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Original Article

Gingival crevicularfluid osteoprotegerin levels in Indian population

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ABSTRACT

Background: Initial research indicated that higher concentration of osteoprotegerin (OPG) is associated with healthy periodontium (protective) and its concentration decreases as the periodontal disease progresses. However, till date, there are no studies to investigate the levels of OPG in gingival crevicular fluid (GCF) after the treatment of periodontitis. Hence, the present study was carried out to assess its concentration in GCF to find out their association if any, and to explore its possible use as a 'novel bone marker' of the host modulation of periodontal disease.

Materials and Methods: Sixty-four subjects were divided into 4 groups (16 each), based on clinical attachment loss (CAL) and radiological parameters (bone loss); healthy (group I), gingivitis (group II), slight periodontitis (group III), and moderate-to-severe periodontitis (group IV). Moderate-to-severe periodontitis subjects, after nonsurgical periodontal treatment, (SRP) constituted group V. GCF samples were collected to estimate the levels of OPG using enzyme-linked immunosorbent assay (ELISA). The Kruskal-Wallis, Man-Whitney U test, and Wilcoxon signed-rank tests were carried out to compare OPG levels among groups. The Spearman rank correlation test was used to correlate OPG levels between the study groups and the clinical parameters; $P < 0.05$ was considered significant.

Results: The highest mean OPG concentration in GCF was obtained for group I (162.47 ± 51.171 pg/μL) and the least for group IV (10.92 ± 1.913 pg/μL), suggesting a negative correlation between OPG concentration and CAL. OPG concentrations in GCF after the treatment of group IV increased from 10.92 ± 1.913 pg/μL to 15.63 ± 4.679 pg/μL.

Conclusion: OPG concentration in GCF was inversely proportional to CAL and not an active progression factor for periodontal disease. Further, after the treatment of moderate-to-severe periodontitis subjects (group IV), OPG concentrations increased. Hence, it can be concluded that OPG could be considered as a 'novel bone marker' the host modulation of periodontal disease.

Key Words: Gingival crevicular fluid, Osteoprotegerin, periodontal disease

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INTRODUCTION

Periodontitis, a chronic inflammatory disease, is characterized by increased expression of various cytokines and other inflammatory mediators resulting in extensive osteoclast formation and bone loss.[1,2] These cytokines affect bone remodeling and play a vital role in both the physiological and pathological regulation of bone.[2,3]

Bone is the specialized connective tissue comprising various cells collagenous and inorganic matrix. The level of bone mass which is essential to execute various functions is maintained by a balanced act of bone formation and bone resorption.[4] This process is highly co-ordinated and regulated by two specialized cells, osteoblasts, the bone-forming cells and osteoclasts, the bone-resorbing cells. These cells are controlled by various hormones, inflammatory mediators, cytokines, and growth factors.[4,5] Recent addition to these regulators is the multifactorial cytokine OPG which has been detected in GCF.[5,6]

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OPG, a key physiological inhibitor of osteoclastic bone resorption,[7] is a glycoprotein which belongs to the tumor necrosis factor receptor (TNF) super family.[5] It is a decoy receptor for receptor activator nuclear factor kappa–B ligand (RANKL)[5] and inhibits cell-to-cell signaling between marrow stromal cells and precursors of osteoclast.[5,8] Thus, it prevents the bone resorption by RANKL, which is a precursor for the production of osteoclasts.[5]

OPG is expressed by various cell types like osteoblasts, osteoclastic stromal cells, T cells, B cells, chondrocytes, and follicular dendritic cells.[9] However, it is also found in organs like the kidney, liver, heart, lung, spleen, thyroid, lymph nodes, thymus, brain, and placenta.[5] It is also found in periodontal and dental tissues like gingiva and periodontal ligament,[10] both in the internal and the external enamel epithelium, as well as in the mesenchyme of the dental papilla during tooth development.[11] Moreover, a prominent expression of OPG in the cartilaginous primordia of developing maxilla, mandible, and hyoid bone has been reported.[5]

Further, OPG has been reported to have a preventive role in rheumatoid arthritis-associated bone erosion in joints[12] and its deficiency in inflamed joints of rheumatoid arthritis confirms this finding.[13]

Recently, OPG was isolated in gingival tissue[14] and GCF[15] with its levels decreasing with the progression of periodontal diseases suggesting that OPG levels in GCF may become a modulator of periodontal disease, especially alveolar bone resorption.[6,15]

However, till date, there have been no studies on the correlation of OPG levels in GCF during periodontal health, disease, and after nonsurgical periodontal therapy (SRP).

Hence in the light of the aforementioned facts, this clinico-biochemical study was designed to estimate the levels of OPG in the GCF of subjects with clinically healthy periodontium, gingivitis, slight periodontitis, moderate-to-severe periodontitis, and of moderate-to-severe periodontitis subjects after scaling and root planing (SRP).

MATERIALS AND METHODS

This study conducted during the period from August to September 2007 involved 64 subjects (32 females, 32 males) aged 30-39 years selected from the outpatient section, Department of Periodontics, Government Dental College and Research Institute, Bengaluru, Karnataka, India. Essential ethical clearance for the study was obtained from the institutional ethical review board, Government Dental College and Research Institute, Rajiv Gandhi University of Health Sciences, Karnataka, India. Those who volunteered were briefed about the study procedure, and a consent form was signed on acceptance by them.

Specific conditions that excluded participation in the study were (i) pregnant, lactating, and postmenopausal women, (ii) patients with diabetes mellitus, ischemic heart disease, or any other conditions contributing to atherosclerosis, malignant tumors, rheumatoid arthritis, bone disorders, and/or on cancer chemotherapy or those on antiresorptive drugs like bisphosphonates, With this disese will hve OPG concentration less because of infl ammation, (iii) smokers and alcoholics, (iv) subjects who received treatment with anti-inflammatory drugs, antibiotics, steroids, or contraceptives in the last six months, and (v) those receiving any periodontal treatment.

The subjects were categorized into groups, each group comprising 16 patients based on modified gingival index (MGI)[16] and clinical attachment loss (CAL)[17] with radiographic evidence of bone loss.

Group I (healthy): Consisted of 16 subjects with clinically healthy periodontium and with no evidence of disease. The score obtained after assessing the gingival status using MGI was zero and with no crestal bone loss as determined from the radiograph. Group II (gingivitis): Consisted of 16 subjects whose gingivae showed clinical signs of inflammation but there was no evidence of CAL, which was zero. The intraoral periapical radiographs did not show any bone loss. A score between 1 and 2 was obtained after recording MGI. Group III (slight periodontitis): Consisted of 16 subjects, who showed clinical signs of gingival inflammation and CAL of 1-2 mm with radiographic evidence of bone loss. MGI score between 2 and 4 was obtained for these patients. Group IV (moderate to severe periodontitis): Consisted of 16 subjects, who showed clinical signs of gingival inflammation and CAL >3 mm with radiographic evidence of bone loss. MGI score between 2 and 4 was obtained for these patients. Group V (after-treatment group): Consisted of group IV subjects treated nonsurgically, and GCF samples were collected from the same sites six to eight weeks after treatment, to constitute group V [Figures 1-5].

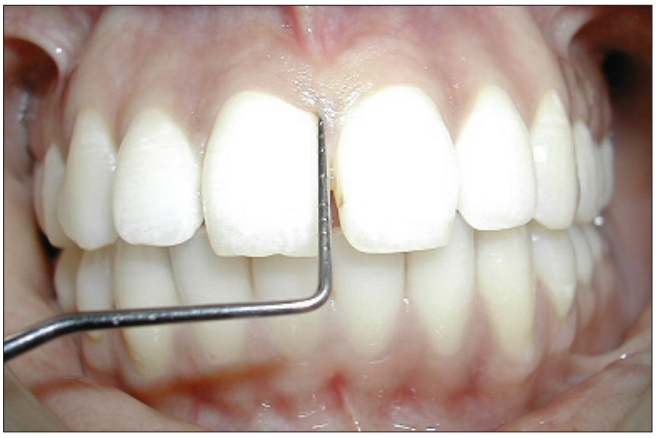

Figure 1a: Clinical picture of a patient of Group I (Healthy) showing CAL of 0 mm on the mesiolabial aspect of 11

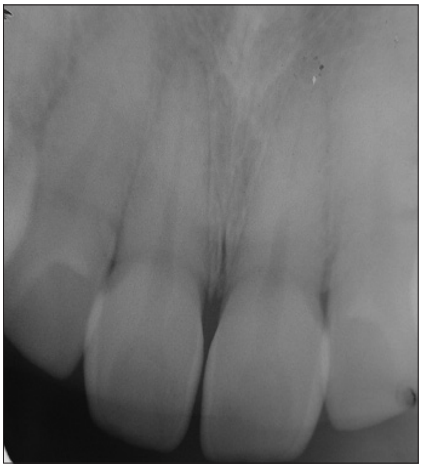

Figure 1b: Radiographic picture of same area

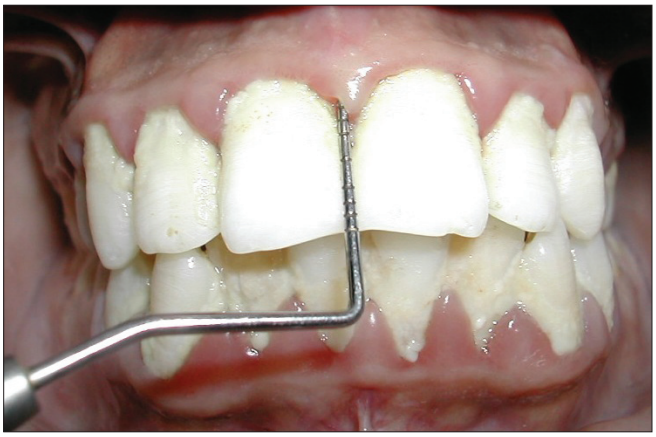

Figure 2a: Clinical picture of a patient of Group II (Gingivitis) showing CAL of 0 mm on the mesiolabial aspect of 11

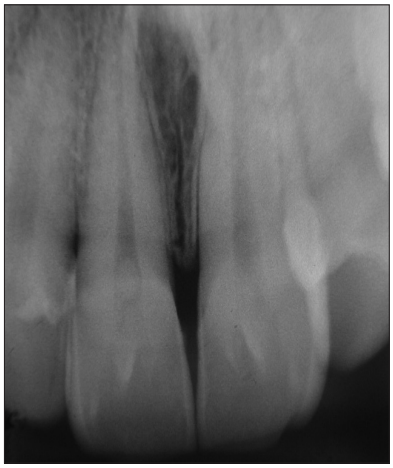

Figure 2b: Radiographic picture of same area

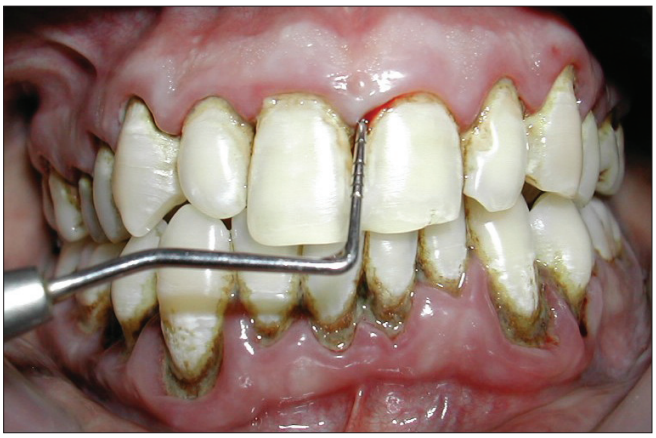

Figure 3a: Clinical picture of a patient of Group III (Slight periodontitis) showing CAL of 1-2 mm on the mesiolabial aspect of 21

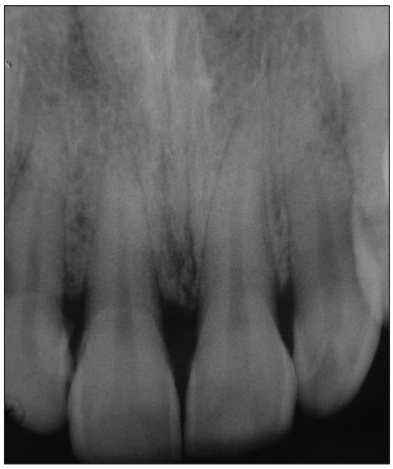

Figure 3b: Radiographic picture of same area

Site selection and fluid collection

The sampling protocol was followed as described previously in the earlier studies by the group.[18] Briefly, clinical and radiological examinations, group allocation, and sampling-site selections were performed by one examiner (PB) and the samples were collected on the subsequent day by the second examiner (MVRP). This was done to ensure blinding of the sampling examiner and to prevent contamination

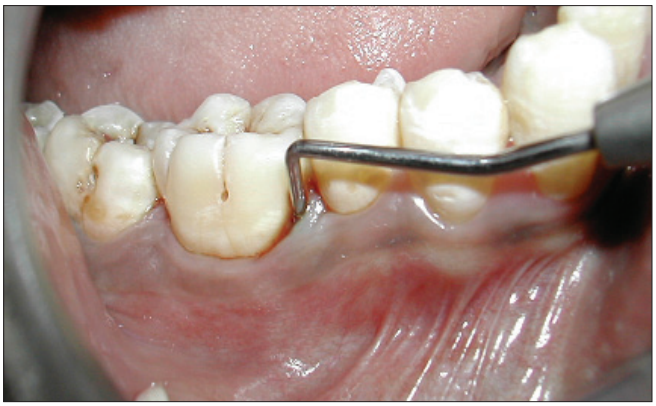

Figure 4a: Clinical picture of a patient of Group IV (moderate to severe periodontitis) showing CAL of >3 mm on the mesiobuccal aspect of 46

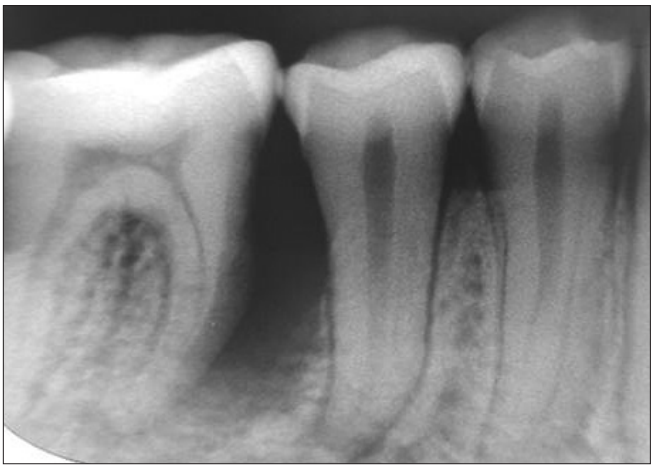

Figure 4b: Radiographic picture of same area

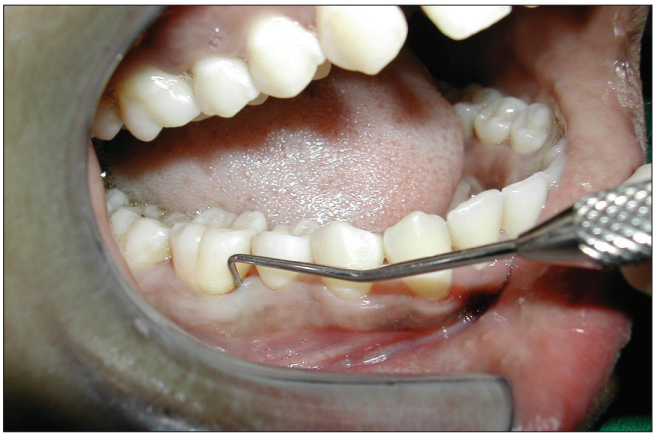

Figure 5a: Clinical picture of a patient of Group V (moderate to severe periodontitis after treatment)

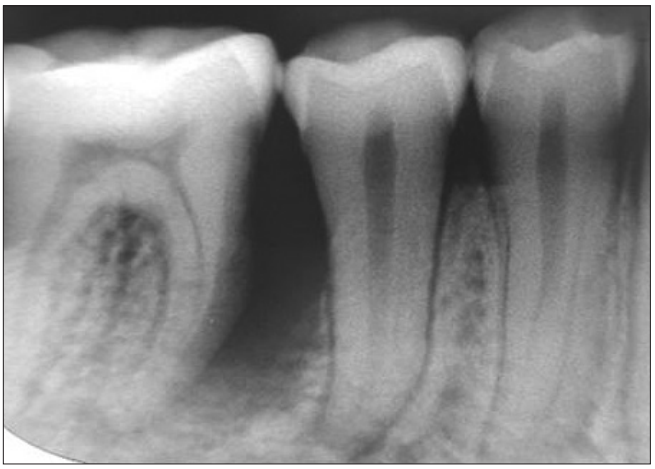

Figure 5b: Radiographic picture of same area after treatment

of GCF with blood associated with the probing of inflamed sites. Only one site per subject was selected as a sampling site. In the healthy group, sampling was predetermined to be from the mesiobuccal region of the maxillary right or left first molar, wherever adequate sample was obtainable. In gingivitis, sites with the highest clinical signs of inflammation were selected. In periodontitis, sites with 1-6 mm CAL were identified using a Williams graduated periodontal probe followed by radiographical confirmation of bone loss and assigned for sampling. On subsequent day, a standardized volume of 1 mL GCF was collected from each predetermined test site using the calibration on color-coded 1-5 mL calibrated volumetric microcapillary pipettes* with an extracrevicular approach (unstimulated). Plastic vials were used to store the GCF-containing micropipettes and stored at −70°C until the assay [Figures 6 and 7].

* Sigma Aldrich Chemicals Company Limited, USA.

Osteoprotegerin assay

After appropriate dilution of GCF samples, a sandwich type of ELISA Development Kit† (ELISA: enzyme-linked immunosorbent assay) was employed for the quantitative determination of human OPG [Figures 8 and 9].

The 96 well-plate kit was prepared the previous day and incubated overnight, as per the manufacturer's kit development instructions. The kit utilizes a capture antibody which was coated on a microtiter plate for immobilization and to bind the human OPG in the standards or sample. Then, 100 µL of diluted sample and standards were added to appropriate wells and covered with adhesive strips followed by incubation for two hours at room temperature. Later, 100 µL (each) of working dilution of streptavidin-horseradish peroxidase (HRP) and substrate solution were added

† Human Osteoprotegerin/TNFRSF11B DuoSet, R&D Systems, USA.

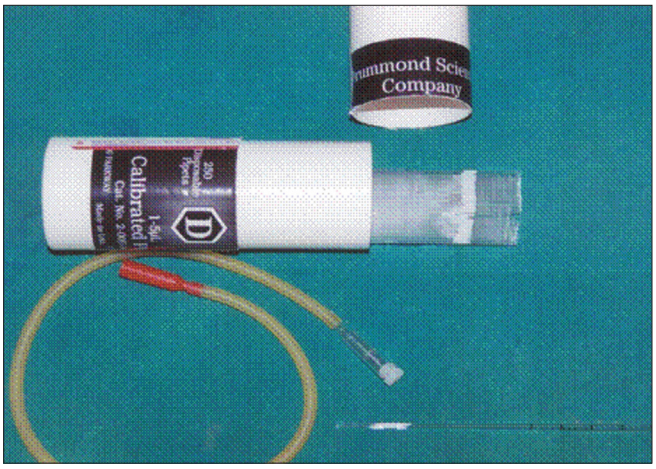

Figure 6: The microcapillary pipettes with aspirator tube

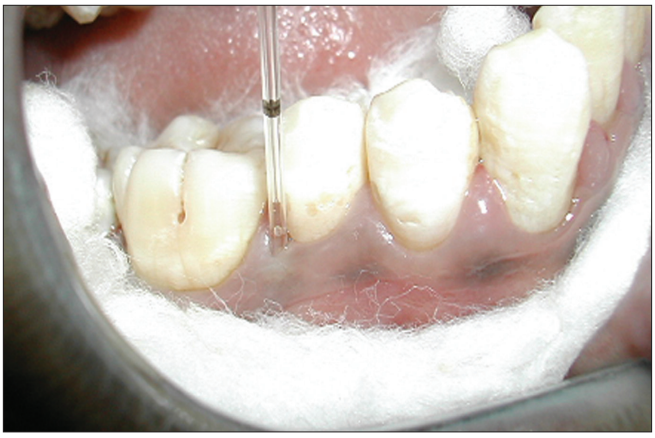

Figure 7: Collection of GCF using extra-crevicular positioning of microcapillary pipette

Figure 8: DuoSet Human OPG ELISA KIT

Figure 9: Contents of OPG ELISA kit

Figure 10: OPG capture Antibody preparation

to each well, after washing the plate three times with wash buffer between each addition, and incubated for 20 minutes at room temperature. Finally, 50 µL of stop solution was added to each well to stop enzyme reaction and the color generated was read at 450 nm. The concentration of OPG in the tested samples was calculated using the standard curve plotted using the optical density values with the standards [Figure 10].

Statistical analysis

A statistical software program was applied to analyze the data obtained.‡ The Kruskal-Wallis, Man-Whitney U test, and Wilcoxon signed-rank tests were carried out to compare OPG levels among groups. The Spearman rank correlation test was used to correlate OPG levels between the study groups and the clinical parameters.

RESULTS

All the GCF samples assayed showed the presence of OPG. The highest mean OPG concentration was noted in group I (162.47 ± 51.17 pg/µL) and lowest in group IV, that is, 10.92 ± 1.91 pg/µL with intermediate values for group II (41.39 ± 16.64 pg/µL),

‡ Systat® version 11 & SigmaStat®, Systat Software, Inc CA, USA.

Table 1: Mean OPG concentration of the study groups

Study group	Mean±SD	Min	Max
Group I	162.47±51.171	100.00	300.40
Group II	40.27±16.639	21.25	70.00
Group III	23.40±1.989	20.20	23.25
Group IV	10.92±1.913	8.12	10.91
Group V	15.63±4.679	10.73	15.38

OPG: Osteoprotegerin, SD: Standard deviation, Min: Minimum, Max: Maximum

Table 2: Kruskal-Wallis test comparing mean GCF OPG levels between groups

Study group	Mean rank	*P* value
Group I	72.50	0.00*
Group II	52.44	
Group III	44.16	
Group IV	12.50	
Group V	20.91	

Significant at $P < 0.05$, OPG: Osteoprotegerin, GCF: Gingival crevicular fluid

Table 3: Pairwise comparison using Mann-Whitney U test for GCF OPG

Study group	Mean rank	Z value	*P* value
Group I	24.38	−4.756	0.00*
Group II	8.63		
Group I	24.38	−4.842	0.00*
Group III	8.50		
Group I	24.38	−4.827	0.00*
Group IV	8.50		
Group I	24.38	−4.829	0.00*
Group V	8.50		
Group II	19.56	−1.873	0.00*
Group III	13.44		
Group II	24.50	−4.833	0.00*
Group IV	8.50		
Group II	24.19	−4.650	0.00*
Group V	8.81		
Group III	24.50	−4.845	0.00*
Group IV	8.50		
Group III	24.19	−4.661	0.00*
Group V	8.81		

$P < 0.05$ significant, OPG: Osteoprotegerin, GCF: Gingival crevicular fluid

group III (23.40 ± 1.99 pg/μL), and group V (15.63 ± 4.68 pg/μL). These results are shown in Table 1 and Figure 11.

The results of Kruskal Wallis test (nonparametric), carried out to evaluate the difference in OPG levels with 5% level of significance ($P < 0.05$), suggested that the mean concentration of OPG differed significantly among the groups tested [Table 2].

Further, multiple comparison using Mann-Whitney U test carried out to find the pair or pairs of groups that differed significantly ($P < 0.05$) suggested that OPG levels in GCF decreased progressively from health to severe periodontitis [Table 3].

Table 4: Wilcoxon signed-rank test comparing GCF OPG concentration before and after treatment

Study group	*n*	Mean OPG Conc (pg/μL)	Z	*P* value
Group IV	16	10.92	-3.516	0.000*
Group V	16	15.63		

*$P < 0.05$ significant, OPG: Osteoprotegerin, GCF: Gingival crevicular fluid, Conc: Concentration

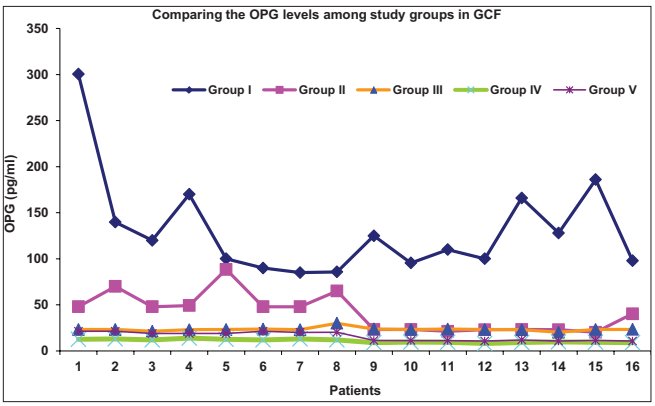

Figure 11: Graph comparing OPG levels among study groups

When group IV (moderate to severe periodontitis) and group V (after treatment of group IV) were compared using Wilcoxon signed-rank test [Table 4], the difference in the concentration of OPG was statistically significant ($P < 0.05$), indicating that after SRP, OPG levels increased considerably in GCF (10.92 pg/μL to 15.63 pg/μL).

Spearman's rank correlation test was done to observe any correlation between the GCF OPG concentration and clinical variables (MGI and CAL). When OPG levels in GCF were tested for correlation with the disease severity measures, that is, MGI (in group I and II) and MGI and CAL (in groups III, IV, and V), a negative correlation was found for GCF OPG concentration as shown in Table 5.

When Kruskal Wallis test was done to compare the mean OPG concentration in GCF at different CAL levels (before and after treatment), there was a significant reduction of CAL after treatment, which was proportional to a statistically significant ($P < 0.05$) increase of OPG levels in GCF as shown in Table 6.

In summary, the mean rank for the group I is the highest and it differs significantly from group II, III,

Table 5: Kruskal -Wallis test comparing CAL and OPG concentrations

Groups	CAL level	*n*	Mean±Std. Deviation	Minimum	Maximum	F	*P* value
Group III	1	9	23.82±2.42	21.250	30.000	0.939	0.349
	2	7	22.85±1.18	20.200	23.700		
Group IV	3	4	12.57±0.45	12.025	13.125	26.419	0.000*
	4	4	12.79±0.95	12.025	14.000		
	5	5	9.14±0.65	8.125	9.800		
	6	2	9.20±0.28	9.000	9.400		
	7	1	9.20	9.200	9.200		
Group V	1	3	20.13±1.15	18.950	21.250	145.623	0.000*
	2	5	20.08±1.17	18.900	21.250		
	3	6	11.01±0.31	10.737	11.400		
	4	2	11.63±0.33	11.400	11.875		

* Significant $P < 0.05$, OPG: Osteoprotegerin, CAL: Clinical attachment loss

Table 6: Results of Spearman's rank correlation (*r*) test comparing GCF OPG and CAL within the groups

Groups	CAL vs. OPG correlation	*P* value
Group III	-0.251	0.349
Group IV	-0.794**	0.000*
Group V	-0.846**	0.000*

*Significant $P < 0.05$, **negative correlation OPG: Osteoprotegerin, GCF: Gingival crevicular fluid, CAL: Clinical attachment loss

IV, and V. Further, these results indicate that GCF OPG concentration decreases from periodontal health to disease.

DISCUSSION

Periodontitis, a chronic inflammatory disease, is characterized by increased expression of various cytokines and other inflammatory mediators resulting in extensive osteoclast formation and bone loss.[1] These cytokines affect bone remodeling and play a vital role in both the physiological and pathological regulation of bone.[2]

OPG, a key physiological inhibitor of osteoclastic bone resorption,[7] is a glycoprotein which belongs to the TNF super family.[5] It is a decoy receptor for RANKL[5] and inhibits cell-to-cell signaling between marrow stromal cells and precursors of osteoclasts.[9] Thus it prevents the bone resorption by RANKL, which is a precursor for the production of osteoclasts.[6]

OPG is expressed by various cell types like osteoblasts, osteoclastic stromal cells, T cells, B cells, chondrocytes, and follicular dendritic cells.[9] Moreover, it is also found in organs and tissues like the kidney, liver, heart, lung, spleen, thyroid, lymph nodes, thymus, brain, and placenta.[5] It is found also in periodontal and dental tissues like gingiva and periodontal ligament and in both internal and external enamel epithelium as well as in the mesenchyme of the dental papilla during tooth development.[10,11] There is a prominent expression in the cartilaginous primordia of developing maxilla, mandible, and hyoid bone.[5]

Earlier, Mogi *et al*. correlated OPG concentration in GCF and explained its possible role in periodontal disease progression.[6] However, till date no other studies have been documented that have compared the OPG levels in GCF of subjects with healthy and diseased periodontium, and those after SRP.

Hence, the present study was undertaken to determine the potential role of OPG as a 'novel bone marker' of periodontal disease modulator. To achieve this objective, OPG levels in GCF were quantified and compared from healthy, gingivitis, slight periodontitis, and moderate-to-severe periodontitis subjects, and from moderate-to-severe periodontitis subjects after treatment.

In our study, the mean concentrations of OPG in GCF were found to decrease progressively from 162.47 ± 51.171 pg/μL in healthy subjects to 8.12 pg/μL in periodontitis subjects, whereas in gingivitis, mean concentration of OPG was 60.50 pg/μL. The results of the present study, with respect to the general trend, are in accordance with those of Mogi *et al*. who reported decreasing OPG levels in GCF with the progression of periodontal disease.[6]

When pairwise comparison was done between healthy and gingivitis group, gingivitis and slight periodontitis group, slight periodontitis and moderate-to-severe periodontitis group, and moderate-to-severe

periodontitis group and after-treatment group, healthy and slight periodontitis group, healthy and moderate-to-severe periodontitis group, and healthy and moderate-to-severe periodontitis group after treatment, the differences were statistically significant in GCF OPG concentration. The mean rank obtained for moderate to severe periodontitis (12.50 pg/μL) was very much lower than that of healthy (72.50 pg/μL), gingivitis (52.44 pg/μL), and slight periodontitis (44.16 pg/μL) groups. The results were significant at $P < 0.05$. This suggests that OPG levels in GCF decrease progressively from health to periodontitis.

In the present study, influence of age and sex of the subjects on the OPG levels was minimized by selecting the subjects within the narrow age group of 30-39 years and including equal number of male and female subjects in each group. Further, this study comprised five groups (healthy, gingivitis, slight periodontitis, moderate-to-severe periodontitis, and moderate-to-severe periodontitis after treatment) as compared to the previous study (Mogi *et al*. 2004) where only four groups namely, healthy controls, slight periodontitis, moderate periodontitis, and severe periodontitis subjects were included, having no provision to evaluate the effect of periodontal therapy on OPG levels in GCF which can further confirm the role of OPG in the modulation of periodontal disease.

The levels of OPG reported by the earlier study were expressed as 'amount of OPG' in pg/μL/site and the levels were higher than those of our study. The mean of OPG levels of our study are half the concentration of OPG levels of the previous study. The reasons for these variations in the levels of OPG may be due to difference of study population, environmental factors, and volume of bone present during the time of GCF collection. Due to these reasons, the numerical values of OPG from the earlier study are not comparable with our study.

The variability of OPG concentration in each group could be due to different stages of the disease process at the time of collection of GCF. The levels of OPG are low in the gingivitis group (group II) compared to the healthy group, which could be due to near conversion of gingivitis lesion to periodontitis lesion that is not detectable clinically either by manual probing or radiography. In the slight periodontitis group, the OPG concentration was less than that of healthy and gingivitis groups.

The moderate-to-severe periodontitis subjects were treated by SRP, and strict measures of oral hygiene were instituted. Eight weeks after the treatment, the GCF samples were collected from group IV (group V). The mean concentration of OPG in GCF in the moderate-to-severe periodontitis group increased from 10.92 pg/μL before treatment to an after-treatment level of 21.25 pg/μL, respectively, which was statistically significant. Recently, similar results were reported when gingival biopsies were quantified for OPG mRNA (mRNA: messenger RNA) and RANKL in healthy and chronic periodontitis patients (after SRP).[14]

Most of the samples of group V (after treatment) fall between group III (slight periodontitis) and group IV, which could be due to modulation of the periodontal disease by OPG where it protects the bone by preventing osteoclastogenesis accelerated by RANKL.

In our study, the mean MGI scores of periodontitis subjects before and after treatment were 2.27 and 1.34, respectively, which commensurate with those of the CAL levels and OPG levels in GCF.

In summary, our study shows that OPG concentration in GCF decreases with progression of periodontal disease. Further, this was accompanied with the decrease in OPG levels that directly correlated with the stage of periodontal disease, that is, the amount of bone loss and CAL. Further, treatment aimed at arresting the progression of periodontal disease resulted in a statistically significant rise in levels of OPG in GCF proportionally, confirming its active role in periodontal attachment gain. The results of the present study, therefore, provide site-specific information on changes in OPG levels as a result of periodontal disease and after treatment.

The source of OPG in GCF seems to be from neighboring tissues including alveolar bone, gingiva, and periodontal ligament in periodontal tissues.[5,10] Further, the concentration of OPG in GCF was increased after periodontal therapy, thus increasing the GCF OPG concentration.

In our study, the mean concentration of OPG in group IV (moderate-to-severe periodontitis group) was 10.92 pg/μL, lower than the concentration described in the earlier study.[14] Based on the above findings, it can be hypothesized that decreased OPG levels due to progressive periodontal disease could act a as risk factor for the development of periodontal

disease. However, this needs to be confirmed by conducting longitudinal, prospective studies involving larger sample size.

CONCLUSIONS

The present study shows that the OPG concentration in GCF decreases proportionally with the progression of periodontal disease, that is, gingival inflammation and CAL. Further, treatment aimed at arresting the progression of periodontal disease resulted in statistically significant increased levels of GCF OPG proportionally, confirming its active role in periodontal attachment gain. Therefore, the results of the present study provide site-specific information on changes in OPG levels as a result of periodontal disease and after treatment. Thus, within the limits of the present study, we can conclude that OPG can be considered as a 'novel bone marker' of the modulation of periodontal disease.

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