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ASSESSMENT OF GINGIVAL CREVICULAR FLUID LEPTIN LEVELS IN OBESE AND NON-OBESE INDIVIDUALS WITH AND WITHOUT CHRONIC PERIODONTITIS

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ABSTRACT

Aim and Objectives: Leptin, a multifunctional adipokine and pleiotrophic hormone produced by the adipose tissue plays a significant role in pathogenesis of periodontitis. The purpose of this study is to compare the gingival crevicular fluid (GCF) leptin levels in obese and non-obese individuals with and without Chronic Periodontitis and to lend greater clarity to the role of leptin as a reliable biomarker for Periodontitis. **Materials and Methods:** Sixty subjects were divided into 4 groups of 15 each based on the clinical evaluation and body mass index (BMI) – Group I (obese with healthy periodontium), Group II (non-obese with healthy periodontium), Group III (obese with chronic periodontitis) and Group IV(non-obese with chronic periodontitis). GCF samples (by microcapillary pipettes) were collected to estimate the levels of leptin using enzyme linked immunosorbent assay (ELISA) kit **Results:** The mean GCF leptin levels in Group I is 715 ± 71pg/ml, Group II is 503 ± 230 pg/ml, Group III is 346 ± 82 pg/ml and Group IV is 137 ± 40 pg/ml. It proved that Group I and II had significantly higher leptin concentrations than Groups III and IV ($p<0.05$). In healthy and periodontitis subjects BMI and leptin were positively correlated. **Conclusion**: The results suggested that leptin being an adipokine is increased in obese subjects and GCF leptin levels are increased in healthy states. Since leptin concentration in GCF is inversely proportional to clinical attachment loss, leptin may be a prognostic marker in periodontal disease progression and in assessing post treatment outcome

Key words: GCF, Leptin, Obesity, Periodontitis

INTRODUCTION

Periodontitis is a polymicrobial and multifactorial infection characterized by a destructive inflammatory processes that leads to loss of tooth-supporting tissues. Although disease progression is episodic in nature at a tooth site level, the risk of developing periodontal disease is principally patient-based rather than site-based. While microorganisms are the primary etiologic agent in periodontitis, current research attributes tissue destruction as a consequence of an exaggerated host immune response.[1,2]

Obesity is associated with a state of chronic low level inflammation, which is characterized by abnormal cytokine production and activation of pro-inflammatory signaling pathways. Adipose tissue is not only a reserve passive organ but also a metabolically active endocrine organ releasing adipokines including Leptin, Adiponectin, Resistin, Visfatin, Serum retinol

binding protein (RBP4) and various other cytokines.[3]

Leptin (Ob), a product of the ob gene is a 16 kDa non-glycosylated peptide hormone, synthesized mainly in adipocytes and in minor quantities by placenta, T cells, osteoblasts and gastric epithelium. It regulates weight control and modulates other physiological functions, such as regulation of neuroendocrine, reproductive, hematopoietic systems, and bone remodeling. It has been classified as a cytokine as it shows structural similarities to the long chain helical cytokine family [interleukin (IL)-6][4] and during inflammation, leptin expression is altered in a manner similar to the cytokine response to infection and injury. Though it has been suggested that leptin modulates the host response by enhancing pro-inflammatory cytokine production and phagocytosis by macrophages,[5] other studies have elucidated its anti-inflammatory role also.[6,7]

Very few studies address the problem of altered leptin levels in periodontal health and disease. Though there are no adipocytes in gingiva, studies by **Johnson & Serio et al 2001[8] and Karthikeyan & Pradeep et al 2007[9]** revealed that "leptin concentration is higher in the healthy gingiva compared to diseased gingiva." It may be due to the entrapment of leptin within gingiva by diffusion from microvasculature. Since, leptin has a role in inflammatory response,[3] an increase in leptin level in healthy gingiva may be a host defense mechanism[10]. However, during gingival inflammation its concentration is decreased due to expansion of vascular network, which possibly increases the net rate of leptin removal from the gingival tissues and could raise serum leptin levels. Elevated serum leptin concentration have been suggested as a risk factor for cardiovascular disease by promoting atherosclerosis and enhancing calcification of arterial walls.[11,26]

Leptin levels and the role of Leptin in the pathogenesis of periodontitis is still a matter to be addressed and debated. Hence, this study is designed to 1) Assess the concentration of human leptin in GCF during periodontal health and disease 2) To find out the possible association between BMI and leptin levels 3) To explain it's potential role in the initiation and progression of periodontal disease.

MATERIALS AND METHODS

Patient selection

A total of 60 adult patients, aged 21-48 years, who reported to the Department of Periodontology (SRM Dental College and Hospital, Chennai, India) were selected and randomly allocated to the 4 different groups of 15 each.

Based on the Plaque index, CPITN index, radiograph evidence of bone loss and body mass index, according to the chart of the World Health Organization 2002[12] patients were categorized into four groups. BMI was computed as weight in kilograms divided by square of height in meters. Group I (obese with healthy periodontium) consisted of 15 patients who had clinically healthy gingiva with no evidence of bone loss and clinical attachment loss ie CAL=0 and BMI >30 kg/m^2 Group II (non-obese with healthy periodontium) consisted of 15 patients who had clinically healthy gingiva with no evidence of bone loss and clinical attachment loss ie CAL=0 and BMI<25 kg/m^2. Group III (obese with chronic periodontitis) comprised of 15 patients who showed clinical signs of gingival inflammation with attachment loss and radiographic evidence of bone loss and BMI >30 kg/m^2. Group IV (non-obese with chronic periodontitis) comprised 15 patients who showed clinical signs of gingival inflammation with loss of attachment and radiographic evidence of bone loss and BMI < 25kg/m^2.

Furthermore, the participants were systemically healthy, without taking any medications affecting the periodontal status, and had received no periodontal therapy in the preceding 6 months. Exclusion criteria includes: aggressive forms of periodontitis; smoking; alcoholism; pregnancy and lactating women. Written informed and verbal consent was obtained from all recruits and the ethical clearance was obtained from the institutional ethical committee.

GCF sample collection

Prior to GCF collection, the Supragingival plaque was scored. The test site for GCF sample collection in chronic periodontitis groups (III & IV) was selected based on the highest scored sites in the oral cavity ie the site with maximum attachment loss. In the healthy groups (Group I & II), to standardize site selection, sampling was predetermined to be from the mesio-buccal region of the maxillary first molar.

The test site selected, was air dried, isolated with a cotton roll and supragingival plaque was removed without contacting the marginal gingiva. From each test site, GCF samples were obtained before probing by placing a black color-coded 1–5 µL calibrated volumetric microcapillary pipettes (Sigma Aldrich Chemical Company USA) extracrevicularly (unstimulated) for 5-20 minutes. A standardized volume of 1 µL was collected, using the calibration on the micropipette. Test sites from which no GCF could be obtained, and the micropipette which was contaminated with blood and saliva, were excluded from the study. The GCF collected was immediately transferred to a plastic vial and frozen at -70°C until the time of assay.

Principles of Leptin assay

The assay was performed using the leptin Elisa kit (Biosource International Inc., Camarillo, CA, USA). The manufacturer's instructions were strictly adhered to and 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 assay uses monoclonal antibodies (MAbs) directed against distinct epitopes of human leptin. The samples were run in duplicate. Standards and samples react with the capture monoclonal antibody (MAb 1) coated on the microtiter well and with a monoclonal antibody (MAb 2) labelled with horseradish peroxidase (HRP) forming a sandwich : coated MAb 1 - human leptin - MAb 2 - HRP. Bound enzyme-labelled antibody is measured through a chromogenic reaction. Absorbance of the substrate color reaction was read on an enzyme-linked immunosorbent assay (ELISA) reader using 450 nm as the primary wavelength. The optical density values obtained with the known samples were used to calculate the quantity of leptin in the other samples.

Assay procedure

50 µl of each calibrator, control, and sample was pipetted into the appropriate wells in the microtitre plate. 100 µl of anti-Leptin-HRP Conjugate and then 50 µl of Incubation Buffer was pipetted into all the wells and incubated for 2 hours at room temperature, on an horizontal shaker set at 700 ± 100 rpm. The liquid was apirated from each well. The plate was washed four times by: dispensing 0.4 ml of BioSource Wash Solution into each well and then aspirating the content of each well. 100 µl of Chromogenic Solution was pipetted into each well within 15 minutes following the washing step. The plate was incubated for 30 minutes at room temperature on an horizontal shaker set at 700 ±100 rpm, avoiding direct sunlight. 200 µl of Stop reagent was pipetted into each well. Absorbances was read at 450 nm and 490 nm

(reference filter: 630 or 650 nm) within 3 hours and results calculated.

Statistical analysis

The Kruskal Wallis one way ANOVA test was used to calculate the P- value and the P value < 0.05 was considered statistically significant. The Mann Whitney U test followed by Bonferroni Correction method was employed to identify the significant groups at 5% level. The Spearmans Rank Correlation Coefficient was calculated to assess the relationship between the various clinical parameters and leptin levels in each study group.

RESULTS

Table 1: Representing the mean demographic data and physical characteristics of the subjects belonging to the different groups

Parameters	GROUP – 1	GROUP – 2	GROUP – 3	GROUP – 4
No. of Subjects	15	15	15	15
Mean age	24.4 ± 3.5	23.2 ± 2.2	40.6 ± 7.6	33.9 ± 8.7
BMI (Kg/M. Square.)	31.8 ± 1.7	22.0 ± 2.0	34.0 ± 3.2	22.6 ± 2.4

Table 2: Representing the clinical parameters of various study groups

Variable	Group	Mean ± S.D.	Overall P-value*	Significant Groups[$]
PI	I	0.66 ± 0.30	<0.0001 (Sig.)	I vs. III I vs. IV II vs. III II vs. IV
	II	0.58 ± 0.24		
	III	2.57 ± 0.34		
	IV	2.37 ± 0.38		
CAL	I	0.00 ± 0.00	<0.0001 (Sig.)	I vs. III I vs. IV II vs. III II vs. IV
	II	0.00 ± 0.00		
	III	6.75 ± 0.91		
	IV	6.43 ± 0.92		
BMI	I	31.8 ± 1.7	<0.0001 (Sig.)	I vs. II I vs. IV II vs. III III vs. IV
	II	22.0 ± 2.0		
	III	34.0 ± 3.2		
	IV	22.6 ± 2.4		
CPITN	I	1.4 ± 0.6	<0.0001 (Sig.)	I vs. III I vs. IV II vs. III II vs. IV
	II	1.2 ± 0.7		
	III	4.1 ± 0.3		
	IV	3.9 ± 0.3		

The mean PI and CPITN index in group III (2.57 ± 0.34) (4.1 ± 0.3) and group IV (2.37 ± 0.38) (3.9 ± 0.3) are significantly higher than the mean PI and CPITN index in group I (0.66 ± 0.30) (1.4 ± 0.6) and group II (0.58 ± 0.24) (1.2 ± 0.7) ($p<0.05$)

The mean CAL in group III (6.75 ± 0.91) and group IV (6.43 ± 0.92) are significantly higher than the mean CAL in group I and II (0.00) ($p<0.05$).On comparison the mean BMI between obese and non- obese groups are found to be statistically significant

Table 3: Representing GCF leptin levels in each group

GROUPS	LEPTIN (pg/ml)	Overall P-value	Significant Groups
GROUP I	715 ± 71	<0.0001 (Sig.)	I vs II I vs III I vs IV II vs IV III vs IV
GROUP II	503 ± 230		
GROUP III	346 ± 82		
GROUP IV	137 ± 40		

The mean leptin level in Group I **(715 ± 71)** was significantly higher than the mean leptin level in Group II **(503 ± 230)**, Group III **(346 ± 82)** and Group IV **(137 ± 40)** ($p<0.05$). Also, the mean leptin level in Group II **(503 ± 230)** and Group III **(346 ± 82)** was significantly higher than the mean leptin level in Group IV **(137 ± 40)** ($P<0.05$). The mean leptin level in group II **(503 ± 230)** are comparatively higher than group III **(346 ± 82)**

Table 4: Representing the relationship between leptin and various clinical parameters within each study group

Group	Clinical Parameters	Correlation Coefficient*	P-value
I	PI	-0.06	0.84 (N.S.)
	CAL	-	-
	BMI	0.75	0.003 (Sig.)
II	PI	-0.41	0.16 (N.S.)
	CAL	-	-
	BMI	0.36	0.23 (N.S.)
III	PI	-0.49	0.09 (N.S.)
	CAL	-0.72	0.006 (Sig.)
	BMI	-0.34	0.26 (N.S.)
IV	PI	-0.52	0.06 (N.S.)
	CAL	-0.16	0.60 (N.S.)
	BMI	0.19	0.54 (N.S.)

*Spearman Rank Correlation Coefficient

Table 3 shows the correlation between Leptin level and other clinical parameters and reveals a positive correlation between BMI and leptin in Group I (r=0.75, P=0.003) and in Group III CAL and Leptin are negatively correlated (r=-0.72, P=0.006)

Table 5 shows the Comparison of mean values between healthy (normal + obese) and periodontitis (normal + obese) subjects

Variable	Healthy	Periodontitis	P-value[$]
	Mean ± S.D	Mean ± S.D	
PI	0.62 ± 0.27	2.47 ± 0.36	**<0.0001 (Sig.)**
CAL	0.00 ± 0.00	6.59 ± 0.91	**<0.0001 (Sig.)**
Leptin	609 ± 199	241 ± 124	**<0.0001 (Sig.)**
BMI	26.9 ± 5.3	28.3 ± 6.4	**0.26 (N.S.)**
CPITN	1.3 ± 0.7	3.9 ± 0.3	**<0.0001 (Sig.)**

[$] Mann-Whitney U- test was used to calculate the p-value. The mean Leptin level in healthy subjects (609 ± 199) is significantly higher than the mean Leptin concentration in Periodontitis subjects (241 ± 124) ($p<0.05$).

DISCUSSION

The recent advances in diagnostics have proven that GCF is a potential source of biomarkers for periodontal diseases and its collection being a minimally invasive procedure, it can be precisely considered the biomedium of choice for diagnostic & prognostic tests. Since host defence is a critical determinant in periodontal disease pathogenesis, the measure of such biomarkers in the GCF had been used to evaluate risk for an individual to develop periodontal disease[13].

The research on the pathophysiology of obesity has recently intensified, due to the breakthrough of leptin, in 1994, that completely changed the traditional view of white adipose tissue which was once considered as only a triglyceride reservoir with a passive or null endocrine role.[4]

The adipokine leptin, is secereted predoinantly by adipocytes and regulates weight in a central manner[4,14]. It is also produced in small percentages by inflammatory-regulatory cells, suggesting that leptin expression could trigger or participate in the inflammatory process through direct para- or autocrine actions.[15]

It enhances body's immune mechanism by inducing chemotaxis, oxidative species production and phagocytosis by stimulated PMNs and macrophages, also in contrast, another study provides evidence that leptin inhibits neutrophil migration in response to classical chemoattractant[16]. It was also reported that leptin directly activates neutrophils and delays spontaneous apoptosis of these cells by inhibiting proapoptotic events.

It activates proinflammatory cells, shifting T-cell responses towards Th1 cytokine type (IL-2 and INF-γ)[16], and mediating the production of the other proinflammatory cytokines, such as TNF-α, IL-2, or IL-6. Moreover, activated T cells themselves have been shown to express and secrete leptin, which sustained their

proliferation in an autocrine loop[17]. Thus the overall leptin action in the immune system is a proinflammatory effect.

In an invtro model leptin results in increased IL-4 production and reduced serum TNF-α or IL-1. Secretion of IL-1Ra by human monocytes (*in vitro* by 1.4 fold) suggests an anti-inflammatory role for leptin.[18]

Further, it is also involved in anti-osteogenic effects by acting centrally on hypothalamus[19] but recently, leptin has also been suggested to play a role in bone formation by virtue of it's direct effect on the osteoblast proliferation, differentiation, and prolonging the life span of human primary osteoblasts by inhibiting apoptosis. The local environment may provide bone cells with signals favouring constant growth, whereas the central negative signal determines the density and length. Thus, leptin in high concentration locally, protects the host from inflammation and infection and maintains bone levels.[20]

However, it is difficult to elucidate the role, if any, of leptin during inflammatory conditions in human patients as different clinical studies have so far yielded inconsistent results, suggesting that leptin has a rather complex role in immune response and inflammation in humans.

Leptin concentrations in healthy and diseased gingiva were evaluated by *Johnson & Serio et al* [8] to define its possible role in periodontal disease progression. Healthy (non-haemorrhagic gingiva adjacent to a <=3 mm gingival sulcus) and inflamed gingiva (haemorrhagic gingiva adjacent to a >3 mm periodontal pocket) was assessed within solubilized gingival biopsies using ELISA method. Leptin concentrations were found to be highest within gingiva adjacent to <=3 mm sulcus. This study showed that human leptin is present within healthy and marginally inflamed gingiva and decreases in concentration as the adjacent PD increases. *Yesim Bozkurt et al* [21] evaluated GCF leptin levels and the influence of long-term heavy smoking on GCF leptin levels in patients with chronic periodontitis. They found that GCF leptin levels were significantly lower in smokers than non-smokers. This shows that smoking may dysregulate leptin levels. Recently, *Karthikeyan & Pradeep et al* [9] assessed the concentration of human leptin levels in GCF from healthy periodontium, chronic gingivitis and chronic periodontitis patients. Results showed that there is a strong negative correlation between the GCF leptin concentration and periodontal disease progression

All of these previous investigations on leptin, have estimated leptin levels in either healthy or diseased state with normal BMI. Hence, an attempt was made to include obesity (BMI ≥ 30) and study the associated changes in leptin concentrations. Thus the present study was the first study aimed at investigating leptin concentration in GCF samples from obese and non-obese patients with healthy and diseased periodontium and assess the relationship between leptin, BMI and periodontal disease. Furthermore to gain an insight into the possible role of leptin in the initiation and progression of periodontal disease.

In the present study broadly, healthy periodontium groups (609±199) had significantly higher leptin levels than chronic periodontitis groups (241±124) which is in accordance to the studies reported by *Johnson et al* [8] & *Karthikeyan et al* [9]

Generally during inflammatory conditions, cytokines such as TNF-α and IL-1ß stimulates leptin production by acting on adipocytes leading to increased leptin levels[8]**.** However during periodontal inflammation there is no local rise in gingival tissue leptin concentration, probably as a result of the absence of adipocytes within the gingiva. Though the exact mechanism for the increase of GCF leptin levels in health is still ambiguous, the possible source could be

from serum (since GCF is considered to be a serum exudate), gingiva (leptin entrapped within gingiva from the microvasculature), [22,23] and from the resident T cells[15] and osteoblast[20] which forms the minor source. It can also be speculated that during inflammation there is expansion of vascular network caused by increasing VEGF levels, which might increase the net rate of leptin removal from the gingival tissues and could raise serum leptin levels.

When obesity was included the obese with healthy periodontium (group I) had significantly higher leptin levels than the other groups and BMI and leptin were positively correlated. On comparing, leptin levels are significantly higher in group I (715 ± 71) than group II (503 ± 230), so also it is higher in group III (346 ± 82) than group IV (137 ± 40) showing the predominant role of obesity and reaffirming leptins role as a prominent adipocytokine with potent immunomodulatory functions.

In contrast, group II with non-obese subjects had higher leptin levels than group III with obese subjects showing the balancing nature of obesity and periodontitis in influencing GCF leptin levels. Obesity is a known risk factor for CVD. The rise in serum leptin levels above 10,000 pg/ml is considered as a risk factor for cardiovascular disease[24,25]. Decreased GCF leptin associated with increased serum leptin levels as suggested by Karthikeyan et al[8] may propose possible mechanisms why obesity and periodontal disease should be considered probable risk factors for CVD. However, this possibility requires further investigation and this could open a new era in the field of periodontal medicine.

Future studies are required to clarify whether leptin in GCF has a protective role in the pathogenesis of periodontitis and its receptor expression could be determined in gingival tissue to elucidate this function. Furthermore studies are required to assess the effect of serum leptin levels on GCF concentrations and the probable mechanism of leptin activity on immune cells.

CONCLUSION

In this study GCF leptin concentration increases substantially in health which extends our knowledge of the protective role of leptin in periodontal health and it can be used as an effective diagnostic tool for monitoring the progression of periodontal disease.

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Fig 1: Collection of GCF from healthy sites using microcapillary pipette

Fig 2: Picture of microtitre plate after addition of the chromogenic solution and final incubation

Fig 3: Picture of microtitre plate after addition of the stop solution