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RESEARCH ARTICLE

CATHELICIDIN (hCAP-18/LL-37) LEVELS IN CHRONIC PERIODONTITIS SUBJECTS WITH SMOKELESS TOBACCO PRODUCTS (STP) CONSUMPTION

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ABSTRACT

Background: Periodontal epithelium has an active role in multi-factorial pathogenesis of periodontal diseases by producing diverse range of antimicrobial peptides, amongst which cathelicidins (LL-37) is the only one of its kind that has been found in humans. Genetic factors such as deficiency of LL-37 and environmental factors such as smokeless tobacco products consumption are found to be associated with severe periodontal destruction. Hence, the present study was conducted to estimate salivary LL-37 levels of chronic periodontitis (CP) subjects who consume smokeless tobacco Products (STP) and also correlate it with clinical parameters.

Methods: 90 subjects distributed into three groups (n=30 each) as Healthy, CP and CP with STP habit participated in the study. Clinical parameters such as Gingival Index (GI), Oral Hygiene Index-Simplified (OHI-S), Pocket Probing Depth (PPD) and Clinical Attachment Level (CAL) were recorded. Unstimulated whole Saliva was collected by draining method. Salivary LL-37 levels were estimated by Enzyme Linked Immunosorbent Assay.

Results: Mean Salivary LL-37 levels (ng/ml) was 24.03±60.59 in Healthy, 235.06±318.8 in CP and 102.95±151.45 in CP with STP group ($p<0.0001$). Spearman correlation analysis revealed a positive correlation of salivary LL-37 levels with GI, OHI-S, PPD and CAL ($p<0.0003$).

Conclusion: Salivary LL-37 levels were markedly reduced in CP subjects who consumed STP compared to CP subjects but were higher than healthy subjects.

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INTRODUCTION

Periodontal diseases are highly complex and multi-factorial in nature. Genetic and environmental factors appear to increase the susceptibility of some individuals in developing this severe inflammatory disease in presence of periodontopathogens such as *Porphyromonas gingivalis (Pg), Aggregatibacter actinomycetemcomitans (Aa)* and *Tannerella forsythia (Tf)* (Feng and Weinberg, 2006). The progression of disease occurs due to a complex interplay between periodontopathic bacteria, pro-inflammatory cytokines, matrix metalloproteinases (MMPs), prostaglandin E2 (PGE_2) and anti-inflammatory cytokines such as interleukin-10 (IL-10), transforming growth factor (TGF-β) and tissue inhibitors of MMPs (TIMPs) (Page, 1997; Gemmell and Seymour, 2004). Periodontal epithelium provides a physical barrier to infection. It has an active role in the innate host defense because they are in constant contact with the bacterial products (Mariano *et al*., 2010). It can participate in the infection by signaling further immune responses. It is now acknowledged that epithelium throughout the human body produces assorted range of antimicrobial peptides (AMP) also known as host defense peptides (HDPs) such as α-defensins, β-defensins, cathelicidins, saposins (Marshall, 2004). These peptides are present in saliva and dento-gingival junction region. These peptides complement the antimicrobial factors of saliva, thereby playing a specific role in the innate host defense in response to an infection (Martinez *et al.*, 2009). The term "cathelicidin" was introduced twenty years ago to describe a subset of HDPs that was first isolated from bovine neutrophils. Cathelicidin family are a picturesque example of molecules with a remarkable diversity concerning size, sequence and structure, yet maintaining the cationic and amphipathic character typical of antimicrobial peptides (Tomasinsig and Zanetti, 2005). Cathelicidin genes consist of four exons and three introns; the first three exons comprise the signal sequence and cathelin prodomain, while the fourth exon encodes the processing site and variable C-terminal

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antimicrobial peptide. All cathelicidins (hCAP-18/LL-37) are a precursor consisting of N-terminal signal peptide, a highly conserved prosequence and a structurally variable C-terminal mature peptide. It is the presence of the evolutionarily conserved prosequence which attributes an antimicrobial function to the cathelicidin class of molecules. Proteolytic cleavage of the inactive precursor molecule to release the mature C-terminal antimicrobial peptide from the cathelin prodomain is accomplished by elastase or proteinase-3 upon degranulation of activated neutrophils (Potturu *et al.*, 2008). The primary function of LL-37 is antibacterial and usually it acts along with other AMPs. LL-37 exhibits broad spectrum antimicrobial activity against gram-negative and gram-positive bacteria. It includes oral microorganisms such as *Streptococcus mutans, Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*. It also inhibits the growth of *Candida albicans* and viruses. The expression of LL-37 is seen to be upregulated in the inflamed sites and is correlated positively with the depth of the gingival crevice, indicating that LL-37 expression is associated with the severity of periodontal diseases (Potturu *et al.*, 2008). The studies have also proved an increased expression of LL-37 in periodontal diseases and smokers.

A study based on National Health and Nutrition Examination Survey III (NHANES III) data conducted in US showed strong association between oral Smokeless Tobacco products (STP) use and severe periodontal disease (Fisher *et al.*, 2005). Studies conducted among Asian populations, especially in India have reported that oral STP users are more vulnerable for periodontal disease (Kamath *et al.*, 2014). The mechanisms behind such outcomes aren't studied extensively yet. Considering the widespread use of STP globally, the influence of such products on the periodontal tissues may be important, especially in Indian Subcontinent. Hence, the present study was undertaken to assess the expression of LL-37 in Smokeless Tobacco users and correlate the same with the severity of periodontal disease.

MATERIALS AND METHODS

Study population

The present study was approved by the institutional ethical committee of P. M. N. M Dental College and Hospital, Bagalkot. A total of 90 subjects, aged 18-60 years, were recruited from the Outpatient Department of Periodontics, P. M. N. M Dental College and Hospital, Bagalkot. The study was conducted from October 2014 to April 2015. A written informed consent was obtained from each subject after explaining the design and need of the study. Subjects were categorized into 3 groups as follows: 30 periodontally healthy subjects showing absence of clinical features of periodontal disease, 30 Chronic Periodontitis who were systemically healthy subjects with periodontal findings according to AAP 1999 classification (Armitage, 1999) and 30 Chronic periodontitis (smokeless tobacco chewers) who were systemically healthy subjects with periodontal findings according to AAP 1999 classification and who chew smokeless tobacco for over a period of $\geq$ 1 year. Subjects who had undergone periodontal therapy or taken any systemic antibiotics, anti- inflammatory and corticosteroid therapy in the previous 6 months, medically compromised and former smokeless tobacco chewers, pregnant or lactating mothers were excluded from the study.

Determination of clinical parameters

The clinical parameters recorded in each subject were as follows: Oral Hygiene index- Simplified (OHI-S) (Greene and Vermillion 1964) (Peter, 2009), Gingival Index (GI) (Loe and Silness 1963) (peter, 2009), Probing Pocket Depth (PPD) (Sture and Lindhe, 2003; Mariano *et al.*, 2007) using William's graduated periodontal probe and Clinical Attachment Level (CAL) (Sture and Lindhe, 2003; Mariano *et al.*, 2007).

Sample collection and processing

Unstimulated whole saliva collection was performed in the morning to avoid circadian periodicity. Subjects were instructed to void the mouth of saliva prior to collection, by rinsing the mouth thoroughly with water. It was collected by draining method wherein saliva was allowed to drip off the lower lip into screw top tubes (Vissink *et al.*, 2008). The samples were immediately frozen at -80ºC. The samples were thawed and cleared by centrifugation at 14,000× g for 5 min before analysis. Salivary LL-37 concentration was determined by enzyme-linked immunosorbent assay (ELISA) using a commercially available kit according to the manufacturer's instructions.

Statistical Analysis

Results on continuous measurements are presented on mean ± sd. Results on categorical measurements are presented in Number (%). Differences were considered to be statistically significant when the p-value was < 0.05. One-way ANOVA had been used to compare the three groups with respect to age and gender. Kruskal Wallis ANOVA had been used to compare the three groups with respect to GI, OHI-S, PPD and CAL. Mann-Whitney U-test had been used for pair wise comparison between any two groups. Spearman's rank correlation had been used to find out correlation between LL-37 levels and OHI-S, GI, PPD and CAL in three groups. The Statistical software namely SPSS 21.0 was used for the analysis of the data. Microsoft word and Excel have been used to generate graphs, tables.

RESULTS

According to demographical data of 90 study subjects, gender distribution was 42 males (46.67%) and 48 females (53.33%) and the mean age of the study subjects was 35.32 ±13.39 years. This data is presented in Table 1 and Table 2 respectively. Mean Salivary LL-37 levels (in ng/ml) was 24.03±60.59 in Healthy group, 235.06±318.8 in chronic periodontitis group and 102.95±151.45 in chronic periodontitis with STP. LL-37 levels were statistically significant lower in healthy when compared to chronic periodontitis group ($p= 0.0001$) and chronic periodontitis with STP ($p= 0.0001$).This data is presented in Table 3. Salivary LL-37 levels of 90 subjects showed a highly significant positive correlation with GI, OHI-S, PPD and CAL at p value <0.0003 as shown in Table 4. Salivary LL-37 levels in Healthy group showed a positive correlation between OHI-S with LL-37 levels. Salivary LL-37 levels in Chronic Periodontitis group showed a positive correlation between GI, OHI-S, Probing depth and CAL with LL-37 levels. Salivary LL-37 levels in Chronic Periodontitis with STP group showed a positive correlation between Probing depth and CAL with LL-37 levels and a negative correlation between OHI-S and LL-37 levels which was statistically significant ($p<0.0274$).

Table 1. Distribution of male and female in three groups (healthy, chronic periodontitis and chronic periodontitis with STP)

Gender	Healthy	%	Chronic periodontitis	%	Chronic periodontitis with STP	%	Total	%
Male	5	16.67	14	46.67	23	76.67	42	46.67
Female	25	83.33	16	53.33	7	23.33	48	53.33

Table 2. Comparison of three groups with respect to mean age by one way ANOVA

Groups	Mean± SD
Healthy	21.70± 2.22‡
Chronic periodontitis	39.00± 11.86‡
Chronic periodontitis with STP	45.27± 9.80‡

SD: Standard Deviation; F-value: 55.4878; ‡p<0.05

Table 3. Comparison of three groups with respect to Salivary LL-37 levels (in ng/ml)

Groups	Mean± SD
Healthy	24.03± 60.59
Chronic periodontitis	235.06± 318.8‡
Chronic periodontitis with STP	102.95± 151.45‡

SD: Standard Deviation; F-value: 26.973; ‡p<0.05

Table 4. Correlation between LL-37 levels with clinical parameters like GI, OHI-S, Probing depth and CAL scores by Spearman's rank correlation method

Variable	Spearman R	t-value	p-level
GI	0.5370	5.9712	0.0001‡
OHI-S	0.3768	3.8165	0.0003‡
Probing depth	0.4566	4.8143	0.0001‡
CAL	0.5553	6.2632	0.0001‡

‡p<0.05

DISCUSSION

The present study, comprising of 90 subjects, demonstrated that Salivary LL-37 levels were significantly higher in chronic periodontitis group compared to healthy group. Salivary LL-37 levels were also higher in chronic periodontitis group when compared to chronic periodontitis with STP habit. Salivary LL-37 levels also positively correlated with the clinical parameters. These results are suggestive of LL-37 playing a fundamental role in maintenance of periodontal health and disease. It also consolidates the data reported by Hosakawa *et al.*, (2006) which showed that cathelicidin LL-37 expression was prominent in the inflammatory lesions compared to healthy gingiva. Turkoglu *et al.* (2009; 2011), in his studies, also found higher levels of LL-37 in GCF of CP patients which he further demonstrated by increased immunostaining of hCAP-18 / LL-37 in epithelium and connective tissue of the gingival tissues in patients with chronic periodontitis than those of healthy controls. We assessed ll-37 in saliva as Murakami *et al.* (2002) proved in their study that cathelicidins are present in saliva thereby contributing to broad spectrum defense mechanisms of the oral cavity. As the principal source of LL-37 is neutrophils, it is rational to assess their levels in saliva. Moreover, saliva being in close proximity with periodontally inflamed sites may contain biological markers reflective of these diseases (Kim *et al.*, 2013). Healthy subjects in the present study had significantly lower levels of LL-37 (24.03±60.59 ng/ml). This finding is in concord with the study done by Puklo *et al.* (2008) who reported that mature LL-37 was detectable in GCF of only 56% of healthy subjects. This can be recognized by the fact that the majority of LL-37 originated from activated neutrophils in healthy tissues whereas neutrophils migrating into the gingival crevice from healthy periodontium were not activated. Another plausible mechanism which is explained by Turkoglu *et al.* (2009; 2011) in his study is that after inflammatory stimulus, hCAP18 is cleaved to LL-37 and cathelin-like domain by proteinase 3. Neutrophils do not become activated in absence of inflammation. Hence, they don't express LL-37. This data is also in agreement with the results of Dale *et al.*, (2001) who suggested that cathelicidin LL-37 was brought into the periodontium via neutrophils that reaches the gingival crevice via transendothelial migration. Therefore, it is reasonable that hCAP-18 / LL-37 expressions were low in healthy periodontium. Salivary LL-37 levels were lower in CP-STP group when compared to CP. The values were broadly different (235.06±318.8 ng/ml vs 102.95±151.45 ng/ml) and the mechanism behind such findings could be attributed to the negative influence of tobacco use on neutrophil activity (MacFarlane *et al.*, 1992; Persson *et al.*, 2001; Giannopoulou *et al.*, 2001). As shown in a study by Takeuchi *et al.* (2011), there exists an inverse relation between salivary LL-37 levels and cotinine thereby demonstrating the negative influence of tobacco smoking on LL-37 levels. Contradictory to such findings, Ertugrul *et al.* (2014) found higher levels in smokers compared to non-smokers which he explained by an increased number of microorganisms colonizing the oral cavity in smokers leading to higher levels of LL-37 in comparison to non-smokers.

We also found a statistically significant positive correlation of salivary LL-37 levels with clinical parameters such as PPD, CAL, GI and OHI-S. This denotes that LL-37 levels are higher in severe periodontal inflammation. This might be accredited to the fact that more the inflammation, and hence more neutrophil activation and subsequent release of ll-37 are associated with severe periodontal inflammation. When salivary LL-37 levels were correlated with clinical parameters in Healthy group, a positive correlation was observed between OHI-S and LL-37 levels only. Higher OHI-S scores usually

denote poor oral hygiene which might lead to subsequent periodontal inflammation. Therefore, this positive correlation is acceptable.

A positive correlation was observed between GI, OHI-S, PPD and CAL with LL-37 levels in chronic periodontitis group. This denotes that LL-37 levels are higher in severe chronic periodontitis. This finding is similar to the findings of Takeuchi *et al.*, (2011) wherein he found that the percentage of teeth with PD ≥ 5 mm was positively associated with high salivary LL-37 levels. This is also reported by Ertugrul *et al.* (2014) who found that gingival crevicular fluid LL-37 levels had positive correlations with the clinical parameters i.e. Gingival Index, probing depth, clinical attachment loss and Bleeding on probing. Contrary to the correlations in chronic periodontitis group, a statistically significant negative correlation was observed between OHI-S and LL-37 levels chronic periodontitis with STP group. The probable rationale could be that STP users have poor oral hygiene owing to local irritation by tobacco ingredients and extrinsic stains developed secondary to tobacco chewing habit. The other parameters positively correlated with LL-37 levels.

The anti-biofilm properties of LL-37, along with its antimicrobial and immunomodulatory activities, provide a feasible platform for the development of therapeutic formulations to fight chronic infections even, chronic periodontitis. It seems practicable to utilize antimicrobial peptides in future therapy of periodontal diseases in order to combat increased antimicrobial resistance. The oral cavity, being easily reached for local application, may be particularly appropriate for such therapy. as in case of any research, our research isn't free from limitations as well. The cross-sectional feature of this study prevents the assessment of causal relationship between chronic periodontitis, Smokeless Tobacco Products (STP) and hCAP-18/LL-37 antimicrobial peptide. In order to establish a relationship between Smokeless Tobacco Products (STP) and LL-37 in periodontal diseases, investigating these levels before and after periodontal treatment and quitting tobacco habit might enlighten the possible interactions occurring between tobacco and LL-37 in periodontal diseases. Moreover, the smaller sample size and various other differences such as race and population variations don't allow generalizing these findings on to the entire population. Therefore, further studies with larger sample size and on a multi-center level including population of various races are required to consolidate the findings of the present study.

Conclusion

Salivary LL-37 levels were lower in CP subjects with STP habit compared to CP subjects. These results are reflective of an immunosuppressive effect of STP contents on neutrophils and hence, LL-37 levels thereby increasing susceptibility to periodontal diseases. The study highlights one of the possible mechanisms of periodontal destruction in STP users. In countries such as India, where the tobacco consumption in the form of oral STP is predominant compared to smoking, the role of oral STP on periodontal health should be considered in the etiology of periodontal diseases. Further studies with larger sample size on a multicenter level including various races and population subjects will be able to enlighten the impact of STP on periodontium and explore possible diagnostic and therapeutic implications of LL-37 in periodontal diseases.

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