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Analysis and Correlation of the Salivary Levels of Hepatocyte Growth Factor (HGF) In Smokers and Non – Smokers with Periodontitis

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Abstract

Introduction: Periodontitis is an inflammatory disease which causes progressive destruction of the supporting structures of the teeth caused by a group of specific microorganisms.

Aim: To estimate Hepatocyte growth factor levels in saliva of smoking and non-smoking group of patients with Periodontitis and a healthy group.

Materials and Methods: Individuals included in the study were patient's groups who were referred to the department of Periodontics for the diagnosis and treatment of Periodontitis. The control included are the post graduates and interns at the dental college. Ethical committee clearance was obtained from Sree Balaji Dental College, Pallikaranai, TN, India. Approximately 2mL of unstimulated whole saliva was collected in a sterile container using the spit-out method. The Sandwich ELISA technique was used to detect the levels of hepatocyte growth factor present in the Saliva to estimate the level of Periodontitis.

Results

A total of 45 systemically healthy, smoking, and non-smoking individuals 15 in each group, were analysed for HGF levels. The overall levels of HGF in non smoking group were elevated compared to smoking with Periodontitis group and the healthy control group.

Conclusion

Within the limitations of the study, the levels of HGF in saliva can be used as a marker of active Periodontitis. The elevated levels of HGF in the saliva of the Periodontitis group compared to the other groups could be due to the effect of cigarette smoke extract on the mesenchymal cells responsible for the secretion of HGF or may be due to the improper selection of smokers based on severity.

Keywords: Periodontal disease, Hepatocyte Growth Factors, Saliva

Introduction

Periodontal disease is one of the major dental diseases that affect human populations all over the world[1]. Periodontal disease belong to a group of chronic inflammatory lesions that involve periodontal tissues[2]. It is well-known fact that periodontal diseases are caused by a combination of periodontopathogens, that grows and invades the supporting structures of the teeth, and due to the impaired host immune response that results in tissue destruction [3].Periodontal destruction is characterized by attachment loss and bone loss around the teeth with the formation of periodontal pockets [4]. Periodontal disease progresses in different stages where there are stages of active destruction followed by stages of remissions. It has been realized in recent times that periodontitis is basically a patient-based rather than a site-based disease. Understanding the risk factors associated with the host could be the key for better understanding the disease progression [5]. The evidence available in recent times has shown periodontal disease as a risk factor to poor oral hygiene, tobacco use and diabetes mellitus. In the old days, periodontal diagnosis involved retrospective studies measuring the attachment loss and bone loss. For these reasons, a majority of recent periodontal studies have been concerned with finding and testing the potential markers of periodontal disease activity [6].In the disease process, potential biomarkers of disease activity are involved in some way or the other during disease progression. Gingival crevicular fluid and Saliva components that are the result of these processes have been the main source for the study [7]. The most specific sign of connective tissue breakdown may be due to the protein concentration in the GCF. The GCF and Saliva contain a variety of markers of periodontal disease activity and complement component cytokines [8].

Hepatocyte growth factor(HGF)is a protein secreted by the mesenchymal cells. It regulates angiogenesis, vascular permeability, cell migration and wound healing. It is also known to be a multifunctional cytokine. The functions of these cells are widely overlapped but each show characteristic properties. It is involved in tissue development, regeneration and wound healing and it also plays an important role in the progression of periodontitis by stimulating the growth of epithelial cells and preventing the regeneration of connective tissue attachment [9]. HGF is a well-known serum marker for a number of systemic diseases such as lung, kidney, liver diseases including periodontitis. The levels of HGF in saliva and gingival crevicular fluid (GCF) of patients with periodontitis is 10 folds higher than healthy patients. Higher levels of HGF in Saliva and GCF has also been observed in periodontally compromised sites [10].The role of HGF in periodontitis was first reported in subjects having loss of connective tissue attachment in periodontitis cases thus suggesting that HGF may be involved in epithelial invasion. Similarly, HGF levels in saliva were found to be elevated in periodontal disease, and were correlated with clinical parameters such as probing depths (PDs) and bleeding on probing[11].In the study conducted by Mehta etal, the role of HGF in epithelial invasion, suggests a synergistic expression of HGF in connective tissue and hepatocyte growth factor activator (HGFA) expression in epithelium contributing to disease progression in periodontitis. It is a well-known fact that cigarette smoking is an important risk factor in the pathogenesis and progression of periodontal disease. Smokers have a greater risk of more extensive and severe alveolar bone loss [12]. Studies have shown that smoking impairs various aspects of the innate and adaptive immune responses, including altered neutrophil function, antibody production, altered fibroblast activity, vascular factors,

and inflammatory mediator production [13]. Hence in the present study, the saliva HGF levels are being estimated to assess periodontal disease activity in smokers compared to non-smokers group of patients with periodontitis.

Materials and Methods

This study was performed at the Department of Periodontology and Oral Implantology at Sree Balaji Dental College, Chennai. The study was reviewed and approved by an Ethical Committee of Bharath University, Chennai.

A total of 45 systemically healthy, smoking, and non-smoking individuals (15 participants in each group), were enrolled in this study. Individuals included in this study include patient's groups who were referred to the dental clinics for the diagnosis and treatment of periodontitis. The controls include the staff and interns at the dental college. The periodontal status of the patient's groups was assessed based on American Academy of Periodontology. Smoking and non-smoking periodontitis were determined based on a specific criterion. [14].

Study population

The study total population (n=45) comprises both male and female patients age (25 to 55).Selected patients were based on three groups

Group A: Systemically healthy without periodontitis (15 samples).

Group B: Non-smokers with periodontitis (15 samples).

Group C: Smokers with periodontitis (15 samples).

Inclusion criteria

Systemically healthy patients with periodontal probing depth > 4mm and clinical attachment loss > 2mm in at least 30% of the teeth were taken in to the chronic periodontitis group. Smokers who smoked more than 20 cigarettes/day for more than 2yrs and patients who never smoked were assigned as non- smokers group.

Exclusion criteria

Pregnant women, chronic use of any medication, presence of aggressive periodontitis, abscess, necrotizing ulcerative gingivitis/ periodontitis, antibiotic therapy for past 3 months and former smokers who had quit smoking were excluded from the study.

Study protocol

The clinical periodontal status evaluated and clinical parameters like gingival index, probing pocket depth and clinical attachment levels are recorded.

Method of Saliva sample collection

After getting informed consent from the patient in each group,2ml of unstimulated whole saliva was collected in sterile cryo vials for 5 minutes after rinsing the mouth thoroughly with distilled water. The saliva samples were kept on ice for an hour. The supernatant middle 1/3 was collected by centrifugation at 3800 rpm for10min and stored at −70°C until analysis.

Salivary HGF levels

The concentration of HGF was determined using an ELISA kit. The intensity of the colour was measured using a microplate reader set to 450 nm. The concentration of HGF in the tested samples was estimated using the reference calibrated standard curve, obtained by plotting the optical density values of the standards against the concentrations.

Determination of hepatocyte growth factor in gingival crevicular fluid and saliva

Kit reagents and saliva samples were brought to room temperature Addition of 150 ml of assay diluent RD1W per well added 50 ml of standard or sample per well, kept for 15 min and then mixed gently, covered with the adhesive strip and incubated for 2 h at room temperature.

After aspiration, the wells were washed for four times with wash buffers to remove unbound molecules and dried the plate with tissue paper. The addition of 200 ul of the HGF conjugate to each well was done and covered with

adhesive strips. Incubation for 2 h was made at room temperature.

Aspiration/washing steps were repeated for four times. The addition of 200 ul of substrate solution made to each well and incubated for 30 min at room temperature and kept at dark. Finally, the reaction was stopped by addition of 50 ul of stop solution to each well and the color change was determined with OD of each well-using microplate readers set to 450 nm.

Statistical Analysis

Statistical analysis of the data was performed using the GraphPad instat and GraphPad Prism software. Means and standard deviations of HGF levels (healthy, non-smoker periodontitis, and smoker periodontitis) were analysed. Differences between the three study groups for all variables were determined by one-way analysis of variance (ANOVA). When an overall ANOVA showed statistical significance, post hoc testing (Tukey-Kramer multiple comparisons test) was performed to explore the differences between any two groups. Values <0.05 were considered significant. Student's -test was used to analyse the mean differences in two periodontitis groups.

Results

Organization of Patients samples in ELISA plates for the detection of HGF levels in human saliva

Periodontitis patient's saliva samples were collected in aseptic conditions as recommended and followed in our hospital (Balaji Dental College and Hospital, Pallikaranai, TN, India). All Periodontitis patients without smoking (n=15) (PNS) salivary samples were loaded in the ELISA plate (Table 1) in column manner followed by periodontitis patients smoking (n=15) (PWS) loaded in same order as the PNS. Healthy control of 15 samples loaded into wells and finally the HGF standards are from 125,250, 500,1000,2000,4000,6000 and 8000 pg/ml.

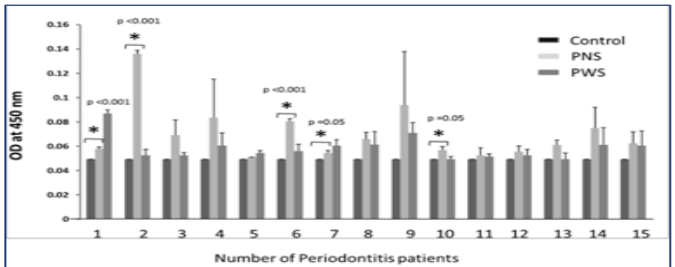
Determination of Standard HGF slop formation in the graph for the comparison of the unknown concentration of Periodontitis patients by sandwich ELISA method

The raw data obtained from individual periodontitis patients with non-smoking were compared to healthy controls and their mean.SD were analysed with significance in optical density. Results shown in (Table 2) states that the colour intensity was significantly increased in few patients with periodontitis with non-smoking, especially patients 1 and 3 showed the significant difference in the development of colour intensity which is the direct indication of HGF levels in these patients were induced.

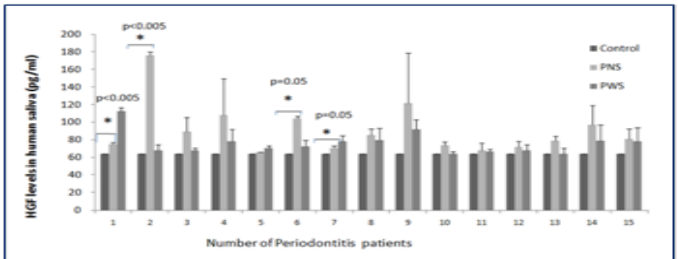
Patients with non-smoking the raw data optical density values were compared to healthy control and there is no significant change in optical density in these patients except few patients such PWS 5,10 and 13.All showed border significance which is not drastic changes in Periodontitis with smoking.

Sandwich ELISA method for detection of HGF levels in periodontitis patients with non-smoking, smoking and healthy controls

The HGF levels detected indirectly by the way of optical density and quantity of HGF detected using standard HGF values calculated the levels in periodontitis with non-smoking and smoking compared to controls as showed in Figure 1 and 2.



[Table/Fig-1]: Optical density of Periodontitis patients with no smoking and smoking compared with healthy controls



[Table/Fig-2]: Human HGF levels of Periodontitis patients with no smoking and smoking compared with healthy controls

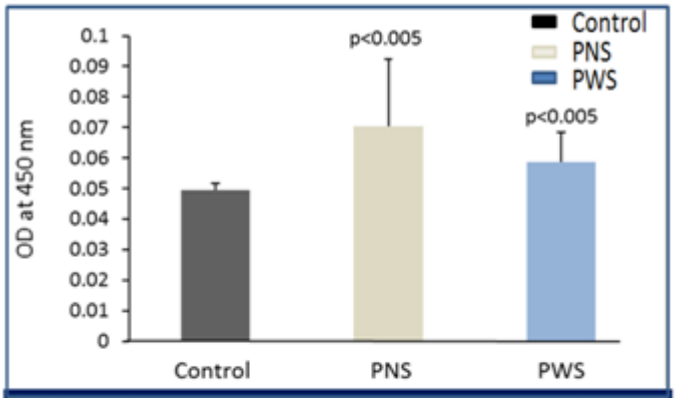
The bar graph has shown the optical and HGF levels follows the same pattern of significant difference between the PNS, controls, and PWS compared to the controls. Figure 2 specifically shows the individual patients (PNS) patient 1,2,6 and 7 were statistically significant in HGF levels induced with non-smoking which is interesting study observation compared to the Periodontitis with smoking.

HGF levels in group comparison of Periodontitis with non-smoking, smoking and Controls

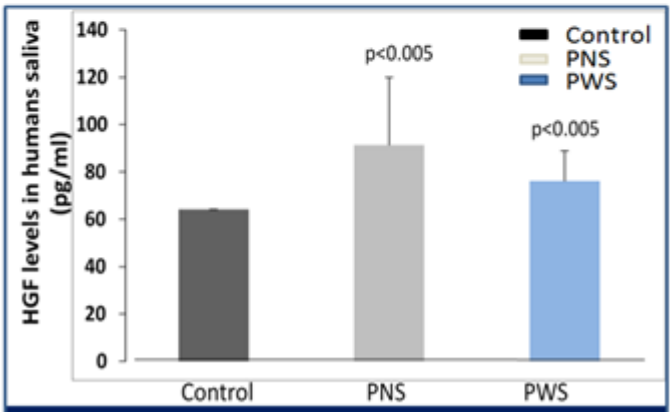
HGF levels in individual patients showed a different profile in both PNS and PWS in previous data analysis but no difference in HGF levels was observed in patients with PWS. Then we compared the HGF levels between groups, to show that significant elevation of HGF could be determined.

	Control	PNS	PWS
Mean	0.0495	0.0704	0.0587
SD	0.0023	0.0220	0.0097
t-test		0.0013	0.0020
P-value		<0.005	<0.005
Comparison		Control vs PNS	Control vs PWS

[Table/Fig-3] Raw data of optical density at 450 nm comparison of Control, Smoking and non smoking < 0.05 as significant against each groups



[Table/Fig-4]: Bar graph with optical density at 450 nm with significant against each group consider P<0.05



[Table/Fig-5]: Bar graph with HGF levels measured at 450 nm with significant against each group consider P<0.05

Analysis of HGF levels in student t-test showed significant changes individual vs controls but we need to see the significance between groups such as PNS and PWS. We used the Graph Pad Prism software to compare the significance the result showed that confirmation of the significance of means between groups was observed.

Table Analysed	One-way ANOVA data		
Data sets analyzed	A: Control	B: PNS	C: PWS
ANOVA summary			
F	3.458		
P value	0.0441		
P value summary	*		
Significant diff. among means (P < 0.05)?	Yes		
R Square	0.1824		
Brown-Forsythe test			
F (DFn, DFd)	2.001 (2, 31)		
P value	0.1522		
P value summary	ns		
Are SDs significantly different (P < 0.05)?	No		
Bartlett's test			
Bartlett's statistic (corrected)	16.07		
P value	0.0003		
P value summary	***		
Are SDs significantly different (P < 0.05)?	Yes		

Table/Fig-6]: One Way ANOVA analysis for the significance between the two different groups with HGF levels

One way ANOVA in Table 2 showed a significant of 0.044, there is a change in means of HGF levels but after passing this analysis the next analysis is post hoc which exactly reveals the significant differences observed between groups.

So, the Turkey analysis Table 2 showed a clear analysis that there is no difference among the groups such as Control vs PNS, Control vs PWS and finally PNS vs PWS indicated that HGF levels were not changed among them.

One-way Analysis of Variance (ANOVA)

The P value is 0.0441, considered significant. Variation among column means is significantly greater than expected by chance.

Tukey-Kramer Multiple Comparisons Test

If the value of q is greater than 3.482 then the P value is less than 0.05.

Table: 3 Tukey-Kramer Multiple Comparisons Test(post hoc analysis) for the significance between the two different groups with HGF levels after ANOVA analysis

Discussion

Human periodontal diseases are inflammatory disorders that give rise to tissue destruction as a result of complex interactions between pathogenic bacteria and the host's immune response. IL-1 and TNF-α are key mediators of chronic inflammatory diseases and have the potential to initiate tissue destruction in periodontal diseases.

Fibroblasts produce Hepatocyte growth factor by inflammatory cytokines, such as IL-1a, IL-1b, TNF-α11, and PGE2.Hepatocyte growth factor (HGF) is a protein secreted by mesenchymal cells, regulates angiogenesis, vascular permeability, cell migration, reepithelialisation, and other wound healing processes.

HGF is also a well-known serum marker for various diseases, including periodontitis. HGF stimulates the growth of gingival epithelial cells into the periodontal pockets, thereby impairing the regeneration of the collagenous structures of the periodontium [15].

With the advent of highly sensitive techniques, traces of markers can be accurately established in body fluids, such as saliva and GCF [16].Most biomarkers in GCF and saliva are indicators of inflammatory events that precede the destruction of the alveolar bone [17]. The use of a sensitive enzyme-linked immunosorbent assay method to quantify HGF from selected sites allowed us to avoid pooling of GCF samples from multiple sites or subjects.

In the study conducted by Pradeep etal 2007, the mean concentrations of HGF in GCF increased progressively from health to periodontitis and the mean concentration of HGF in gingivitis was intermediate between these two groups[18].The results of this study, with respect to a general trend, are in accordance with those of reported increasing HGF levels in the GCF with the progression of

periodontal disease. When a pair-wise comparison was done using theTurkey test, a statistically significant difference was observed between healthy and chronic periodontitis group, gingivitis and chronic periodontitis groups, and chronic periodontitis after-treatment groups (P <0.05). The results of the study are in contrast with a recent study by Kakimoto et al 2002 which did not find a statistically significant decrease in HGF concentrations.

In the study conducted by Sukumaran etal, a significantly higher level of salivary HGF was found in smokers with periodontitis and non-smokers with periodontitis compared to the control group. The study validates the results by stating that the HGF levels in the GCF and saliva increase substantially due to the overall burden of smoking and bacterial load.

In case of non-smokers, the HGF value proportionally increases with the periodontal disease progression which is similar to [18].

The observations from my current study reveal a high HGF level in the saliva of non-smoking groups with periodontitis when compared to smoking group and the healthy group. This could be due to the fact that cigarette smoke extract impairs the mesenchymal cells which are responsible for the secretion of hepatocyte growth factor [19].

A pair-wise comparison done using the Turkey-Kramer test inferred that there was no statistically significant difference between the HGF concentration in the saliva of the three groups. However, further correlative studies with a greater sample size are required to confirm the role of HGF as a potential marker for the periodontium in health and disease.

The main drawback of our present study could be the improper selection of smokers. We have not classified the smokers based on severity (mild, moderate, severe) this could have deviated our results.

Conclusion

In the present study, a significantly higher level of salivary HGF was found in non-smokers with periodontitis and smokers with periodontitis compared to the control group. These observations were not correlated with other studies.

HGF plays an important role in mesenchymal epithelial interactions, which contributes to wound healing. During inflammatory conditions, such as periodontitis, it could be due to bacterial load that the HGF production by oral fibroblasts is enhanced.

The significantly higher levels of HGF in non- smokers with periodontitis might be due to the fact that cigarette smoke extract impairs the mesenchymal cells which are responsible for the secretion of hepatocyte growth factor or improper selection of smokers based on the severity of smoking. Hence further studies are required to help establish the association between the severity of periodontitis and the HGF levels in saliva of non-smokers in South Indian populations.

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