

Original Article

Comparison of matrix metalloproteinase-3 and tissue inhibitor of matrix metalloproteinase-1 levels in gingival crevicular fluid in periodontal health, disease and after treatment: A clinico biochemical study

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ABSTRACT

Background: Considering the role of matrix metalloproteinase-3 (MMP-3) and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) in the pathogenesis of periodontitis, the present study is to estimate the levels of MMP-3 and TIMP-1 in gingival crevicular fluid (GCF) in periodontal health, disease and to evaluate the effect of periodontal therapy on MMP-3 and TIMP-1 concentrations in GCF.

Materials and Methods: A periodontal examination and collection of GCF by extra-crevicular method was performed in 30 subjects selected randomly and categorized into 3 groups. Group I consists of 10 subjects Group II consists of 20 patients and Group III consists of 20 patients of Group II. Non surgical periodontal therapy was performed, and GCF was collected after 8 weeks from the same site of 20 chronic periodontitis patients who are considered as Group III. MMP-3 and TIMP-1 levels were estimated in GCF-samples by using enzyme-linked immunosorbent assay. The findings were analyzed using the software and descriptive statistical methods such as Mann-Whitney U-test and Kruskal-Wallis test. *P* value < 0.001 was considered significant.

Results: MMP-3 and TIMP-1 was detected in all samples. Highest mean MMP-3 concentrations in GCF were obtained for Group II (7.490 ng/ml) while the lowest concentrations were seen in Group I (0.344 ng/ml) and Group III (2.129 ng/ml). This suggests that MMP-3 levels in GCF increases proportionally with the progression of periodontal disease and decreases after treatment. Lowest mean TIMP-1 concentrations in GCF were obtained for Group-II (1.592 ng/ml), while the highest concentrations were seen in Group-I (8.78 ng/ml) and Group-III (6.40 ng/ml). This suggests that TIMP-1 levels in GCF decreases proportionally with progression of periodontal disease and increases after treatment.

Conclusion: There is a substantial increase in the concentrations of MMP-3 and decrease in TIMP-1 as periodontal disease progress. Since MMP-3 and TIMP-1 levels in GCF are positively correlated with gingival index, probing pocket depth, and clinical attachment loss, MMP-3, and TIMP-1 may be considered as a "Novel Biomarkers" in periodontal disease. However, controlled, longitudinal studies are needed to confirm this possibility.

Key Words: Gingival crevicular fluid, matrix metalloproteinase-3, periodontal disease, scaling and root planning, tissue inhibitor of matrix metalloproteinase-1

Received: April 2012
Accepted: May 2013

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INTRODUCTION

Periodontitis is a chronic inflammatory disease causing destruction of the attachment apparatus of teeth. Progression of the periodontal lesion during the inflammatory process is the consequence of breakdown of the collagenous Sharpey's fibers anchored in the root

cementum.^[1,2,3] Degradation of the collagenous matrix involves the activity of a group of enzymes known as matrix metalloproteinases (MMPs).^[4,5]

These MMPs are important sub family of zinc and calcium dependent endopeptidases secreted or released by a variety of host cells such as polymorphonuclear leucocytes, macrophages, fibroblasts, bone, epithelial, and endothelial cells that function at neutral pH and utilize the various constituents of the extracellular matrix as their substrates, and are responsible for their remodeling and degradation.^[6]

Stromelysin-1 (MMP-3) is with broad substrate specificity that has been linked to tissue destruction associated with chronic inflammatory diseases such as periodontitis.^[6,7]

MMPs are counteracted by tissue inhibitor of matrix metalloproteinases (TIMPs), which inhibit MMP activity there by restricting extracellular matrix (ECM) breakdown. The balance between MMPs and TIMPs play an important role in maintaining the integrity of healthy tissues. A disturbed balance of MMPs and TIMPs is found in various pathologic conditions, such as rheumatoid arthritis, cancer, and periodontitis.^[6,8]

This clinico biochemical study is aimed to estimate the levels of MMP-3 and TIMP-1 in gingival crevicular fluid (GCF) in periodontal health, disease and to evaluate the effect of periodontal therapy on MMP-3 and TIMP-1 concentrations in GCF.

MATERIALS AND METHODS

Study population

This clinic-biochemical study population consists of 30 subjects in the age group of 20-50 years attending

the out-patient section, Department of Periodontics, C.K.S. Teja Institute of Dental Sciences and Research, Tirupathi based on the following criteria: All the patients had not received any periodontal treatment in previous 6 months. The exclusion criteria should include diabetes mellitus patients, hypertension, psoriasis, tumors, smoking, Sjogren's syndrome, delayed hypersensitivity, cardio vascular disease, subjects who have received anti-inflammatory drugs, antibiotics and neo vascularization inhibitors in the previous 6 months and rheumatoid arthritis.

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.

Subjects were selected for each group after a brief and precise case history recording. All clinical measurements were carried out by a single operator. Test site for GCF sample collection was selected based on the highest scored sites in the oral cavity, i.e., the site showing greatest amount of attachment loss (in chronic periodontitis cases), and the same test site for after treatment group was selected.

GCF sampling and phase I therapy

After making the subjects sit comfortably in an upright position on the dental chair, the selected test site was air dried and isolated with cotton rolls. Without touching the marginal gingiva, supragingival plaque was removed to avoid contamination and blocking of the micro-capillary pipette. GCF was collected by 1-3 μ l calibrated volumetric micro-capillary pipettes obtained from Sigma Aldrich Chemical Company, USA (Catalog No. p0549) as shown in Figures 1 and 2.



Figure 1: Armamentarium for gingival crevicular fluid collection



Figure 2: Gingival crevicular fluid collection by micro-capillary pipettes

By placing the tip of the pipette extra-crevicular (unstimulated) for 30 s, a standardized volume of 3 μ l GCF was collected using the calibration on the micro-pipette from each test site. The test sites, which did not express standard volume (3 μ l) of GCF, and micro-pipette contaminated with blood and saliva were excluded. Samples of GCF were collected at the initial visit in Group II patients.

Periodontal treatment (scaling and root planing [SRP]) was performed for periodontitis patients at the same appointment after GCF collection. After 8 weeks, GCF was collected from the same site of these subjects who were considered as Group III. For this 8 week period, subjects were called at 1-week interval and plaque control measures were performed. The GCF collected was immediately transferred to aliquots and stored at -70°C until the time of the assay.

Enzyme-linked immunosorbent assay (ELISA) for MMP-3, and TIMP-1 analysis in GCF

This assay employs the quantitative sandwich enzyme immunoassay technique with a catalog number DMP300 and DTM100 from R and D systems. A polyclonal antibody specific for MMP-3 and TIMP-1 has been pre-coated onto a micro-plate. Standards and samples are pipetted into the wells, and any MMP-3 and TIMP-1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for MMP-3 and TIMP-1 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells, and color develops in proportion to the concentrations of total MMP-3 (pro- and/or active) and TIMP-1 bound in the initial step. After the color development is stopped then the intensity of the color is measured.

Significance of the method used in this study

In the present study, GCF collection was carried out using micro-capillary pipettes. MMP-3 and TIMP-1 concentrations were analyzed by ELISA. Whereas the earlier studies used filter paper strips and periotron 8000 and 6000, which can result in non-specific

attachment of the analyte to filter paper fibers ensuing in a false reduction in the detectable MMP-3 and TIMP-1 levels, which underestimates the correlation of MMP-3 and TIMP-1 levels to the periodontal disease.

Statistical analysis

The following methods of statistical analysis have been used in this study. The results were averaged (mean \pm standard deviation) for each parameter are presented in Table 1. Multiple comparisons using Mann-Whitney U-test were carried on to find out which pair or pairs differ significantly. Kruskal-Wallis test was used to compare mean concentrations of GCF MMP-3 with respect to clinical attachment loss (CAL). *P* value < 0.001 was considered significant.

RESULTS

Clinical parameters

Clinical parameters such as gingival index, probing pocket depth, CAL were recorded for all 30 subjects. MMP-3 and TIMP-1 concentrations obtained as a result of biochemical analysis were included for statistical analysis shown in Table 1.

The mean gingival index of Group I was 0.112 with SD 0.018, Group II was 1.666 with SD 0.233 and Group III was 0.820 with SD 0.047. The mean Gingival Index was significantly higher in Group II, i.e., 1.666; SD 0.233, when compared with Group I (i.e., 0.112, SD 0.018) and Group III (i.e., 0.820, SD 0.047), which was statistically significant (*F* value 378.20-; *P* value < 0.001).

The mean probing pocket depth of Group I was 1.100 with SD 0.316, Group II was 5.550 with SD 0.686 and Group III was 3.700 with SD 0.571. The mean probing pocket depth was significantly higher in Group II, i.e., 5.550, when compared with Group I (i.e., 1.100, SD 0.316) and Group III (i.e., 3.700, SD 0.571), which was statistically significant (*F* value 195.65-; *P* value < 0.001).

The mean CAL of Group I was 0.00 with SD 0.00,

Table 1: The mean values of age distribution, gingival index, probing depth, CAL and MMP-3 and TIMP-1 concentrations of the study population

Study group	Mean age	Mean gingival index	Mean probing depth	Mean CAL	Mean MMP-3	Mean TIMP-1
Group I	23.50	0.112	1.100	0.00	0.344	8.7810
Group II	32.45	1.666	5.550	3.450	7.490	1.592
Group III	32.45	0.820	3.700	1.800	2.129	6.408

CAL: Clinical attachment loss; MMP: Matrix metalloproteinase; TIMP: Tissue inhibitor of matrix metalloproteinase

Group II was 3.450 with SD 0.686 and Group III was 1.800 with SD 0.615. The mean CAL was significantly higher in Group II, i.e., 3.450, when compared with Group I (i.e., 0.00, SD 0.00) and Group III (i.e., 1.800, SD 0.615), which was statistically significant (F value 119.82-; P value < 0.001).

BIOCHEMICAL ANALYSIS

MMP-3 levels

All the samples, in each group tested positive for the presence of MMP-3. The mean concentration of MMP-3 in Group I was 0.344 ng/ml with SD 0.131 and with the highest value, i.e., 0.566 ng/ml and lowest value of 0.114 ng/ml. The mean MMP-3 concentration in Group II was 7.490 ng/ml with SD 1.963 and with the highest value, i.e., 9.940 ng/ml and lowest value of 4.900 ng/ml. The mean MMP-3 concentration in Group III was 2.129 ng/ml with SD 1.101. The highest value of MMP-3 concentration in Group III was 3.901 ng/ml and lowest value of 0.745 ng/ml. When we compare in between groups the mean MMP-3 concentrations in GCF was observed to be highest in Group II, i.e., 7.490 ng/ml and lowest in Group I, i.e., 0.344 ng/ml. The mean MMP-3 concentration in Group III (2.129 ng/ml) fell between the highest and lowest values. The P value is statistically significant with < 0.001 .

Further, multiple comparisons using Mann-Whitney U-test was carried on to find out, which pair or pairs differ significantly. When Groups I and II, II and III and I and III were compared, the differences were statistically significant with mean ranks between Group I and Group II, i.e., 5.50 and 20.50, between Group II and Group III, i.e., 30.50 and 10.50 and between Group I and Group III, i.e., 5.50 and 20.50 with $P = 0.05$.

When Kruskal-Wallis test was carried out to compare the mean MMP-3 concentration in GCF at different CAL levels (before and after treatment), i.e., in Group II with CAL 3, 4 and 5 had mean MMP-3 concentrations of 6.175, 9.417, and 9.920 with SD values of 1.159, 0.647, and 0.028 respectively, with $P = 0.00$, which is statistically significant. There was a reduction in CAL after treatment, with 1, 2, and 3 values, which had mean MMP-3 concentrations of 1.501, 2.155, and 3.456 with SD values of 0.978, 0.948, and 0.516 respectively, with $P = 0.06$, which was not statistically significant as shown in Table 2.

TIMP-1 levels

All the samples, in each group tested positive for

Table 2: Descriptive statistics (results of Kruskal-Wallis test comparing mean concentrations of GCF MMP-3 with respect to CAL)

Study group	CAL	N	Mean	SD	Min	Max	P value
Group II	3	13	6.175	1.159	4.900	8.760	0.000
	4	5	9.417	0.647	8.324	9.885	
	5	2	9.920	0.028	9.900	9.940	
Group III	1	6	1.501	0.978	0.745	2.887	0.060
	2	12	2.155	0.948	0.879	3.787	
	3	2	3.456	0.516	3.091	3.822	

GGF: Gingival crevicular fluid; CAL: Clinical attachment loss; MMP: Matrix metalloproteinase

the presence of TIMP-1. The mean concentration of TIMP-1 concentration in Group I was 8.781 ng/ml with SD 0.584 and with the highest value, i.e., 9.61 ng/ml and lowest value of 8.04 ng/ml. The mean TIMP-1 concentration in Group II was 1.592 ng/ml with SD 0.623 with the highest value, i.e., 2.93 ng/ml and lowest value of 1.02 ng/ml. The mean TIMP-1 concentration in Group III was 6.408 ng/ml with SD 0.386. The highest value of TIMP-1 concentration in Group III was 7.52 ng/ml and lowest value of 4.42 ng/ml. When we compare in between groups the mean TIMP-1 concentrations in GCF was observed to be highest in Group I, i.e., 8.781 ng/ml and lowest in Group II, i.e., 1.592 ng/ml. The mean TIMP-1 concentration in Group III (6.408 ng/ml) fell between the highest and lowest values.

Further, multiple comparisons using Mann-Whitney U-test was carried on to find out, which pair or pairs differ significantly. When Groups I and II, II and III and I and III were compared, the differences were statistically significant with mean ranks between Group I and Group II, i.e., 20.5 and 8.00, between Group II and Group III, i.e., 8.00 and 23.00 and between Group I and Group III, i.e., 20.50 and 8.00 with $P = 0.000$.

To test the hypothesis of equality of means among the three groups non-parametric Kruskal-Wallis test was carried out. The mean ranks obtained for Groups I-III are 35.50, 8.00, and 23.00 for GCF with $P = 0.00$. Therefore, the hypothesis of equality of means is rejected at 5% level of significance ($P < 0.05$), which indicates that the means differ significantly as shown in Table 3. To summarize, the results of the study suggest that the mean concentrations of MMP-3 in GCF were highest for Group II, whereas the same group shown lowest TIMP-1 concentrations. Thus, the result of the study shows that MMPs are counteracted

Table 3: Results of Kruskal-Wallis test comparing mean TIMP-1 concentration in GCF between three groups

Study group	No. of samples	Mean rank	P value
Group I	10	35.50	0.000
Group II	20	8.00	
Group III	20	23.00	

TIMP: Tissue inhibitor of matrix metalloproteinase; GCF: Gingival crevicular fluid

by TIMPs, which inhibit MMP activity there by restricting ECM breakdown. Hence, the balance between MMPs and TIMPs play an important role in maintaining the integrity of healthy tissues.

DISCUSSION

Periodontitis is an inflammatory disease of supporting tissues of the teeth, characterized by a loss of collagen fibers and destruction of ECM constituents in periodontal tissues resulting in pocket formation.^[8,9] A possible mechanism for the degradation of periodontal ECM is the independent and/or cooperative action of both human and bacterial proteinases.^[10,11]

Periodontal tissue destruction is mediated by the MMPs derived from the host cells. The MMP family includes 23 human MMPs. Each MMP has distinct and overlapping property, which can cleave all the components of extracellular matrix and basement membrane. The activities of most MMPs are low in normally healthy periodontium, whereas more MMP concentrations can be detected in diseased and inflammatory conditions of periodontal tissues.^[12,13]

MMP-3 is a broad spectrum MMP and has a pivotal role in activating latent MMPs including pro-MMP-1, -8 and -9. Hence, the regulatory effects of MMP-3 is important in the overall regulation of connective tissue degradation in both physiologic and pathologic conditions.^[13,14] Previously, MMP-3 expression and increased amount of MMP-3 mRNA in periodontal lesions have been demonstrated (Ingman *et al.*, 1996) and it has been suggested that MMP-3 may act as a marker stromal cell in the tissue degradation process. Therefore, we have investigated the MMP-3 levels in GCF of healthy, chronic periodontitis, and after treatment groups.

This study presents two interesting findings. One is the positive correlation of clinical parameters, i.e., gingival index, probing pocket depth, CAL, MMP-3,

and TIMP-1 concentrations in diseased state. The second is the relatively low or reduction in concentrations of MMP-3 and increase in TIMP-1 concentrations in GCF after phase-1 therapy.

In this study, the MMP-3 and TIMP-1 levels in GCF were determined in healthy and chronic periodontitis patients. The principle finding of this study was the active levels of MMP-3 in GCF were significantly higher than the healthy subjects. In addition, there was a significant reduction in MMP-3 and increase in TIMP-1 levels of same sites after scaling and root planing.

In the present study, the mean concentrations of MMP-3 in GCF were found to increase progressively from healthy (i.e., 0.344 ng/ml) to periodontitis group (i.e., 7.490 ng/ml) with $P = 0.000$. These results are in accordance with Tüter *et al.*^[15] and Haerian *et al.*^[16] whereas the TIMP-1 in GCF were found to decrease progressively from healthy (i.e., 8.7810 ng/ml) to periodontitis group (i.e., 1.592 ng/ml) with $P < 0.001$, these results are in accordance with Tüter *et al.*

To inhibit disease progression successfully, it is important to reduce the MMP activity in the diseased state by phase-1 periodontal therapy. In the present study, chronic periodontitis subjects were treated by SRP and strict oral hygiene measures were instituted. The mean MMP-3 concentrations in GCF in chronic periodontitis group reduced from 7.490 ng/ml to an after treatment levels of 2.129 ng/ml, which were statistically significant with value $P < 0.001$. The mean TIMP-1 concentrations in GCF in chronic periodontitis group increased from 1.592 ng/ml to an after treatment levels of 6.408 ng/ml. The results are in accordance with Haerian *et al.*^[16,17]

Although the principle findings of this study are mostly congruent with previous studies, the most significant results may be obtained by the use of micro-capillary pipettes in this study for GCF collection and determination of MMP-3 and TIMP-1 levels by ELISA. In the previous studies, GCF was collected by paper points, and the molecule concentrations were analyzed by periotron, which can result in non-specific attachment of the analyte to the filter paper fibers ensuing in a false reduction in the detectable molecular levels, which underestimates the correlation of molecular levels to disease.

CONCLUSION

In conclusion, within the limits of our study, it can

be postulated that, greater the extent of periodontal destruction, increase in the concentrations of MMP-3 and decrease in the concentrations of TIMP-1 in GCF were seen. When chronic periodontitis subjects were treated by SRP, the mean GCF MMP-3 concentrations reduced, and TIMP-1 levels increased significantly. However, further longitudinal studies are needed to evaluate the concentrations of MMP-3 and TIMP-1 levels in the periodontal disease tissues and GCF and then it will be beneficial in clarifying the role in the pathogenesis of periodontitis and to validate MMP-3 and TIMP-1 as “Novel Biomarkers” of periodontal disease.

ACKNOWLEDGMENTS

We thank Prof. Dr. K. Krishna Murthy and Prof. Dr. D. Roopa for critically reviewing the manuscript. Support for this research was provided by Dr. Supraja, C.K.S TEJA Institute of Dental Sciences. Without their help, this project would not have been possible. We also acknowledge Mr. and Mrs. Sreedhar, Mr. and Mrs. Chandra Mohan, Mr. Vinod and Mrs. Usha Rani for partly funding this research. We also acknowledge Mr. Diwakar for his extreme support. No conflicts of interest related to this study.

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How to cite this article: Kumar PM, Reddy NR, Deepa A, Babu DM, Kumar AK, Chavan V. Comparison of matrix metalloproteinase-3 and tissue inhibitor of matrix metalloproteinase-1 levels in gingival crevicular fluid in periodontal health, disease and after treatment: A clinico biochemical study. *Dent Res J* 2013;10:434-9.

Source of Support: Nil. **Conflict of Interest:** None declared.