Journal of Advanced Clinical & Research Insights (2019), 6, 72–77

ORIGINAL ARTICLE

Investigation of leptin levels in periodontal wound healing: A preliminary study

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Keywords:
Gingival crevicular fluid, leptin, periodontal disease, periodontal wound healing

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Received: 14 March 2019;
Accepted: 24 April 2019

doi: 10.15713/ins.jcri.264

Abstract

Background and Objectives: Leptin *(Ob)* is an obese protein and is known to maintain tissue integrity. Off late, there are reports that leptin aids in dermal wound healing. However, to date, there are no reports on whether leptin has a similar role in periodontal wound healing. Hence, the present study was undertaken to explore whether leptin has a similar role in periodontal wound healing.

Materials and Methods: A total of 15 patients (10 males and 5 females) diagnosed as periodontitis were selected who fell under the age range of 30–39 years. The gingival crevicular fluid was collected for leptin analysis from the study group at baseline (who acted as pre-treatment controls), 30th day following non-surgical periodontal therapy (which coincides with the surgical intervention, i.e. open flap periodontal surgery), and at the 3rd day, 14th day, and 35th day following surgical intervention which corresponds to the various phases of periodontal wound healing, acted as post-treatment tests groups. Leptin levels were determined from individual gingival crevicular fluid samples by enzyme-linked immunosorbent assay.

Results: There was a little increase in gingival crevicular fluid leptin levels following surgical intervention which was statistically not significant ($P > 0.01$). One-way analysis of variance and Tukey test were applied for statistical significance.

Conclusion: Within the limitations of our study, it can be concluded that there was a little and insignificant raise of leptin at various stages of periodontal wound healing. Hence, it is not clear whether leptin aids in periodontal wound healing and maintains tissue integrity and homeostasis. At the same time, we cannot categorically deny the role of leptin in periodontal wound healing.

Introduction

Periodontitis is a chronic inflammatory disease involving destruction of the supporting structures of the teeth caused due to specific interaction between the Gram-negative anaerobic bacteria and host immune response.[1] The resolution of inflammation and wound healing depends on the balance between the pro- and anti-inflammatory cytokine. Among the various classical cytokines, which are known to mediate the resolution of the inflammation and aids in wound healing, leptin *(Ob)* is one such molecule which has drawn considerable substantial research attention and has been a subject of recent interest.[2]

Leptin is a product of obese *(Ob)* gene which is a 16-kDa, non-glycosylated polypeptide synthesized primarily by adipocytes[3] and in minor quantities by placenta,[4] T cells,[5] osteoblasts,[6] and gastric epithelium.[7] It has multiple physiological effects and plays a pivotal role in the control of body growth, hematopoiesis[8] angiogenesis,[9] reproduction,[10] regulation of various endocrine axis,[11] lipid,[12] and bone metabolism.[13] Increasing research in this area is gradually revealing the interplay of leptin in maintaining periodontal tissue homeostasis.[14] It has been found that the concentrations of leptin are higher in healthy periodontal tissues and its level declines as periodontal disease progresses, suggesting that leptin plays an important role in immune defense mechanism of the host and is a protective molecule which helps in maintaining tissue homeostasis.[14-17]

There is an accumulating substantiation that leptin plays a key role in the regulation of wound healing and repair by

aiding the wound closure through promoting angiogenesis[9,18,19] reepithelization (mitogenesis of keratinocytes),[20] induction of keratinization,[21] as well as fibroblast proliferation and collagen synthesis.[22]

The accumulating evidence have proven beyond doubt that leptin is necessary to orchestrate the major events involved in the biological process of wound.[23,24] It has also been shown that leptin-deficient *ob/ob* mice have impaired wound healing and on application of leptin both systemically and topically, significantly accelerated the wound healing in a dose-dependent manner reversing the atrophic morphology of the wound margins into a well-organized hyperproliferative epithelium, strongly suggesting the importance of leptin in major key events in wound healing.[20,25]

Based on the above studies, it is reasonable to presume that leptin might have a similar effect in periodontal tissue wound healing and its levels may be upregulated. However, to date, there is no direct/valid evidence of the involvement of leptin in periodontal wound healing and has remained unexplored till date. Hence, the present study is designed, which is the first of its kind to determine the role of leptin if any, by estimating and correlating its levels in gingival crevicular fluid at various stages of periodontal wound healing.

Materials and Methods

Selection of patients

For this study, a total of 15 patients (10 males and 5 females) with an age range of 30–39 years, who visited the outpatient section of the Department of Periodontology (Krishnadevaraya College of Dental Sciences and Hospital, Bengaluru, India) for periodontal treatment, were selected for the study from May 2018 to November 2018.

All eligible patients, who volunteered, were informed of the nature, potential risks, and benefits of their participation in the study, and a written signed informed consent was obtained from whom who agreed to participate. The clearance for the study was obtained from the ethical committee of Krishnadevaraya College of Dental Sciences and Hospital, affiliated to the Rajiv Gandhi University of Health Sciences, Bengaluru, India.

The periodontitis patients were selected if they had signs of clinical inflammation, modified gingival index (MGI) of ≥2,[26] plaque index (PI),[27] gingival bleeding index,[28] relative attachment level (RAL) ≥8 mm of at least 10 teeth, and radiographic evidence of bone loss. Afterward, each subject underwent an initial full-mouth periodontal probing and charting. Probing pocket depth and RAL were measured using a UNC-15 periodontal probe (Hu-Friedy Manufacturing Co., Chicago, IL, USA).

The patients were recruited for the study if they had at least 20 functioning teeth (excluding third molars and teeth missing as a result of exodontia) and were systemically healthy. The exclusion criteria included aggressive periodontitis, bleeding disorders, anomalies of the immune system, pregnancy, lactation, obesity, body mass index ([BMI] chart for Asian population given by the WHO in 2002 (18.5–22.9 kg/m^2),[29] smoking, and periodontal treatment or the usage of antibiotics within the past 6 months. The systemic conditions elicited by a medical history likely to influence the inflammatory response (cardiovascular disease, respiratory infections, rheumatoid arthritis, diabetes, etc.) were also excluded from the study.

Site selection

The gingival crevicular fluid samples were obtained only from the buccal and palatal sites. For a distinct standardization of the sampling site, gingival crevicular fluid sample was collected at the second appointment from the interproximal sites, among which, the site showing greatest probing depth, RAL along with radiographic conformation of alveolar bone loss, was selected for gingival crevicular fluid sample collection.

Patient preparation

All participants were instructed to avoid eating or brushing their teeth 90 min before gingival crevicular fluid sampling, to avoid interference with the gingival crevicular fluid volume. For the sample purpose, all the samples were obtained between 10:00 AM and 11:00 AM. Patients were seated comfortably in an upright position on the dental chair and the light was focused to illuminate the area to be examined. Before gingival crevicular fluid sampling, gentle removal of supragingival plaque was completed using dry gauze, without touching the marginal gingival. The sites were then isolated by cotton rolls, rinsed gently with water, and dried with a gentle air spray directed perpendicular to the gingival margin. A saliva ejector was used to avoid salivary contamination of the samples.

Gingival crevicular fluid sample collection

The gingival crevicular fluid was collected for leptin analysis from the study group at baseline, i.e., before non-surgical periodontal therapy (who acted as pre-treatment controls), 30th day following non-surgical periodontal therapy (which coincides with the surgical intervention, i.e., open flap periodontal surgery. In a quadrant of 8 teeth, at least 4–5 teeth with RAL ≥8 mm were included for open flap debridement), and at the 3rd day, 14th day, and 35th day following surgical intervention which corresponds to the various phases of periodontal wound healing (acted as post-treatment tests groups). The gingival crevicular fluid was collected and analyzed for the levels of leptin using enzyme-linked immunosorbent assay (ELISA). The time periods were selected to capture the critical periods during the three phases of wound healing. To prevent any error in recording the clinical parameters, a well-trained and a calibrated examiner (kappa value for interexaminer calibration was >0.8) did all the recordings. Further, professional prophylaxis was performed throughout the study during recall intervals in addition to reinforcement of oral hygiene instructions. This was done in an attempt to promote wound healing and maintain plaque control at an acceptable level throughout the study.[30]

Unstimulated gingival crevicular fluid sample was collected from the patients, for the purpose of which, a sterile Periopaper strip (Periopaper®, Amityville, NY, USA)™ was gently inserted into the entrance of the selected site until the first sign of resistance was felt with 1 mm depth being the maximum. The strip was held in place for 30 s. Attempts were made to avoid the insertion of the strips to the full depth of the pocket, to minimize the risk of contaminating the gingival crevicular fluid with blood. Strips contaminated with blood were discarded and alternate site with equable parameters was sampled. The measurement of the gingival crevicular fluid was performed using Periotron 8000 (Periocol, Oraflow Inc., USA)™ and the data were indicated in Periotron units (PUs). Periotron calibration for the Periotron 8000 was done due to the introduction of a software program which accompanies the device called MLCONVERT. The program will automatically convert inputted data volumes (μl) and according to the manufacturer's instruction manual has two main functions: (1) It will fit a 4th order polynomial function to standard Periotron calibration data for each of the sampling paper used (i.e., Periopaper, Sialopaper) and (2) it will read a patient's file output and convert the PUs to volumes using the 4th order polynomial equation derived from the standard curve.

Gingival crevicular fluid sample preparation

Gingival crevicular fluid samples were placed in aliquots containing 200 μL of phosphate-buffered saline. To elute (extract) the gingival crevicular fluid from paper strips, the following technique was used: The aliquots were centrifuged for 5 min at 5800 g to remove plaque and cellular elements. The strips were removed and the elutes and the samples were stored at −80°C until further use.

Method of estimation of leptin

The levels of leptin were estimated using an Invitrogen Hu Leptin kit (BioSource International Inc., Camarillo, CA, USA) which is a solid-phase sandwich ELISA, according to the manufacturer's instructions. Each plate was checked before use to ensure the calibration curve measured leptin standards (0–1000 pg/mL) within the stated limits of the assay. The kit made use of biotin conjugate and human leptin antibody. All the samples were run in duplicate. An ELISA reader (molecular dynamics, Sunnyvale, CA, USA) with 405 nm as the primary wavelength was used to measure the absorbance of the substrate color reaction. The optical density values obtained with the known samples were used to calculate the quantity of leptin in the other samples.

Statistical analysis

Shapiro–Wilks test was done to analyze if the results were parametric or non-parametric. For intragroup comparison of gingival crevicular fluid leptin levels in the study groups, parametric one-way analysis of variance (one-way ANOVA) test was carried out at 5% level of significance. Further, Tukey test was used for pairwise comparison of the mean differences among the pre-treatment controls and post-treatment tests groups. $P < 0.05$ was considered statistically significant. Spearman's rank correlation test was done to check for the presence of any correlation between the gingival crevicular fluid leptin levels and the clinical parameters including MGI, PI, gingival bleeding index, and RAL. Data analysis was performed using the Statistical Package for the Social Sciences (SPSS)-15 for Windows (SPSS Inc., Chicago, IL, USA).

Results

The highest mean leptin levels in gingival crevicular fluid were obtained for post-surgical intervention (open flap periodontal surgery) at the 35th day (430.06 pg/ml) and least mean leptin level was obtained for post-surgical intervention at the 3rd day (228.45 pg/ml). The mean gingival crevicular fluid leptin levels in the pre-treatment controls and post-treatment tests (30th day following non-surgical periodontal therapy and surgical intervention therapy evaluated at the 3rd day and 14th day) were intermediate between the highest and lowest values. These results are shown in Table 1.

For intragroup comparison of gingival crevicular fluid leptin levels in the study groups, parametric one-way ANOVA test was carried out at 5% level of significance and the results for these are tabulated in Table 1. Further, Tukey test was used for pairwise comparison of the mean differences among the pre-treatment controls and post-treatment tests groups for which the results are shown in Table 2. Spearman's rank correlation test was done to check for the presence of any correlation between the gingival crevicular fluid leptin levels and the clinical parameters including MGI, PI, gingival bleeding index, and RAL Table 3. The results demonstrated that the gingival crevicular fluid leptin levels possessed non-significant and negative correlation with MGI, PI, gingival bleeding index, and RAL.

Discussion

Collectively, the growing evidence has led to the suggestion that leptin expression is upregulated after dermal injury and is necessary for normal healing process to occur.[31] With regard to periodontal wound healing, we speculate that leptin would have

Table 1: Results of one-way ANOVA test comparing mean leptin levels in gingival crevicular fluid between the test and control

Visit	*n*	Mean±SD	Min.	Max.	"F" value	"*P*" value
At baseline	15	258.91±65.784	142.96	363.09	26.637	<0.001*
Post-NSPT	15	366.13±80.132	214.73	474.46		
Post-surgery						
3rd day	15	228.45±42.617	181.44	307.60		
14th day	15	333.45±49.261	252.47	417.85		
35th day	15	430.06±60.384	332.39	525.14		

Since, the *"*P*" value is <0.05, the hypothesis of similarity of means is rejected and further multiple comparisons are performed using one-way ANOVA test. NSPT: Non-surgical periodontal therapy, ANOVA: Analysis of variance

Table 2: Pair-wise comparison between the test and controls using one-way ANOVA test for gingival crevicular fluid

Visit	Mean difference	"*P*" value
At baseline	−107.219	<0.001*
Post treatment		
Post-surgery		
3rd day	30.460	0.651
14th day	−74.538	0.011
35th day	−171.151	<0.001*
Post-NSPT		
3rd day	137.679	<0.001*
14th day	32.681	0.588
35th day	−63.932	0.042
Post-surgery 3rd day		
Post-surgery		
14th day	−104.998	<0.001*
35th day	−201.611	<0.001*
Post-surgery 14th day	−96.613	<0.001*
Post-surgery		
35th day		

Tukey test was used for pair-wise comparison of the mean differences among the pre-treatment controls and post-treatment tests groups. NSPT: Non-surgical periodontal therapy. *"*P*" value <0.05. Thus, stating the rejection of null hypothesis

Table 3: Results of Spearman's rank correlation (r) test to compare GCF leptin levels with clinical parameters MGI, PI, BOP, and RAL

		MGI	PI	{(BOP %) Gingival bleeding index}	RAL
GCF leptin (pg/ml)	Correlation	−0.280[a]	−0.531[a]	−0.245[a]	−0.450[a]
	N	15	15	15	15

GCF [a]If the "r" value is between −0.5 and 0, there is a weak negative correlation; if the "r" value is between 0 and 0.5, there is a weak positive correlation; if the "r" value is between 0.5 and 1, there is a strongly positive correlation; and if "r" is 1, there is 100% positive correlation between the two sets of data compared. Gingival crevicular fluid leptin levels possessed non-significant and negative correlation with MGI, PI, BOP (gingival bleeding index), and RAL, GCF: Gingival bleeding index, MGI: Marginal gingival index, PI: Plaque index, RAL: Relative attachment level

a similar protective effect on tissue integrity and homeostasis in the gingiva, and there might be active upregulation of leptin synthesis in response to tissue insult which could enhance the periodontal wound healing. To the best of our knowledge, no literature has documented whether leptin has any role in periodontal wound healing similar to dermal wound healing. Since gingival crevicular fluid reflects local tissue activity, investigating the leptin levels in gingival crevicular fluid at various phases of periodontal wound healing can throw a light on whether leptin has any role in periodontal wound healing. Hence, the present study which is first of its kind was undertaken to determine the role of leptin, if any, at various stages of periodontal wound healing, by estimating and correlating the leptin levels in gingival crevicular fluid.

In the present study, 15 patients (10 males and 5 females) were selected who fell under the age range of 30–39 years. The gingival crevicular fluid was collected for leptin analysis from the study group at baseline (who acted as pre-treatment controls), 30th day following non-surgical periodontal therapy (which coincides with the surgical intervention, i.e., open flap periodontal surgery), and at the 3rd day, 14th day, and 35th day following surgical intervention which corresponds to the various phases of periodontal wound healing (acted as post-treatment tests groups). The 3-day point corresponds to the peak of inflammatory phase, 14-day point to the peak in granulation tissue formation, and the 35-day point corresponds to the matrix formation phase. All the participants were under a stringent oral hygiene programme. The gingival crevicular fluid was collected and analyzed for the levels of leptin using ELISA.

Collection of accurately quantitated, site-specific gingival crevicular fluid samples was done using the standardized PerioPaper strips to ensure a traumatism and accurate quantification of the molecule. Instead of gingival biopsies, we chose gingival crevicular fluid as a sample to estimate the leptin levels for making the sample collection atraumatic. Moreover, to avoid leptin derived from obese patients biasing the estimation of leptin levels, they were excluded from the study by selecting patients who fell in normal BMI chart for Asian population given by the WHO in 2002 (18.5–22.9 kg/m^2).[29] The use of sensitive ELISA to identify leptin from single sites allowed us to avoid pooling of gingival crevicular fluid samples from multiple sites or patients since periodontitis is a site-specific disease.

The results of our study demonstrated a mean gingival crevicular fluid leptin concentration at baseline (who acted as pre-treatment controls) was 258.91 pg/ml, the 30th day following non-surgical periodontal therapy (which coincides with the surgical intervention, i.e., open flap periodontal surgery) was 366.13 pg/ml. Following the surgical intervention, i.e., at the 3rd, 14th and 35th days, the mean gingival crevicular fluid leptin concentrations were 228.45 pg/ml, 333.45 pg/ml, and 430.06 pg/ml, respectively. These multiple time points are necessary for studying the periodontal wound healing process, due to the complexity and dynamic nature of this process. In this study, to capture periodontal wound healing events, we used three different time points that are important in wound healing processes.

There was a raise in the gingival crevicular fluid leptin concentration from baseline value of 258.91 pg/ml to 366.13 pg/ml.[15-17,31] This shows that there is a statistically significant ($p < 0.05$) raise in leptin levels between the baseline and non-surgical periodontal treatment. The results of our study are in accordance with several authors who have studied the role of leptin in periodontal health and disease and effect of periodontal treatment on leptin.[16,17,31,32] Further, it was noted that from the surgical intervention day with open flap periodontal surgery (i.e., the 30th day of non-surgical periodontal

therapy) to the 3rd day following surgical intervention, there was a decrease in gingival crevicular fluid leptin levels from 366.13 pg/ml to 228.45 pg/ml which was statistically significant (mean, $P < 0.01$). This could be possibly due to any persistent residual inflammation which could have lowered the leptin levels as it is known that leptin levels are lower when there is inflammation, which might have increased the net rate of leptin removal from the gingival tissue. It may also be a result of leptin being used up as substrate during inflammation or it may be an artefact.[16,17]

Later, from the 3rd day (228.45 pg/ml) to the 14th day (333.45 pg/ml) and up to the 35th day (430.06 pg/ml) following surgical intervention, there was a little increase in gingival crevicular fluid leptin levels, i.e., 201.61 pg/ml. This shows that there was only a mild raise in the leptin levels at the post-surgical wound healing periods, and the results did not reach the level of statistical significance (mean, $P > 0.01$). This non-significant raise in leptin concentration at periodontal wound healing sites may be attributed due to several reasons and limitations of our study. First, as the leptin level depends on the gender and healing pattern, in our study, the patients were not gender matched which could have influenced the leptin levels. Second, there may be variation in leptin levels in patients from heterogeneous Indian population and ethnicity. Third, in our study, the storage time was around 5 months and there is a possibility that during the storage time, leptin molecule might have developed a stronger bond with other molecules. Thus, to ensure effective gingival crevicular fluid leptin analysis, it is suggested that the storage time should be reduced. Fourth, since gingival crevicular fluid collection was done using PerioPaper, there is a possibility of molecule strongly binding to the analytic paper and could not have been recovered during elution as recovery assay of leptin from analytical paper was not done in our study. Fifth, as the surgical time period influences the healing process, in our study, due to any prolongation of the surgical time which was not standardized could have raised the inflammation and, in turn, led to a decrease in the leptin levels. Sixth, there might be a fluctuation in the leptin levels in gingival crevicular fluid and at gingival tissue, since it is not clear whether leptin originates from the gingiva or the presence of leptin in gingival crevicular fluid is the result of transport from circulation. In our study, we used gingival crevicular fluid for sampling instead of gingival tissue specimens which could have influenced the leptin levels [20] Seventh, leptin produced might be bound to the receptor and may not be present in a free form in the gingival crevicular fluid [33] Eighth, statistical significance could not be achieved due to the relatively small number of patients in our study.

Although the raise in leptin levels was not statistically significant at the wound healing phase, as against our initial assumption, we cannot categorically deny its role in periodontal wound healing due to the above limitations. To overcome the limitations of our study and to explore the role of leptin in various phases of periodontal wound healing, future studies should be performed to determine the origin of leptin and its receptor in gingiva and its expression pattern in health and disease. Prospective studies with a larger sample size could be designed to consider the ratio of leptin with key cytokines such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α (TNF-α). There is a need for subgrouping of the study population according to the serum lipid levels (normolipidemic, hyperlipidemic, hypercholesterolemic, etc.), the different BMI values (normal, obese, morbid obese, etc.), and body fat mass to prevent the systemic health influencing the periodontal tissue leptin levels. In addition, to avoid the microbial influence on gingival crevicular fluid leptin levels, gingival biopsies should be harvested and analyzed for the presence of leptin and its receptor expression in various phases of periodontal wound healing.

Conclusion

Within the limitations of our, it can be concluded that leptin was present and detected in all the samples of gingival crevicular fluid. However, there was a little and insignificant raise of leptin at various stages of periodontal wound healing. Therefore, it is not clear whether leptin aids in periodontal wound healing and maintains tissue integrity and homeostasis. At the same time, we cannot categorically deny the role of leptin in periodontal wound healing.

Acknowledgments

The authors wish to thank all the patients who wholeheartedly participated in the study. The authors report no conflicts of interest related to this study.

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How to cite this article: Bharadwaj CKS, Varadhan KB, Venkatesh PML, Gautam RS. Investigation of leptin levels in periodontal wound healing: A preliminary study. J Adv Clin Res Insights 2019;6: 72-77.

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