as biochemical markers of periodontal inflammation and tissue degradation because the components in GCF increase in accordance with the severity of periodontitis and then correlate with the degree of periodontal inflammation and disease activity.

Recently, several bone-related proteins were identified in GCF which includes osteocalcin (OCN), osteonectin (ON), bone phosphoprotein and telopeptides of type I collagen, which may be important as markers of bone resorption in periodontal disease.

Osteopontin (OPN) is a non-collagenous, highly phosphorylated, sialic acid rich, calcium binding glycosylated phosphoprotein, which has an arginine-glycine-aspartic acid (RGD) sequence. It is an extracellular-matrix cell-adhesion protein, which is abundant in bone and synthesized mainly by preosteoblasts, osteoblasts, and osteoclastic cells that are localized in the mineralized phase of bone matrix.

Osteopontin appears to play a role in the action of osteoclasts by binding them to the mineralized matrix of bone. Osteoclasts synthesize osteopontin and osteopontin might be involved in the regulation of bone resorption, following osteoclast adhesion to bone matrix. In the inflammatory process, OPN is known to be a multifunctional cytokine and is chemotactic for various cell types including monocytes/macrophages. It is essential for the cell-mediated immunity and normal Th-1 cytokine response during granuloma formation.[1]

Recently, in the studies done by **Kido et al (2001)**[2] and **Sharma et al (2006)**[3], the presence of the OPN molecule was reported in GCF, and the increase in its levels was found commensurate with the progression of periodontal disease, suggesting that OPN levels in GCF may be considered a marker of alveolar bone destruction.

This clinico-biochemical study was designed to estimate the levels of osteopontin (OPN) in gingival crevicular fluid (GCF) of subjects with clinically healthy periodontium and subjects with gingivitis and chronic periodontitis and also to investigate the effect of treatment (scaling and root planing) on osteopontin levels in GCF in chronic periodontitis subjects.

MATERIALS AND METHODS

A total of 45 subjects in the age group of 20-45 years were selected from the outpatient section, Department of Periodontology, Meenakshi Ammal Dental college and Hospital, Chennai, based on the criteria for subject grouping as given below. Ethical clearance for the study was obtained from the ethical committee of the MAHER University.

Subjects with aggressive forms of periodontitis, history of diabetes, subjects who need antibiotic prophylaxis and taking drugs affecting bone metabolism like bisphosphonates, steroids, contraceptives, anti-inflammatory drugs or antibiotics (in the last 6 months) or receiving any periodontal treatment, anomalies of blood and immune system, smoking and alcoholism and bone disorders were excluded from the study.

Subjects underwent brief case history recording which included patient's chief complaint, medical and dental history, oral examination with full-mouth periodontal probing and charting and radiographic examination. Clinical measurements recorded were, Modified Gingival Index (Lobene et al, 1986)[4], clinical Attachment Level and radiographic bone loss. Radiographic bone loss was recorded dichotomously to differentiate chronic periodontitis patients from other groups.

The subjects were divided into three groups based on Modified gingival index (MGI), clinical attachment level (CAL) and radiographic evidence of bone loss. Group I consisted of 15 subjects with clinically healthy periodontium and with no evidence of bone loss with scores of MGI < 1 and no attachment loss (CAL=0).Group II (Gingivitis) consisted of 15 subjects who showed clinical signs of gingival inflammation with scores of MGI ≥ 1, but there was no evidence of attachment loss i.e., CAL=0. The intraoral periapical radiographs did not show any bone loss. Group III (Chronic periodontitis) consisted of 15 subjects who showed clinical signs of gingival inflammation and attachment loss with radiographic evidence of bone loss. The scores recorded were, MGI ≥ 1and CAL ≥ 2 mm. Group IV (After Treatment group) consisted of 15 subjects of group-III, who were treated with non-surgical approach. GCF was collected by the same examiner, the next day, to prevent contamination of the sample with blood associated with the probing of inflamed sites.

The patients were explained about the study and written informed consent was obtained from those who agreed to voluntarily participate in this study.

Site selection and GCF collection:

Test site for GCF sample collection was based on the highest scored sites in the oral cavity i.e., the site showing most severe inflammatory signs (in gingivitis cases-Group II) or greatest amount of attachment loss (in chronic periodontitis cases-Group III). The same test sites of chronic periodontitis group were selected for the after-treatment group (Group IV). In the healthy group (Group I), to standardize site selection, sampling was predetermined to be from the mesio-buccal region of the maxillary right first molar.

After making the subjects sit comfortably on the dental chair, the selected test site was air dried and isolated with cotton rolls. Without touching the marginal gingiva, the supragingival plaque was removed to avoid contamination of the GCF sample and blocking of the microcapillary pipette. Then GCF was collected using the black colour-coded 1-5µl calibrated volumetric microcapillary pipettes obtained from Sigma-Aldrich Chemical Company, USA. By placing the tip of the microcapillary pipette extracrevicularly (unstimulated) for 5-20 minutes, a standardized volume of 1µl GCF was collected using the calibration on the microcapillary pipette. (Fig 1 shows the collection of GCF). The test sites which did not express standardized volume (1µl) of GCF were excluded from the study and the micropipettes contaminated with blood or saliva was discarded.

Samples of GCF were collected at the initial visit in Group I, Group II, Group III subjects. Scaling and root planing was performed for chronic periodontitis patients at the same appointment after GCF collection. After 8 weeks, GCF was collected from the same site of the subjects in Group III. The GCF collected was immediately transferred to plastic vial and stored at -70°C till the time of the assay.

Osteopontin assay:

The concentration of osteopontin was determined using a human osteopontin enzyme immunometric assay (EIA) kit (**ENZO Life Sciences**) as instructed by the manufacturer. The kit uses a monoclonal antibody to human OPN immobilized on a microtiter plate to bind the human OPN in the standards or sample. All standards, controls and samples were run in duplicate. Briefly, after appropriate dilution of GCF samples and the addition of standard to the wells, 100 µL of the samples was pipetted into the appropriate wells. The plate was sealed and incubated at room temperature on a plate shaker for 1 hour at 500rpm. After washing the plate four times, 100 µL of yellow antibody was pipetted into each well, except the blank and incubated at room temperature on a plate shaker for 1 hour at 500 rpm. The plate was washed again four times and 100 µL of blue conjugate was added to each well, except the Blank and the plate was incubated at room temperature on a plate shaker for 30 minutes at 500 rpm. The plate was washed four times and 100 µL of substrate solution was pipetted into each well and the plate was incubated for 30 minutes at room temperature on a plate shaker at 500 rpm and 25µL stop solution was pipetted to each well. The plate reader was blanked against the Blank wells and the optical density was read at 405 nm.

Statistical analysis:

One-way ANOVA F-test was used to calculate the overall P-value. Independent t-test was used for pair wise comparison between the study groups. Paired t-test was used to compare the clinical parameters and osteopontin levels in the chronic periodontitis group, before and after treatment. Pearson correlation test was used to correlate OPN levels and the clinical parameters within each study group.

RESULTS

Clinical parameters:

A comparison of the mean values of the clinical parameters in the various study groups is depicted in table1.

Table 1: Comparison of mean values of clinical parameters in various study groups

Variable	Group	Mean	S.D.	Overall P-value	Significant Groups
MGI	I	0.300	0.169	<0.001 (Sig.)	I vs. II I vs. III II vs. III
	II	3.067	0.651		
	III	3.117	0.462		
CAL	I	0.00	0.00	<0.001 (Sig.)	I vs. III II vs. III
	II	0.00	0.00		
	III	5.6	0.737		

The mean MGI in Group III (3.117) was significantly higher than the mean MGI in Group I (0.300) and Group II (3.067) ($p<0.05$). Also, the mean MGI in Group II (3.067) was significantly higher than the mean MGI in Group I (0.300) ($p<0.05$).

The mean CAL in Group III (5.6) was significantly higher than the mean CAL in Group I (0.00) and Group II (0.00) ($p<0.05$).

The osteopontin levels in the study groups are depicted in table 2.

Table 2: Descriptive statistics of OPN levels in various study groups

Group	N	Mean	Std. Dev	P - value	Significant groups
Group-I	**15**	**1.946**	**0.868**	**<0.001**	**I vs II I vs III II vs III**
Group-II	**15**	**7.457**	**2.049**		
Group-III	**15**	**12.733**	**0.968**		

The mean osteopontin level in Group III (**12.733**) was significantly higher than the mean osteopontin level in Group I (**1.946**) and Group II (**7.457**) ($p<0.05$). Also, the mean osteopontin level in Group II (**7.457**) was significantly higher than the mean osteopontin level in Group I (**1.946**) ($P<0.05$).

A comparison of various clinical parameters and osteopontin levels between group III and group IV is shown in table 3, which showed a significant difference, suggesting a significant reduction in the MGI, CAL and OPN levels after treatment.

Table 3: Comparison of various clinical parameters and osteopontin levels between group III and group IV

Variable	N	Group III		Group IV		P - Value
		Mean	Std. Dev	Mean	Std. Dev	
MGI	15	3.117	0.462	0.717	0.265	<0.001
CAL	15	5.600	0.737	3.200	1.612	<0.001
Osteopontin (µg/ml)	15	12.733	0.969	5.690	1.887	<0.001

The relationship between osteopontin level and various clinical parameters within each study group is shown in table 4, which showed a significant correlation between MGI and OPN level in Group I. Also, there was a significant correlation between CAL and OPN level in Group III. However, the remaining clinical parameters did not show significant relationship when compared with their corresponding osteopontin levels.

Table 4: Relationship between osteopontin level and various clinical parameters within each study group

Group	Clinical Parameters	Correlation Coefficient*	P-value
I	MGI	0.614	0.015 **(Sig.)**
	CAL	-	-
II	MGI	0.333	0.224
	CAL	-	-
III	MGI	0.376	0.168
	CAL	0.659	0.009 **(Sig.)**
IV	MGI	-0.052	0.855
	CAL	0.115	0.683

DISCUSSION

GCF has been extensively investigated for the release of host response factors. Of the 50 or more different components in GCF and saliva evaluated to date for periodontal diagnosis, most lack specificity to alveolar bone destruction and essentially constitute soft tissue inflammatory events.

As recently reviewed by ***Loos and Tjoa***,[5] more than 90 different components in GCF have been evaluated to date for periodontal diagnosis. Bone-related biomarkers from oral fluids associated with periodontal diseases include ALP, cathepsin B, collagenase-2 (MMP-8), gelatinase (MMP-9), collagenase-3 (MMP-13), calprotectin, osteocalcin, pyridinoline cross-links (ICTP), osteonectin and osteopontin.

OPN known to act as an anchor for osteoclasts can be one of the principal mediators of alveolar bone destruction in progressing periodontal disease**.**[2] OPN is a single-chain polypeptide having a molecular weight of approximately 32,600. OPN has been found in the kidney, blood, mammary gland, salivary glands, sweat glands, hypertrophic cartilage, dentin, cementum and bone.[1] Recent studies have revealed increased osteopontin expression in various clinical states such as chronic stable angina, cardiac fibrosis, tumourigenesis, glomerulonephritis, tubulointerstitial nephritis, allergic inflammation, asthma and rheumatoid arthritis.

OPN is involved in osteoclast adhesion and motility. In bone matrix, OPN is highly concentrated at sites where osteoclasts are attached to the underlying mineral surface, that is, the clear zone attachment areas of the plasma membrane.[6,7] OPN plays a role in bone homeostasis by inhibiting bone mineralization, by promoting differentiation of osteoclasts and by enhancing osteoclast activity.[8]

Reinholt et al, 1990[6] reported that osteopontin is a natural ligand for the vitronectin receptor in bone and suggested that calcitriol mediated bone resorption is exerted by inducing osteopontin production. OPN stimulate osteoclast migration through $\alpha_v\beta_3$ and CD44 and also increases CD44 expression on osteoclasts. ***Chelliah et al, 2003***[9] reported that that osteopontin stimulated osteoclast motility and bone resorption. All of the above studies definitely confirm the role of OPN in osteoclast differentiation and activity.

In bone remodeling, OPN mRNA expression is highest in osteoclasts and

they are capable of synthesizing their own OPN to facilitate anchorage to the bone matrix prior to resorption ***(Merry et al, 1993).***[10] OPN mediate the attachment of osteoclasts to bone. ***Uemura et al, 2001,***[11] in his review regarding OPN function in bone remodeling and its application to tissue engineering, suggested that OPN acts as a trigger for osteoblast early differentiation and activates osteoclast resorption.

Osteopontin has been recently found in GCF. Since GCF is an inflammatory exudate that reflects ongoing events in the periodontal tissues that produce it, various GCF components serve as potential diagnostic or prognostic markers for the progression of periodontitis.

Recently the presence of the OPN molecule was reported in GCF and the increase in its levels was found commensurate with the progression of periodontal disease, suggesting that OPN levels in GCF may be considered a marker of alveolar bone destruction.[1]

In this study, osteopontin (OPN) levels was estimated in GCF of subjects with clinically healthy periodontium, gingivitis and chronic periodontitis, and after scaling and root planing of periodontitis subjects.

The mean MGI and CAL levels increased progressively with increase in the severity of periodontal disease. The mean MGI and CAL levels in chronic periodontitis subjects (Group III) showed statistically significant reduction following treatment. The mean OPN levels were highest in Group III (12.733µg/ml) and lowest in Group I (1.946 µg/ml). Differences in mean OPN concentration were statistically significant in all groups, suggesting that OPN levels progressively increased from healthy to periodontitis states. The results are in accordance with that of ***Kido et al, 2001.***[2] However the OPN levels obtained in each group in this study is higher when compared to the previous study done by ***Kido et al.***[2] This difference could be attributed to the use of different sampling methods of GCF i.e, use of filter paper strips and the time-bound (30 seconds) strategy used to collect GCF, unlike the volume based approach used in this study and study done by ***Sharma et al.***[3]The chronic periodontitis subjects treated by scaling and root planing resulted in the reduction of mean OPN level from 12.733µg/ml to after-treatment level of 5.690µg/ml, which was statistically significant. These results are in accordance with study done by ***Sharma et al.***[3]

There was a significant correlation between MGI and OPN level in Group I. Also, there was a significant relationship between CAL and OPN level in Group III. However, the remaining clinical parameters did not show significant relationship when compared with their corresponding osteopontin levels. This is in contrast to the study done by ***Sharma et al,***[3] in which they reported significant positive correlation between GCF osteopontin concentration and all clinical parameters within each study group. However, in this study, there was a significant relationship between CAL and OPN level in Group III which is accordance with the study done by ***Sharma et al,***[3] who also reported that there was a statistically significant increase of GCF OPN levels as CAL progressed.

The source of OPN in GCF seems to originate from neighboring tissues such as alveolar bone, cementum, dentin, macrophages in periodontal tissues, blood and salivary glands. This explains the presence of OPN in GCF of patients with clinically healthy periodontium.

Sharma et al, 2007[12] analyzed the plasma and crevicular fluid osteopontin levels in periodontal health and disease. The mean

concentrations of OPN were found to increase progressively from health to gingivitis to periodontitis, both in GCF and, proportionately in plasma. This could be caused by spillage or overflow of OPN from diseased periodontal tissues, or produced by the circulating activated macrophages. Osteopontin play an important role in the onset and progression of disease in human coronary atheroma and, ultimately to alter vessel compliance ***(Fitzpatrick et al, 1994).***[13] Further the role of OPN in pathogenesis of atherosclerosis has been proved by various studies ***(Ohmori et al, 2003;***[14] ***Minoretti et al, 2005***[15]***)***. Therefore, a rise in serum OPN levels due to progressive periodontal disease could possibly act as a risk factor for coronary artery disease. But this needs to be confirmed with further longitudinal prospective studies involving larger populations.

Very recently, ***Tawfiq et al, 2010***[16] analyzed the levels of OPN in GCF of subjects with clinically healthy periodontium, chronic periodontitis and aggressive periodontitis. The highest mean level of Osteopontin (OPN) was found in the aggressive periodontitis group, followed by chronic periodontitis group and the least mean level was found in the healthy group.

The results of our study indicate that osteopontin levels in GCF are positively associated with periodontal disease and may be an indicator of periodontal disease activity. Further, treatment aimed at arresting periodontal disease progression resulted in a statistically significant reduction in the levels of GCF osteopontin which confirms its active role in periodontal attachment loss.

CONCLUSION

Within the limitations of this clinical study, the role of OPN as a diagnostic biomarker of periodontal disease progression in gingival crevicular fluid could be elucidated. However, further long-term prospective studies with larger sample sizes are needed to fully establish the role of OPN as a predictor of periodontal tissue destruction.

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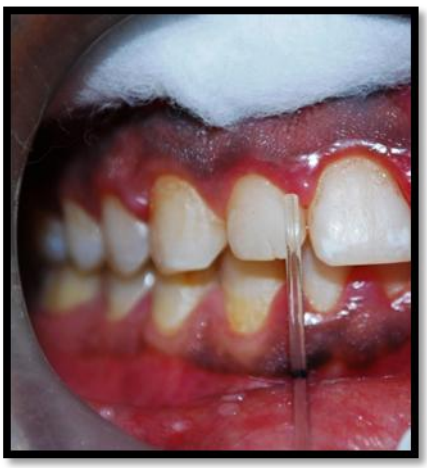

Fig 1 - Collection of GCF using black colour-coded 1-5µl calibrated volumetric microcapillary pipette.

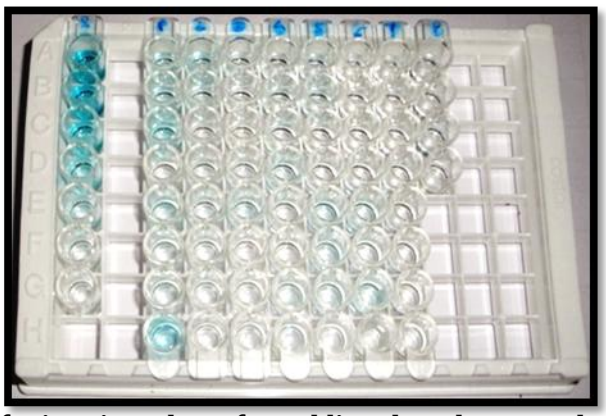

Fig 2 - A view of microtitre plate after adding the substrate solution and final incubation

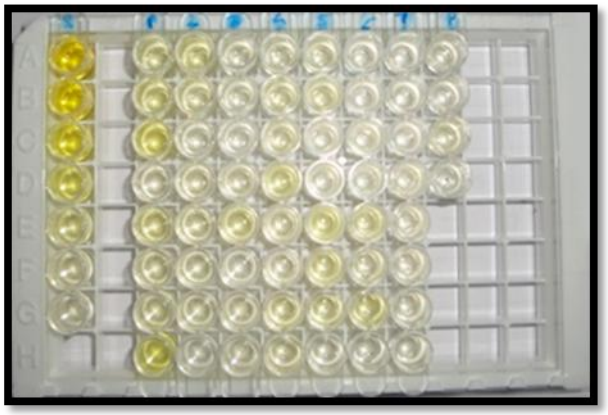

Fig 3 - A view of microtitre plate after adding the stop solution