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Effect of Smoking on the Expression of Human Beta-Defensin-2 in Gingival Crevicular Fluid after Non Surgical Periodontal Therapy

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ABSTRACT

Aim: This study was designed to evaluate the effect of smoking on the expression of hβD-2 in the GCF after non-surgical periodontal therapy. **Subjects & Methods:** Ten non-smokers patients with chronic periodontitis and ten smoker patients with chronic periodontitis with age ranged between 25-40 years were selected for this study. All patients were examined with clinical periodontal parameters. Patients in both groups underwent nonsurgical periodontal therapy combined with a maintenance program (including brushing with regular toothpaste). GCF samples were collected all patients at baseline, one month as well as 3 months after periodontal therapy. Quantification of β-defensin-2 in human samples was measured using hβD-2 ELISA test. **Results:** Non-surgical periodontal therapy resulted in relative improvement in all clinical parameters as well as an increase in hβD-2 levels. In addition, GCF levels of hβD-2 were higher after non-surgical treatment in non-smoker groups than smokers. **Conclusions:** Deficiency of hβD-2 possibly could be related to host/microbial interaction and Smoking could modulate secretion of hβD-2, which represents a local defense dysfunction.

KEYWORDS

Beta defensins-2; Chronic periodontitis; Smoking.

INTRODUCTION

Chronic periodontitis is the most prevalent form of periodontitis that is directly related to the longstanding plaque and calculus accumulation. However, environmental factors such as smoking may modify the host's immune response to the dental biofilm so that periodontal destruction becomes more progressive [1, 2].

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Smoking is one of the main and most prevalent risk factors for chronic periodontitis. Smokers have demonstrated a decreased inflammatory response to plaque accumulation and reduced gingival bleeding[3,4]. This altered inflammatory response has been attributed to alteration in the gingival vasculature which includes decreased vascular density, lumen area of gingival vessels, and epithelial thickness [5,6]. Furthermore, smoking produces a suppressive effect or impairment on various immune cells such as monocytes, neutrophils, lymphocytes and natural killer (NK) cells[7]. In addition, smoking diminishes the phagocytic uptake of both bacteria and apoptotic cells and induces qualitative and quantitative defects in circulating NK cells which are important in host viral and anti-tumor responses[8,9]. On the other hand, smoking also had a role in modulating the expression of pro-inflammatory cytokines in periodontal ligament and fibroblast cells[10] as well as in oral keratinocytes and GCF [11-13].

The goals of today's treatment of periodontitis are to reduce infection, resolve inflammation and create a clinical condition, which is compatible with periodontal health [14]. Non-surgical periodontal therapy consists of scaling and root planing (SRP) combined with oral hygiene instructions and their efficacy directly related to the ability of treatment to lower levels and prevalence of one or more pathogenic bacterial species. Typically, this results in attachment gain and pocket depth reduction due to a resolution of the inflammation [15-17].

Antimicrobial peptides (AMPs) are multifunctional peptides whose fundamental biological role has been proposed to be the elimination of a diverse spectrum of microorganisms [18].The most important antimicrobial peptide group in humans is *defensins*. *Defensins* have the ability to inactivate many bacteria, fungi, and some enveloped viruses. In humans, *defensins* can be subdivided into two families: *alpha-defensins* and *beta-defensins* [19, 20].

The human *beta-defensins* (hβDs) are small, cationic AMPs made primarily by epithelial cells and expressed in all human epithelia [21]. The hβDs are secreted in biological fluids, including urine, bronchial fluids, nasal secretions, saliva and gingival crevicular fluid [22-24].

Among different AMPs, in the oral cavity, hβDs-2 was found to be 10-fold more potent than hβD-1 and exhibited microbicidal activities against gram-positive and gram-negative bacteria, fungi, and some parasites [25-27]. Furthermore, hβD-2 mainly secreted in response to stimulation. This stimulation does not necessarily come only from bacteria since proinflammatory cytokines such as TNF- α, interferon (IFN)-gamma, IL-1β, IL-17, and IL-22, stimulate hβD-2 secretion. Otherwise, anti-inflammatory cytokines such as IL-4, and IL-10, suppress its production[28]. Besides, hβD-2 brings blood cells to the site of infection by acting as chemotactic agents[28, 29]. HβD-2 also was found to trigger fibroblast proliferation[30]. Additionally, hβD-2 has a strong impact on the maturation of premature osteoblasts which might be effective in bone tissue regeneration [31]. The aim of the present study was to investigate the effect of smoking on the expression of hβD-2 in the GCF after non-surgical periodontal therapy.

SUBJECTS AND METHODS

Subjects:

Twenty patients (age ranged 25-40 years) were selected from those attended to the Outpatient Clinics of Oral Medicine, Periodontology, Oral Diagnosis & Radiology department, Faculty of Dental Medicine, Al-Azhar University (Girls' Branch), clinically diagnosed as having chronic periodontitis according to the classification of periodontal diseases by ***Armittage (1999)*** [32]. The criteria for inclusion in the current study were including patients free from any systemic conditions that affect periodontium or interfere with periodontal treatment, diagnosed as having moderate to advance chronic periodontitis [32], did not receive any periodontal treatment in the past

six months before the examination, and not receive antibiotics or anti-inflammatory therapy in the six months before the examination, for female patients, no pregnancy or lactation was included.

All individuals were informed about the procedures of the study and benefits of their participation in the study. A satisfactory written consent was obtained from all the patients denoting they're convinced about the schedule research program design. Ethical committee meeting approved the study protocol. The smoking history of the patients was evaluated using questionnaire, after which the patients were divided into two groups based on their smoking history. If the patient smoked more than 10 cigarettes per day, then he/she was classified as a smoker; if he/she had never smoked, then he/ she was classified as a non-smoker [33].

Each patient's periodontal status was evaluated by measuring the Plaque Index (Pl) [34], Gingival Index (GI) [35] Probing Depth, Clinic Attachment Loss (CAL); at the baseline, 1-month, and at three months intervals by using *Michigan* 'O' *Probe* With *Williams* graduated periodontal probe. Ramfjord teeth (16,21,24,36,41,44) or their substitutes were the target teeth for recording these parameters [36].

Collection of samples:

Samples of gingival crevicular fluid were collected at baseline, one month and three months after periodontal therapy. The samples were pooled from four periodontal sites with attachment loss of 4mm or more (in the four different quadrants). The sampling area was isolated with cotton rolls and carefully cleaned supragingivally with sterile cotton pellets. A sterile absorbent paper point was inserted into the gingival crevice or pocket until resistance was felt. The paper point was held in place for the 30s. The samples were immediately placed in Eppendorf tubes, transported to the laboratory and stored at -80°C. The collected samples analyzed using the enzyme-linked immunosorbent assay (ELISA) technique of human beta defensin-2 kit.

Non-Surgical Periodontal Therapy:

All patients in both groups were treated with nonsurgical periodontal therapy, which included the following: Supragingival and subgingival scaling and root debridement were performed with an ultrasonic device with iPiezo engine (NSK Varios 970, Japan)**,** Chlorhexidine mouthwash was prescribed twice daily for one week post periodontal therapy and Oral hygiene instructions included brushing teeth with soft dental brush three times daily and using dental floss once a day.

Quantification of Human beta defensin-2 using ELISA technique:

Quantification of hβD-2 in human samples was measured using Bioneovan Inova hβD-2 ELISA kit. The kit is suitable for testing a variety of sample types *in-vitro* and Purchased from Bioneovan Inova Co. Beijing, China. The kit assayed hβD-2 level in the sample, using a Purified hβD-2 antibody to coat microtiter plate wells, made a solid-phase antibody, then added hβD-2 to wells, Combined HBD2 antibody which With HRP labeled, become antibody-antigen - enzyme-antibody complex. After washing Completely, Added TMB substrate solution, TMB substrate becomes blue color At HRP enzyme-catalyzed, reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of hβD-2 in the samples is then determined by comparing the O.D. of the samples to the standard curve.

Statistical analysis

Values were presented as mean and standard deviation (SD) values. Data were explored for normality using Kolmogorov-Smirnov test of normality. The results of Kolmogorov-Smirnov test indicated that most of the data were normally distributed (parametric data), so one-way analysis of variance ANOVA test was used to compare between different intervals within the same group, followed

by Tukey's post hoc test when the difference was found to be significant. Unpaired t-test was used to compare both groups (non-smokers, smokers). The significance level was set at $p \leq 0.05$. Statistical analysis was performed with SPSS 16.0 (Statistical Package for Scientific Studies, SPSS, Inc., Chicago, IL, USA) for Windows.

RESULTS

Table (1) showed the changes in the scores and measurements of PD and CLA from baseline, 1-month and 3-months after non-surgical periodontal therapy in the chronic periodontitis of smoker and non-smoker patients.

HβDs-2 ELISA analysis showed that in both groups, hβD-2 level increased after non-surgical therapy, to reach the highest mean value after 3 months (Figure-1). One-way analysis of variance revealed that there is a statistically significant increase by time in both groups (p<0.0001). Tukey's post hoc test revealed no significant difference between mean values recorded at baseline (69.18±9.30), after one month (75.89±13.41) and after three months (135.77±32.83) in the non-smokers group. However, in the smokers group, there was a significant difference between baseline (61.99±12.97), after one-month (79.90±13.33) and after three month observation times (117.64±17.77).

At a baseline, a slightly greater mean hβD-2 level was recorded in non-smokers group (69.18±9.30) than smoker group (61.99±12.97), with no statistically significant difference (p=0.1714). At one-month, a slightly greater mean hβD-2 level was recorded in smokers group (79.90+_13.33) than non-smokers (75.89±13.41), with no statistically significant difference (p=0.0511). At three months, a slightly greater mean hβD-2 level was recorded in non-smokers group (135.77±32.83) than smoker (117.64±17.77) with no statistically significant difference (p=0.1420).

Table (1) *Probing depth (mm) & Clinical attachment level (mm) in both groups.*

PD	Baseline		1 month		3months	
	Non-smokers	Smokers	Non-smokers	Smokers	Non-smokers	Smokers
Mean	3.17	3.33	2.55	3.01	1.84	2.53
SD	0.50	0.48	0.52	0.42	0.54	0.51
T value	1.7881		5.3306		7.1957	
P value	0.0763^{ns}		<0.0001*		<0.0001*	
CAL	Baseline		1 month		3months	
	Non-smokers	Smokers	Non-smokers	Smokers	Non-smokers	Smokers
Mean	3.77	3.74	3.11	3.42	2.42	2.94
SD	0.70	0.57	0.78	0.52	0.75	0.57
T value	0.2574		2.5615		4.2758	
P value	0.7973^{ns}		0.0117*		<0.0001*	

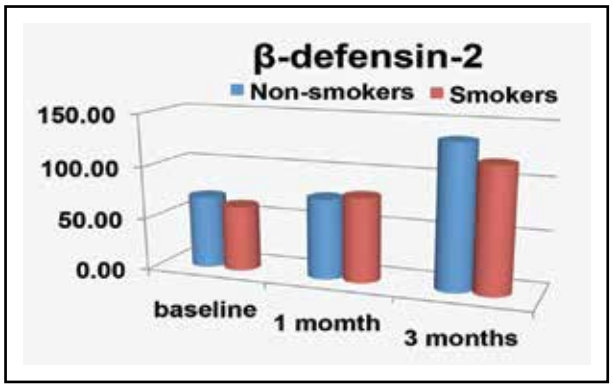

Fig. (1) Column chart showing mean hβD-2 levels in both groups.

DISCUSSION

The complex effects of smoking on periodontal and oral diseases, and the mechanisms that mediate these diseases, are still considerably important. However, establishing a link between cigarette smoking and abnormal levels of antimicrobial peptides will provide a new insight into the epidemiology of the less favorable response following non-surgical periodontal therapy.

Human beta *defensin-2* considered one of the important AMPs in epithelial innate immunity, and their differential expression is associated with periodontal health and diseases. The hβD-2 has a significant role as chemotactic, trigger fibroblast proliferation and has a strong impact on the maturation of premature osteoblasts, which might be effective in bone tissue regeneration. As such, the understanding of their role will undoubtedly unfold their clinical application in periodontal diseases.

According to ***Fan et al. (2015)*** [37] the expression level of hβD-2 and hβD-3 in GCF among the smoking group was significantly lower than that in the non-smoking group. Also, The mRNA expression level of hβD-2 and hβD-3 in the smoking group was weakened compared with that in the non-smoking group indicating that smoking may have a negative effect on the immune defense system of the periodontal host, however, this study is in agreement with another study by ***Wang et al. (2015)*** [38] demonstrating that the whole cigarette smoke (WCS) exposure remarkably attenuated hβD-1 expression and secretion while clearly enhanced hβD-2 and hβD-3 expression levels, suggesting a link between cigarette smoke and abnormal levels of antimicrobial peptides.

The current study demonstrated that after non-surgical periodontal therapy in both groups, hβD-2 level increased after non-surgical periodontal therapy, to reach the highest mean value after 3 months. However, at baseline, a slightly greater mean hβD-2 level was recorded in non-smokers group, with no statistically significant difference. At one-month, a slightly greater mean hβD-2 level was recorded in smokers group, with no statistically significant difference. At three months greater mean hβD-2 level was recorded in non-smokers group, with no statistically significant difference.

At baseline, the low level of hβD-2 in smoker's group could be explained by the fact that periodontopathogenic microorganisms mainly *P.gingivalis*, which had a specific role in β-*defensins* degradation was found in smokers more than in non-smokers [39, 40]. Moreover, bacteria with resistance to β-*defensins*, such as *T. denticola* and *P.gingivalis*, survive and colonize on epithelial surfaces, and eventually, invade gingival tissues [41]. With the bacterial invasion, β-*defensins* stimulate the secretion of chemokines, such as IL-8 and MCP-1, from dendritic cells, and, also, act as chemoattractants, which bring phagocytes and lymphocytes to the site of infection. Correspondingly, the activated immune response limits innate response and, hence, secretion of β-*defensins* [42]. Our findings were in agreement with previous studies shown that smoking down-regulates hβD-2 expression [37, 39, 43].

On the other hand, the proteolytic enzymes, which is produced by periodontal pathogens and the host in different ways such as (trypsin-like proteases and gingipains of *P.gingivalis*) potentially degrade and inactivate hβD-2 *in-vitro* conditions [44-47].

The improvement noticed in terms of clinical parameters after one and three months of non-surgical periodontal therapy when compared to baseline could be explained by the positive effect of non-surgical periodontal therapy suggesting the relationship between host/bacterial factors of chronic periodontitis and hβD-2 levels in GCF.

In conclusion, the GCF levels of human hβD-2 among chronic periodontitis patients could be changed depending on some factors such as smoking. Smoking might also affect the different clinical parameters including; PI, GI, PD and CAL. Moreover, the non-surgical periodontal therapy may lead to increased levels of hβD-2 in GCF among both smokers and non-smokers. However, The discrepancies of hβD-2 slight greater in smoker group after therapy, which could represents, a local defense dysfunction.

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