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GCF levels of human beta defensins 1 and 3 in diabetic and non diabetic patients with chronic periodontitis

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Abstract---Background: Human beta defensins (HβDs) expression in healthy tissues may aid in preventing the onset and/or progression of inflammatory disease. Type 2 diabetes mellitus (type 2 DM) and periodontal disease encompass inflammatory changes. HβDs in periodontitis patients is differentially regulated by periodontal pathogens, commensal organisms, host derived factors and inflammatory mediators. Additionally hyperglycaemia might influence this inflammatory state. Aim: To evaluate the role of the expression of human beta-defensin 1 and 3 levels in gingival crevicular fluid samples of gingivitis and chronic periodontitis patients with various glycaemic status. Methods and Material: In this study a total number

of 140 patients were divided into five groups (Group I-V) consisted of 28 patients under each group. Group I consisted of 28 Type 2 Diabetes mellitus (DM) patients with well controlled glycemic status ($>6.5 < 7\% \text{HbA}_{1c}$) and chronic periodontitis (CP), Group II comprised of 28 type 2 DM-CP patients ($7-8\% \text{HbA}_{1c}$), Group III had 28 Type 2 DM-CP patients ($>8\% \text{HbA}_{1c}$). The concentrations of H β D-1 and H β D-3 were estimated using ELISA. Results: H β D-1 and 3 levels are higher in gingivitis subjects than periodontitis. Significantly higher levels of H β D-3 expression were found in the healthy tissues compared to the diseased ones. Elevated levels of H β D-1 and H β D-3 in DM-CP patients with worsening glycaemic status. Conclusion: H β D-1 & 3 may serve as novel diagnostic markers for patients with type 2 diabetes and chronic periodontitis.

Keywords---H β D-1&3, Periodontitis, type 2 diabetes mellitus, HbA $_{1c}$, gingival crevicular fluid.

Introduction

In the periodontal tissues, the gingival epithelium provides the primary physical barrier against microbial invasion ^[1]. However, when the microbes gain entry into the epithelium, the recognition of specific microbial molecules by host immune cells leads to activation of the innate immune response. ^[2] This leads to the secretion of cytokines as well as chemokines to activate the influx of neutrophils and other immune cells into the sulcular area ^[3] and most importantly it is a source of antimicrobial products termed Epithelial Antimicrobial Peptides (EAPs). ^[4]

Several families of antimicrobial peptides have been identified in the oral cavity which includes defensins, calprotectin, adrenomedullin, histatins, and cathelicidins. Among the defensins, according to the spacing of the cysteines, the alignment of the disulfide bridges, and the overall molecular structure, The human defensins family are subdivided into two groups: alpha-defensins and beta-defensins (hBD-1, -2, -3, -4) based on the length and the folding of the peptide chains and location of cysteines residues in the amino acid sequence ^[5]. Human beta defensin (H β Ds) are cationic peptides that participate in the innate immune response. They are present in the gingiva, tongue, salivary glands, and mucosa and constitute the major antimicrobial factors of saliva. ^{[6][7]}

Human beta-defensin 1 (H β D -1) is active against gram-negative bacteria with limited activity against gram-positive bacteria and is mostly found to be elevated in the epithelium. ^[8] Previous studies have shown that H β D -1 is constitutively expressed in both healthy and inflamed tissues. ^[9] The expression of H β D contributes to the maintenance of periodontal homeostasis by its antimicrobial and immunomodulatory activities. ^[10]

H β D3s has gained priority over other members in the defensin family due to their broad spectrum of antimicrobial action against gram-negative and gram positive organism, various fungi, and viruses especially it exhibits greater antimicrobial

activity against *A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum* (red complex)^[11] Moreover, H β D3 expresses a strong chemotactic activity for innate immune cells and also amplifies the adaptive immune response thus bridging the innate and adaptive immune responses.^[12] Clinical studies have shown that in periodontal disease, the gingival tissue and gingival crevicular fluid samples exhibited reduced expression of H β D3.^{[13][14]}

When the microorganisms come in contact with the gingival epithelial cells, there is secretion of H β D-1, which when activated by epithelial cells, induces the further release of H β D-3.^[15] In a study, H β D 1 and/or H β D 3 mRNAs levels within the gingival biopsy samples were found to be lower than or similar to the levels in inflamed tissues compared with healthy tissues. On the contrary, some investigators have found higher levels of H β D-1 and/or H β D -3 in gingival biopsies of patients with periodontitis compared with periodontally healthy patients.^[16] A negative correlation was observed between GCF H β D 3 levels and numbers of periodontopathic bacteria suggesting that H β D 3 could be directly or indirectly involved in limiting the colonization of the oral cavity by high pathogenic bacterial species, thus contributing to the maintenance of periodontal health status.^{[17][18]}

There is a well-established two-way relationship between diabetes and periodontitis. Beta-defensins are released in patients treated with insulin compared with patients who are not receiving such treatment.^[19] Additionally, it has been determined that high levels of hyperglycemia reduce the expression and function of H β D-3 in epithelial cells. Few studies have analyzed the relationship among H β Ds, DM and periodontal disease,^[20] in a study by erutrugul et al, the hBD-1 and hBD3 levels in the DM groups were significantly higher than in the non-DM groups.^[21]

Although, differential expression patterns for H β Ds during gingivitis and chronic periodontal disease in diabetic patients have been previously studied, the precise role of H β D in a hyper-inflammatory state like diabetes and periodontitis still unclear. Additionally, no studies are comparing the influence of different glycaemic statuses on expression levels of H β D-1 and H β D-3. Thus, the current study focuses on the role of the expression of human beta-defensin 1 and 3 levels in gingival crevicular fluid samples of gingivitis and chronic periodontitis patients with various glycaemic statuses.

Materials and Methods

Study population and design:

This study was a case-control study, approved by Scientific and Ethical committee review board of SRM Dental College, Ramapuram SRM/MHS/SRMDC/2013/M.D.S-PG student/501. The study was carried out from December 2014 to July 2015.

A routine preliminary clinical examination followed by a detailed case sheet documentation including medical history was performed by a single calibrated examiner. A total of 140 patients were selected from the outpatient Department of Periodontics, SRM Dental College & Hospital and Hariharan Diabetic and Heart

Care Hospital, Chennai. All the participants were explained about the study verbally and a written informed consent was obtained.

Inclusion and exclusion criteria:

Subjects who satisfied the following criteria were included in the study, (1) Individuals aged from 30 to 70 years were included and Non – obese individuals with Body mass index (Kg/m^2) ≤ 29.9 (2) Patients with type 2 diabetes were included if they presented with a history of type 2 DM for the past 1 year or more and treated with oral hypoglycemic drugs. The glycemic status was evaluated using HbA_{1c} and categorized based on the American Diabetic Association classification 2003 for diagnosis of type 2 diabetes. (3) Patient with generalized CP, exhibiting pocket depth of ≥ 4 mm and clinical attachment level (CAL) of ≥ 3 mm in more than 30% of diseased sites with a minimum of 15 teeth (according to the American Academy of Periodontology 1999 World Workshop classification of periodontal diseases).

Individuals were excluded if they had, (1) Systemic conditions influencing the course of periodontal disease other than type 2 diabetes. (2) Type 1 diabetes, hemoglobinopathies, bleeding, and clotting disorders. (3) History of periodontal treatment within the previous 6 months. (4) Under antimicrobial therapy, immunosuppressant, or lipid-lowering drugs within the past 3 months. (5) Habit of smoking and alcoholism. (6) Pregnant and lactating women.

Sample size and study groups:

The sample size was assumed using statistical software based on the results obtained from a study by Sharadha Jaganath et al 2011^[22] With 90% power and 1% a-error, Based on the results of the above study, in order to obtain a power of 90% with a error of 0.05, the current study required a sample size of 140.

Study groups:

The study population consisted of 84 type 2 diabetes mellitus subjects and 56 non diabetic individuals. Type 2 diabetes mellitus subjects associated with chronic periodontitis were recruited from outpatient of the department of periodontics & Hariharan diabetic and heart care hospital.

Based on their glycaemic status the subjects were categorized under three groups as follows:-

Group I: Type 2 Diabetes mellitus patients with well controlled glycemic status ($> 6.5 < 7\% \text{HbA}_{1c}$) and chronic periodontitis (n=28)

Group II: Type 2 Diabetes mellitus patients with moderately controlled glycemic status ($7-8\% \text{HbA}_{1c}$) and chronic periodontitis (n=28)

Group III: Type 2 Diabetes mellitus patients with uncontrolled glycemic status. ($> 8\% \text{HbA}_{1c}$) and chronic periodontitis (n=28).

Non diabetic subjects were recruited from outpatient of the department of periodontics SRM dental college and hospital, Chennai. Based on their periodontal status they were further categorized under two groups.

Group IV: Non-Diabetic subjects with Gingivitis ($\text{HbA}_{1c} < 6.0$) (n=28),

Group V: Non-Diabetic subjects with chronic periodontitis ($\text{HbA}_{1c} < 6.0$), (n=28).

Recruitment of participants:

A customized case sheet was prepared including the detailed medical history and complete periodontal examination. Clinical parameters assessed for the study were:

1. Plaque index (Silness and Loe 1964)
2. Gingival index (Loe and Silness 1963)
3. Sulcular bleeding index (Muhlemann and Son 1971)
4. Probing pocket depth
5. Clinical attachment loss.

Based on clinical examination, a site is considered as

1. Gingivitis site if the clinical signs of gingival inflammation, Gingival index ≥ 1 , Probing pocket depth ≤ 3 mm, Clinical attachment level = 0
2. Periodontitis site: Probing pocket depth ≥ 3 mm with clinical attachment loss ≥ 3 mm. If more than five sites were coming under similar criteria, sample sites were selected based on greater amount of gingival inflammation, probing pocket depth and clinical attachment level.

For all diabetic patients, detailed history of onset of diabetes mellitus, duration, symptoms, glycaemic control records of last year and hypo glycaemic drug usage was recorded. Height and weight was recorded and Body mass index calculated: BMI calculation formula = Weight (Kg) / Height (m^2)

Method of GCF collection:

Five deepest probing sites were selected per patient with a minimum probing depth of >5 mm. The sample collection site was isolated and supragingival plaque was removed with a curette without touching the marginal gingiva. A calibrated microcapillary pipette (calibration 1–5 mL; Sigma-Aldrich Co.) was placed atraumatically in the sulcus by slightly penetrating the gingival crevice for 1 min and GCF sample was collected. The collected samples were transferred into a graduated Eppendorf microcentrifuge tube (Sigma-Aldrich Co.) containing 200 mL phosphate-buffered saline buffer (pH 6.5) as diluent to obtain a single pooled GCF sample for each patient. Pooled GCF sample was labeled Eppendorf tubes collected and stored for biochemical analysis. The containing GCF samples were transported to deep freezer to be stored at -80 until analyzed.

Measurement of HBD-1 and HBD-3:

The concentrations of HBD-1 and HBD-3 were estimated using Sandwich ELISA Kit (commercially available ELISA kits manufactured by peprotech's kits) for beta defensins 1 and 3. Minimum detection limits for H β D-1 and H β D-3 were 4–1000 pg/mL and 63–4000 pg/mL respectively. All assay procedures were conducted according to the manufacturer's instructions.

Results

In the present study a total number of 140 patients who were divided into five groups (Group I–V) consisted of 28 patients under each group. Mean levels of H β D-1 and H β D-3 in GCF were measured and compared between the groups. The table 1 depicts the mean values of HbA1c, BMI, clinical parameters like PI, GI, SBI, PPD, CAL and HBD-1, HBD-3

Table 2, 3 shows the statistical significance relationship in between all the studied groups. Plaque index scores were statistically significant only between group I and IV, group II and IV, group III and IV and group IV and V respectively. BMI was not significant within all the five groups. HbA_{1c} was highly significant in between all groups except group IV and V, group I and II. Clinical attachment level and probing pocket depth scores were significant in between all groups except group I and group V. Gingival index and sulcular bleeding index was significant in between all groups except group II and V. H β D-1 expression was not statistically significant between all groups except group II and IV. H β D-3 levels and periodontal clinical parameters was statistically significant in between all groups except groups I and V, group II and IV, groups II and III, Group III and IV respectively.

Discussion

The ability of gingival sulcus and junctional epithelia to control bacterial challenge and prevent tissue destruction is crucial for maintenance of periodontal health. A recent study has shown the expression of human β -defensins in oral and sulcular epithelia as well as α -defensins and LL-37 in junctional epithelium, suggesting defensins serve different roles in various regions of the periodontium.^{[23][24]}

Studies show that there is an increased expression of all human beta defensins in non-inflamed oral tissues and less in inflamed tissues, therefore their expression in healthy tissues may assist in preventing the onset and/or progression of disease.^[25] H β Ds 1–3 have been isolated and found to be expressed in human gingival epithelial cells (HGEC).^[17] H β D-1 is constitutively expressed, whereas both H β D-2 and H β D-3 are differentially induced by infectious or inflammatory stimuli.^[26] H β Ds in gingival epithelial cells from periodontal patients is differentially regulated by periodontal pathogens such as *Actinobacillus actinomycetemcomitans* (*A. actinomycetemcomitans*), *Porphyromona gingivalis*, and *Fusobacterium nucleatum* induce upregulate depression of h β Ds in oral keratinocytes, commensal organisms, host derived factors and inflammatory mediators.^[27] There is production and also degradation of HBD degradation by the enzymes produced by these periodontal bacteria.^[28]

In diabetic patients, the degree of glycaemic control is an important variable since the severity of gingival inflammation and periodontal destruction being seen in those with poor control.^[31] As a result of the observed vascular and cell activity changes that occur within patients diagnosed with DM, periodontal diseases become more severe. These changes hinder the migration and the ability of chemotactic factors and leukocytes to protect periodontal tissues from the effects of microorganisms. In order to eliminate microorganisms, the epithelial cells in patients with DM may release more H β D1 and H β D3 into the gingival crevicular fluid. Additionally, it has been determined that high levels of hyperglycaemia reduce the expression and function of H β D-3 in epithelial cells.^[32]

A BMI of ≤ 29.9 was included in the present study according to the classification of overweight and obesity. Overweight range from 25.0–29.9 was taken to exclude obese subjects whose BMI ranges from 30.0–34.9. Since obesity is a known risk

factor for type 2 diabetes mellitus and can influence the level of inflammatory biomarkers such as TNF- α , IL-6 which can in turn influence the pathogenesis of periodontitis.^[33]

Studies that evaluate antimicrobial peptides in patients with periodontal disease and/or DM are available in the literature. A. S. Ertugrul et al 2012^[21] compared the total levels of H β D -1 and H β D-3 in the gingival crevicular fluid and found that both H β D -1 and H β D -3 was expressed more in diabetic than non -diabetic subjects and healthy periodontitis had more expression than Healthy gingivitis group. Whereas Dommisch H et al 2004^[26] showed a differential expression of human β -defensins (H β D-1, 2 and 3) in tissues with inflammatory gingival disease. Lu Q et al 2005^[34] estimated appropriate expression of H β D-3 peptide may contribute to the maintenance of periodontal homeostasis, possibly through its antimicrobial effect and promotion of adaptive immune responses.

Determining the amount of H β D -1 and H β D -3 in the gingival crevicular fluid of patients with and without DM will help to elucidate the relationship among H β D -1, H β D -3, DM and periodontal disease. However, there is a paucity of information about H β D-1 and H β D-3 expression in various glycaemic status. The present study hypothesized that different glycaemic status in DM could play an important role in the secretion and stimulation of H β D-1 and H β D-3 from the epithelial tissue in gingival crevicular fluid within chronic Periodontitis patients. This is the first study to compare the H β D-1 and H β D-3 levels in gingival crevicular fluid with clinical periodontal parameters in diabetic patients, especially with varying glycaemic status.

In the current study group-I (good control DM-CP) showed negative correlation between BMI and H β D-3 which was statistically significant ($p \leq 0.04$). This could be attributed to the fact that majority of individuals in group I had a BMI value of 27.6 ± 1.11 suggestive that they were overweight. Also in Group I mean plaque index scores was 2.57 ± 0.54 , mean gingival index scores of 1.65 ± 0.18 and PPD of 5.18 ± 0.13 suggestive of periodontal disease. The results of the present study are comparable to the results of the previous study done by Al-Zahrani et al 2013^[35] in which there was no association between the expression of H β D-1 and -2 and obesity and periodontitis.

In group II that comprised of moderately controlled DM-CP patients, there was no statistically significant correlation observed between the mean clinical parameters like plaque index score, gingival index score, bleeding sulcular index, probing pocket depth, clinical attachment level, body mass index with the expression of H β D- 1 and H β D-3.

In group III, poorly controlled DM-CP, there was negative correlation between H β D-1(86.02 ± 0.5) and mean plaque index scores (0.6 ± 0.1) which was statistically significant ($p \leq 0.03$). It has been established that periodontal diseases are more severe in uncontrolled diabetic patients than in patients with good glycaemic control ^[36] moreover the cytokine expression levels are also higher in such patients. ^[37]

In group IV, non - diabetic patients with gingivitis, there was no statistically significant correlation observed between the clinical parameters like plaque index score, gingival index score, bleeding sulcular index, probing pocket depth, clinical attachment level, body mass index with the expression of H β D- 1 and H β D-3.

In group V, There was a statistically significant correlation between H β D-3(86.02 $\pm$ 0.5) and plaque index scores (0.6 $\pm$ 0.1) and clinical attachment levels (0.6 $\pm$ 0.1). Among non-diabetic patients, H β D-3 expression was least in group V .The findings of the present were similar to a previous study done by John Bissell et al 2004 ^[17], where there was significantly higher levels of H β D-3 (P = 0.012) in the healthy tissues as compared to the diseased ones. However, the results of the present study was contrary to a previous study done by Ertugrul Al et al 2012 ^[21]who observed increased levels of H β D-1 and H β D-3 in non-diabetics chronic periodontitis group compared to non-diabetic gingivitis group.

The elevated H β D-1 and H β D -3 in chronic periodontitis with worsening glycaemic status may be due to the exaggerated immune and inflammatory response.^[38] On the other hand, H β D-1 and 3 levels are higher in gingivitis subjects than periodontitis subjects suggestive of the protective role of defensins in innate immune response. While pro-inflammatory mediators associated with diseased tissues are known to induce the expression of defensins, inflammatory mediators in healthy tissues are present as growth factors which induce expression of β -defensins in healthy gingival tissues.^[39] The decrease in defensins levels in periodontal disease can be attributed to the degradation and inactivation by cysteine proteases such as cathepsin B, L and S suggesting an increased degradation of H β D 2 and 3 with resultant bacterial colonisation and infection.^[40] The limitations of the present study are the smaller sample size owing to the number of groups and the post treatment changes in defensins level could have reflected the exact protective or tissue destructive role of defensins.

Since the severity of hyperglycemia is a major consideration to induce defensin function, within the limits of the study, we have intended to evaluate the potential role of human beta defensin 1 and 3 as an early marker of inflammation in varying glycaemic statuses. To the best of our knowledge, there are no previous works on the same.

Conclusion and scope for further research

From the findings of the present study, it Human beta defensin 1 and 3 can be considered as early diagnostic and potential prognostic markers in chronic inflammatory diseases such as diabetes and periodontitis. Further research on understanding the molecular basis for induction and regulation of H β D-1 and H β D-3 may lead to development of potential target therapies for control of inflammation.

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Tables

Table 1: Descriptive Data Of The Parameters

Mean Parameters	Group I Mean	Group II Mean	Group III Mean	Group IV Mean	Group V Mean
HbA _{1c}	6.58	7.60	10.24	5.50	5.42
PI	2.57	2.60	0.60	1.68	2.04
GI	1.65	1.95	2.56	1.11	4.96
SBI	2.25	1.92	2.73	1.50	1.88
PPD	5.18	5.91	7.22	2.42	5.16
CAL	5.10	5.78	6.80	1.68	4.96
BMI	27.66	27.78	27.85	27.76	27.88
H β D-1	61.99	79.26	86.02	69.17	69.74
H β D-3	3.86	1.52	1.50	4.00	3.81

Table 2: Comparison Of Parameters Between Groups

PARAMETERS	Groups I&II	Groups I&III	Groups I&IV	Groups I&V	Groups II&III
HbA _{1c}	0.16	0.00*	0.00*	0.00*	0.00*
PI	0.99	0.25	0.00*	0.56	0.36
GI	0.01*	0.00*	0.00*	0.00*	0.00*
SBI	0.00*	0.00*	0.00*	0.00*	0.00*
PPD	0.00*	0.00*	0.00*	0.99	0.00*
CAL	0.00*	0.00*	0.00*	0.13	0.00*
BMI	0.98	0.92	0.99	0.87	0.99
H β D-1	0.64	1.00	0.12	0.88	0.78
H β D-3	0.00*	0.00*	0.00*	0.32	0.65

* p values ≤ 0.01 was highly significant and p values ≤ 0.05 was significant

Table 3: Comparison Of Parameters Between Groups.

PARAMETERS	Groups II&IV	Groups II&V	Groups III&IV	Groups III&V	Groups IV&V
HbA _{1c}	0.00*	0.00*	0.00*	0.00*	0.99
PI	0.00*	0.71	0.00*	.981	0.00*
GI	0.00*	0.84	0.00*	.000*	0.00*
SBI	0.00*	0.99	0.00*	.000*	0.00*
PPD	0.00*	0.00*	0.00*	.000*	0.00*
CAL	0.00*	0.00*	0.00*	.000*	0.00*
BMI	1.00	0.99	0.99	1.00	0.98
H β D-1	0.05*	0.78	0.09	0.89	0.11
H β D-3	0.51	0.00*	0.51	0.00*	0.00*