Estimation of lactoferrin levels in gingival crevicular fluid before and after periodontal therapy in patients with chronic periodontitis

Anasuya V. Kivadasannavar, Pushpa S. Pudakalakatti[1], Sanjeevini A. Hattarki[1], Soumya S. Kambali[1]

Abstract

Background: The lactoferrin (LF) is an iron binding protein present specifically and in abundance in the secondary granules of polymorphonuclear leukocytes (PMN's). It has been suggested that LF in crevicular fluid is a useful marker of PMN activity. Hence, this study aimed to estimate the levels of LF in gingival crevicular fluid (GCF) before and after surgical therapy in chronic periodontitis patients to assess the validity of LF in monitoring of treatment results. **Materials and Methods:** A total of 30 patients with chronic periodontitis having probing depth of ≥5 mm who were scheduled for periodontal surgery were included in the study. The clinical parameters were recorded and GCF samples were obtained 2 weeks after scaling and root planing and 2 weeks after conventional flap technique. The samples collected were then assayed for LF using Enzyme-linked immunosorbent assay (ELISA). **Results:** The results showed that LF levels decreased significantly from 266.53 ± 75.86 to 195.47 ± 74.53 after scaling and root planing. There was further significant reduction in LF levels to 90.42 ± 32.89 following 2 weeks of periodontal surgery, indicating decrease in inflammation. **Conclusion:** There is a significant reduction in GCF LF levels following periodontal surgery. Hence, LF levels in GCF could serve as a useful marker for monitoring of periodontal treatment results.

Keywords: Gingival crevicular fluid, lactoferrin, periodontal flap surgery, periodontitis, scaling and root planing

Introduction

Periodontitis is a chronic infectious disease caused by the interaction of micro-organisms with the host.[1] Recent evidence implicates that periodontitis progresses episodically and thus, there is a need to develop more reliable markers of disease activity than clinical criteria.[2] Qualitative assessment of crevicular fluid might therefore be helpful in determining the state of active disease, response to therapy, and the nature of the process occurring within the supporting tissues.[3]

Acute phase proteins are products of acute episodes of inflammation and tissue destruction. The five acute phase proteins that have been most examined in gingival crevicular fluid (GCF) are α 2 macroglobulin, α 1 proteinase inhibitor, transferrin, lactoferrin (LF), and C reactive protein.[4] LF is an iron binding protein present specifically and, in abundance, in the secondary granules of polymorphonuclear neutrophils (PMN's), but not in other leukocytes and only in trace amounts in serum.

The PMN is the predominant leukocyte within the gingival crevice in both health and disease and play an important role, being the first cellular host defense against bacterial invasion. However, they are also involved in the process of tissue destruction. PMN secondary granules are more numerous than primary granules and their contents are released earlier and more readily during inflammatory responses.

Studies have suggested that LF in crevicular fluid correlates strongly with the number of PMNs in crevicular fluid and LF could be used as a marker for secondary granule release from PMN. Therefore, the quantification of GCF LF can be a sensitive and objective method of detecting the degree of periodontal inflammation.[5]

LF is an important antimicrobial protein. It has high affinity for iron and it acts on bacteria by causing iron depletion and thus reduction in bacterial cell division rate, glucose metabolism, and macromolecular synthesis. Besides bacteriostatic activity, LF also has bactericidal effects independent of iron deprivation. In addition, LF facilitates phagocytosis of plaque bacteria by reducing their hydrophobicity and preventing their adherence. LF may also be implicated in the inflammatory response by enhancing PMN adhesiveness and chemotaxis by scavenging iron, which could catalyze free hydroxyl radical formation.[6]

Department of Dentistry, General Hospital, Bailhongal,
[1]Department of Periodontics, Maratha Mandal's Nathajirao G. Halgekar's Institute of Dental Sciences and Research Center, Belgaum, Karnataka, India

Correspondence: Dr. Sanjeevini A. Hattarki,
Department of Periodontics, Maratha Mandal's Nathajirao G. Halgekar's Institute of Dental Sciences and Research Center, Belgaum - 590 016, Karnataka, India.
E-mail: drsanju_amar@yahoo.com

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Several studies have reported higher levels of LF in the crevicular fluid of gingivitis and periodontitis patients compared to healthy subjects and correlation of these levels with clinical parameters.[5] Recent evidence suggests a significant reduction of LF levels in the crevicular fluid following the surgical periodontal treatment.[7]

Hence, the present study was undertaken to estimate the levels of LF in gingival crevicular fluid in patients with chronic periodontitis and compare these levels before and after periodontal surgical therapy in an attempt to assess the validity of LF as a plausible marker in monitoring of periodontal treatment results.

Materials and Methods

This study was conducted in the Department of Periodontics, KLES's Institute of Dental Sciences, Belgaum, Karnataka. The laboratory procedures were carried out in the Department of Molecular Biology and Immunology, Maratha Mandal's Institute of Dental Sciences, Belgaum. An ethical clearance was obtained before the study by the Ethical Committee, KLES's Institute of Dental Sciences, Belgaum. A total of 30 patients (18 males and 12 females) with chronic periodontitis age ranging between 28 years and 52 years (mean age 40.5 years) were included in the study. The procedure was explained and a written consent was obtained from the patients.

Inclusion criteria

Age group: 28-52 years; patients should have 14 or more natural teeth; chronic periodontitis patients with probing depth of 5 mm or more; patients without history of any periodontal treatment in the last 6 months.

Exclusion criteria

Patients with history of any systemic diseases, pregnant women or those using hormonal contraceptives, patients who had received antibiotics in the past 3 months, and smokers.

Site selection

The sample sites were selected based on probing pocket depth of ≥5 mm and positive for bleeding on probing. The maxillary quadrants indicated for conventional surgical flap procedures were included in the study. GCF was collected from the anterior teeth to avoid contamination and for ease of collection. The site having the deepest probing depth of the quadrant was selected. The following clinical parameters were recorded at the test sites: Plaque index, bleeding on probing, probing pocket depth. The pocket depth was recorded in mm using graduated William's periodontal probe.

Procedure for collection of Gingival Crevicular Fluid

The clinical parameters were recorded and supragingival scaling was done with hand scaling instruments. Test sites were carefully dried with a gentle stream of compressed air. The calibrated microcapillary tube (0-5 µl range) was placed extra crevicularly at the deepest site of the tooth. A standardized volume of 3 µl was collected in the microcapillary tube. If debris or blood clogged into the microcapillary tube, the GCF collection was rejected and repeated. The 3 µl of collected GCF was then transferred to small plastic vials containing 97 µl of normal saline to make 100 µl of sample volume. The samples were then analyzed for LF the same day.

Then, following the sample collection, thorough scaling and root planing was done and patients were recalled after 2 weeks. The GCF samples were collected from the same test sites and clinical parameters were recorded. After which, conventional flap surgery was performed at the test sites on the same day. Sutures of 3-0 mersilk were placed and the treated site was covered with non-eugenol (Coe-Pack) dressing. Post surgical instructions were given. The patients received a course of anti-inflammatory drugs for a period of 3 days post-operatively and Chlorhexidine mouthwash was prescribed. The sutures were removed after 7 days. The patients were recalled 2 weeks (14 days) after the surgical procedure. Plaque index was recorded and GCF sample was collected from the same test sites. The GCF sampling was done in the same site throughout the study period.

The collected GCF sample was analyzed using Enzyme-linked immunosorbent assay (ELISA) technique.

Biochemical laboratory procedure

Principle

GCF samples diluted in the ratio of 1:33 were incubated in the microplates coated with the specific antigen. The unbound antigen was washed off in the following step. Later, antihuman immunoglobulins conjugated to horseradish peroxidase were incubated and made to react with the antigen-antibody complex of the samples in the microplates. Unbound conjugate was washed off in the following step. Addition of 3,3',5,5'-Tetramethylbenzidine (TMB)-substrate generates an enzymatic colorimetric (blue) reaction, which is stopped by diluted acid (color changes to yellow). The rate of color formation from the chromogen is a function of the amount of conjugate bound to the antigen-antibody complex, and this is proportional to the initial concentration of the respective antibodies in the patient sample.

Enzyme-linked immunosorbent assay procedure

The collected GCF sample was analyzed using ELISA technique (AESKU Diagnostics). Each patient's 100 µl of diluted GCF samples were taken in the designated microwells. In each of these wells, 100 µl calibrators were added. A plate cover was placed on wells and incubated for 30 minutes at room temperature (20-26°C). After 30 minutes, liquid from each well was aspirated. 300µl of washing solution was dispensed into each well. Contents of each well were aspirated again and the above steps

repeated 2-3 times. Then, 100 µl of conjugate solution was added to all the wells and incubated for 15 minutes at room temperature. Microwell strips were washed as mentioned above. Into each of these wells, 100 µl of ready to use TMB substrate solution was pipetted. Addition of substrate changed the solution to blue and the wells were again incubated for 15 minutes at room temperature. The substrate reaction was stopped by addition of 100 µl of stop solution containing Hydrochloric acid and incubated for 5 minutes at room temperature. Then, the absorbance was read at 450 nm with ELISA reader immediately. The machine recorded the optical density of each calibrator and converted this into values by comparison with standard graph. Each of these values were then multiplied by the dilution factor (33) to obtain LF concentration in U/ml of the given sample.

The study was statistically analyzed using Wilcoxon's sign rank test by the aid of statistical software to compare the values at various intervals of time. The level of significance of '*P* value at 95% confidence interval was calibrated as follows: Significant (S): $P < 0.05$

Results

The levels of LF (U/mg) were measured in 30 chronic periodontitis patients who were scheduled for conventional surgical flap procedure. The LF levels were measured at various intervals of time. The levels were recorded at baseline, 2 weeks after scaling and root planing and 2 weeks after periodontal surgery. The following results were obtained [Table 1].

The mean and standard deviation were calculated for LF levels and plaque index scores at various intervals during the study period. The mean and standard deviation of LF levels at baseline were 266.53 ± 75.86, 2 weeks after scaling and root planing were 195.47 ± 74.53 [Table 2] and 2 weeks after periodontal surgery were 90.42 ± 32.89 [Tables 3 and 4]. The mean and standard deviation of plaque index scores at baseline were 1.50 ± 68.2, 2 weeks after scaling and root planing were 0.30 ± 0.47 and 2 weeks after periodontal surgery were 0.07 ± 0.25. The results thus obtained were compared statistically using Wilcoxon sign rank test at different intervals of time.

The values obtained at baseline were compared with values obtained 2 weeks after scaling and root planing. The LF levels reduced from 266.53 U/ml to 195.47 U/ml and when the values were compared statistically there was significant reduction with $P < 0.0002$ [Table 2]. Thus, the results indicated that there was significant reduction in LF levels 2 weeks after scaling and root planing. There was further reduction in LF levels after surgery from 195.47 to 90.42 with $P < 0.0001$ suggesting statistically significant reduction following surgery [Table 3]. When the values obtained 2 weeks after surgery were compared with baseline, the results were highly significant. Thus, the results indicated that there was significant reduction in LF levels following periodontal surgical therapy.

The plaque index scores obtained 2 weeks after scaling and root planing and 2 weeks after surgery were compared

Table 1: Levels of lactoferrin (U/ml) in GCF at baseline, 2 weeks after scaling and root planing and 2 weeks after surgery

Baseline	After scaling and root planing	After surgery
320.1	221.1	72.6
188.7	128.7	95.7
221.1	211.2	79.2
221.2	221.1	244.2
332.4	211.2	105.6
363	244.2	69.3
415.8	359.7	124.9
320.1	244.2	112.2
267.3	183.9	52.8
194.7	165	85.8
260.7	196.8	69.3
287.1	112.2	72.6
450.1	432.3	124.9
221.1	118.8	69.3
250.8	194.7	69.3
320.1	244.2	112.2
194.7	165	95.7
267.3	128.7	85.8
250.8	138.4	75.9
188.1	112.2	79.2
250.8	149.8	52.8
359.7	244.2	105.6
221.1	194.7	72.6
165	105.6	69.3
312.5	240.3	79.2
194.7	125.6	85.8
188.1	165	69.3
211.2	194.7	72.6
363	245.6	112.2
194.7	165	95.7

GCF: Gingival crevicular fluid

Table 2: Comparison of mean lactoferrin levels between baseline and 2 weeks after scaling and root planing

Time intervals	Mean	SD	*P* value	Significance
Baseline values	266.53	75.86	<0.0002	HS
After scaling and root planing	195.47	74.53		

SD: Standard deviation; HS: Highly significant

with baseline values. The plaque scores reduced from 1.50 to 0.30 after scaling and root planing, and when these values were compared statistically, there was significant reduction with $P < 0.0001$ [Table 5]. There was further reduction in plaque scores when both the values were compared statistically. There was significant reduction in plaque index scores suggesting significant reduction in periodontal inflammation.

Discussion

Periodontitis is a chronic infectious disease caused by the interaction of microorganisms with the host. The PMN is the principal cell within the gingival sulcus during the initiation and progression of periodontal disease.

LF is one of the acute phase proteins present specifically and in abundance in the secondary granules of PMN's. Sources of LF in GCF include PMN's that lies in the crevice and the epithelial cells that are desquamated or otherwise damaged with inflammation and adjacent connective tissue. During active phases of periodontal disease, cell death occurs and intracellular contents are released.[8] As a result, LF released during this process will pass with the inflammatory exudate into GCF. Therefore, GCF levels of LF provides an effective marker of crevicular PMN's.

Hence, the present study was undertaken to estimate the levels of LF in GCF and to compare these values before and after surgical periodontal therapy in an attempt to assess the validity of LF in monitoring of treatment results.

Table 3: Comparison of mean lactoferrin levels between 2 weeks after scaling and root planing and 2 weeks after surgery

Time intervals	Mean	SD	*P* value	Significance
After scaling and root planing	195.47	74.53	<0.0001	HS
After surgery	90.42	32.89		

SD: Standard deviation; HS: Highly significant

Table 4: Comparison of mean lactoferrin levels between baseline and 2 weeks after surgery

Time intervals	Mean	SD	*P* value	Significance
Baseline values	266.53	75.86	<0.0001	HS
After surgery	90.42	32.89		

SD: Standard deviation; HS: Highly significant

Table 5: Comparison of plaque index scores between baseline and 2 weeks after scaling and root planing

Time intervals	Mean	SD	*P* value	Significance
Baseline values	1.50	0.68	<0.0001	HS
After scaling and root planing	0.30	0.47		

SD: Standard deviation; HS: Highly significant

The rationale behind GCF collection in our study included its non invasive means of collection and also that the lesion sites could be sampled. The extracellular method for collection of GCF was employed in our study using microcapillary tubes with 1-5 µl range. A standardized volume of 3 µl was collected from the sites. Sites from the maxillary arch were selected so as to prevent or minimize contamination of saliva.[9]

The time required for collection of GCF varied during the course of the study. The time required for collection during first visits was less compared to the next visits which could be due to increase in capillary permeability associated with inflammatory response in the periodontal tissues.

The baseline recordings of the clinical parameters and LF values were the highest at the test sites as compared to the values obtained at the other visits during the course of the study. The mean LF levels at baseline were 266.53 ± 75.86 U/ml. These findings are in agreement with the investigation carried out of by authors who determined the relationship between the levels of LF in GCF and clinical parameters. They reported higher levels of LF in periodontitis patients compared to healthy subjects and that these levels highly correlated with clinical parameters and GCF volume.[10] This view supports our results that the considerable amount of periodontal destruction had occurred. This may have resulted in the disintegration of epithelial lining of the periodontal pocket that may have contributed to the elevated levels of LF in GCF.

Another possible mechanism for increased levels of LF could be due to PMN emigration or activation in the crevice. The contents of neutrophils, on being released, have the potential for intensifying the inflammatory response and/or inducing host tissue damage. These findings are in accordance with the study done by authors who examined the levels of LF and its correlation with PMN numbers. They reported that LF levels and PMN numbers correlated positively and thus concluded that crevicular LF provides effective marker of PMN numbers.[5] This data supports our results that the increased LF could be due to increased PMNs in inflammation.

The increased bacterial load present at baseline (mean plaque index = 1.50 ± 0.68) correlates with the increased LF levels. These findings were similar to that obtained by Wei *et al*.,[11] who investigated the essential role of LF in free radical production associated with inflammatory periodontal disease. They suggested higher levels of LF in periodontitis sites which correlated with plaque index and the pro inflammatory cytokine interleukin (IL)-1β.

The authors concluded that the tissue damage of reactive oxygen species in periodontitis is due to the chronic insults of periodontopathogens. The potential action of these bacteria may lead to tissue destruction, which may also have contributed to the elevated LF levels.

Two weeks after scaling and root planing, at the second visit a profound reduction in plaque index scores was observed. Mean plaque index scores of 0.30 ± 0.45 suggest significant reduction in the degree of inflammation. The mean LF levels were 195.47 ± 74.53 U/ml. When these values were compared with baseline, there was statistically significant reduction in the LF levels ($P < 0.0002$). This significant fall can be credited to the resolution of gingival inflammation and some amount of healing and restitution of new epithelial attachment following phase I therapy.

The mean LF levels 2 weeks after surgery were 90.42 ± 32.89 U/ml. These values were the least obtained in our study. There was statistically significant reduction in the values when compared to baseline and scaling and root planing.

The mean LF levels 2 weeks after surgery were 90.42 ± 32.89 U/ml. These values were the least obtained in our study. There was statistically significant reduction in the values when compared to baseline and scaling and root planing. Thus, LF levels being lowest after the pathology from the tissues have been eliminated by surgical therapy is convincing. Thus, our results are in favor of improved periodontal condition after surgical therapy.

The probable mechanism for the reduction in LF levels could be due to the down regulation of local PMN activity following surgical periodontal therapy. These findings are in agreement with the study done by Buchmann *et al*.,[12] who examined the local PMN response in untreated and treated chronic periodontitis patients. The levels of GCF lysosomal enzyme activities were analyzed as indicators of PMN associated tissue destruction. They indicated that there was significant reduction in lysosomal enzyme activity 6 months after the surgical therapy.

The results of the present study are in accordance with the investigation done by Jentsch *et al*.,[7] who examined whether the crevicular and salivary variables reflect the changes after periodontal treatment. The results indicated significant reduction of LF levels 14 days after the surgical treatment both in saliva and GCF. These results support the findings of our study, where we also obtained significant reduction of LF levels 14 days after the periodontal flap surgery.

GCF being a potential source for biochemical analysis and that the LF could be used as a useful marker for the assessment of periodontal disease activity. Further studies are required to substantiate our findings. There were certain limitations, as the GCF sample has to be carried to the laboratory we expect some reduction in enzyme activity with time which may result in somewhat subdued values. The GCF sample had to be taken from only the anterior segment of the dentition due to difficulty in placement of microcapillary tubes in the posterior region. Also, mandibular arch could not be utilized for collection of GCF due to the inability of microcapillary tubes to collect GCF against gravity.

Further research needs to be directed towards developing a chairside diagnostic kit to collect and evaluate the concentration of LF in the clinic. This would further enable the clinician to utilize the results and modify the treatment accordingly.

Conclusions

The results of the study conclude that a statistically significant difference exists between the LF levels before and after scaling and root planing and periodontal flap surgery. A correlation has been found between the LF levels in the GCF and the treatment modalities carried out. LF in GCF can be used as a plausible marker for monitoring of treatment effects in periodontal disease. However, long term studies on the predictability and sensitivity of LF as a biochemical marker is required.

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