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Assessment of Macrophage Inflammatory Protein - Iα Levels in Saliva and Gingival Crevicular Fluid in Patients with Chronic Periodontitis.-Original Research

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ABSTRACT
Introduction: *Macrophage inflammatory protein- I α is a member of the cystine-cystine chemokine family, secreted by inflammatory cells and is primarily associated with cell adhesion and migration. It stimulates monocytes and / or osteoclast progenitor cells to become active osteoclasts. Elevated levels of MIP-I α have been found to be associated with bone remodelling and tissue destruction. Hence, the aim of this study was to investigate whether MIP-I α can be used as an inflammatory marker to assess the progression of Periodontal disease.*
Methods: *Thirty patients in the age range of 25-50 were selected and divided into three equal groups based on periodontal clinical parameters as follows ; Group 1-periodontally and systemically healthy subjects, Group 2- Mild to moderate Periodontitis, Group 3- Severe Periodontitis. Saliva and GCF samples collected from all participants were analyzed for estimation of MIP-I α using ELISA. The results were subjected to statistical analysis for comparison of MIP-I α between the groups and for correlation of clinical parameters with MIP-I α.*
Results: *MIP-I α levels were found to be significantly higher in severe Periodontitis followed by mild to moderate Periodontitis group than the healthy controls (P<0.001). There was a positive correlation between all clinical parameters and MIP-I α levels in saliva and GCF (P<0.001)*
Conclusion: *From the findings of the study it can be concluded that MIP-I α can be used as an inflammatory marker to assess the progression of periodontal disease,*
Keywords: *Chronic Periodontitis, Gingival crevicular fluid, inflammatiory marker, macrophage inflammatory protein, saliva Total word count = 250*
Main Body Of The Manuscript

I. INTRODUCTION

Periodontitis is a polymicrobial disease that results from complex interplay between oral bacteria and the host inflammatory response. This interaction triggers a cascade of inflammatory events, which in turn promote connective tissue destruction and alveolar bone remodelling .[1] These unique biological events contain signatures of the microbial ecology, as well as downstream events involving inflammation, attachment loss and bone destruction. It is likely that identification of dominal signatures for each of these biological phases could provide insight into biomarkers of periodontal disease in oral fluids.[1]

The diagnosis of active phases of periodontal disease and the identification of patients at risk for active disease are challenges for clinical investigators and practitioners alike .[2] Optimal innovative approaches would

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correctly determine the presence of current disease activity, predict sites vulnerable for future breakdown and assess the response to periodontal interventions.[2]

Bio markers, whether produced by normal healthy individuals or individuals affected by specific systemic diseases, are tell-tale molecules that could be used to monitor health status, disease onset, treatment response and outcome.[2]

Researchers involved in periodontal diagnosis have successfully investigated the possible use of oral fluids, such as saliva, for disease assessment.

Whole saliva represents a promising diagnostic fluid for the screening of periodontal disease. It is a fluid that contains constituents of exocrine glands in the oral cavity and gingival crevicular fluid (GCF). Saliva is readily available and easily collected without specialized equipment. Several mediators of chronic inflammation and tissue destruction have been detected in whole saliva of periodontitis patients .[3]

Gingival crevicular fluid (GCF) is a physiological fluid as well as an inflammatory exudates, originating from the gingival plexus of blood vessels in the gingival corium, subjacent to the epithelial lining of the dento gingival space. As GCF transverses through the inflamed periodontal tissues en route to the sulcus, biological markers are gathered from the surrounding areas and are subsequently eluted into the whole saliva.[2]

Macrophage inflammatory protein-I α (MIP-I α) / CCL3 is a member of the cystine-cystine chemokine family, which is secreted by macrophages, neutrophils, dendritic cells, lymphocytes and epithelial cells.[1,4]

MIP-I α stimulates monocytes and /or osteoclast progenitor cells to become active osteoblast in dose dependent manner and acts upstream as an activator of osteoclastogenesis within resorption lacunae.[1,5] It also induces synthesis of other pro inflammatory cytokines such as IL-1, IL-6 and TNF-α from fibroblasts and macrophages in addition to mediation of granulocyte adhesion and migration.[5]

Hence, the purpose of this study was to investigate whether MIP-I α can be used as an inflammatory marker to assess the progression of periodontal disease by comparing the levels of this biomarker in healthy controls and Periodontitis patients and by correlating the salivary and GCF levels of MIP-I α with clinical periodontal parameters.

II. MATERIALS AND METHODS

This cross sectional study was carried out in the Department of Periodontics, Rajah Muthiah Dental college & Hospital, Annamalai University from September 2014 to January 2015. A total number of 30 human male subjects in the age range of 25-50 years were selected from the outpatient department of Periodontics. Ethical clearance for the study was obtained from the Institutional human ethical committee of Rajah Muthiah dental medical college (M18/RMMC/2014). All the participant subjects were clearly informed about the study and they signed an informed consent form.

Sample size

The sample size for the present study was determined by using the power analysis following a pilot study.

Inclusion study

1. systemically healthy males
2. non smokers
3. those with healthy Periodontium and those with generalized chronic Periodontitis.

Exclusion criteria

1. Recent history of any drug that might influence the outcome of the study.
2. Any form of periodontal therapy in the preceding six months.

Study design

The subjects were selected based on inclusion and exclusion criteria and divided in to three groups Group 1 (10 subjects) Periodontally healthy-no clinical signs of inflammation, no bleeding on probing, sulcus depth $\leq$ 3 mm and no clinical attachment loss.[8] Group 2 (10 subjects) Mild to moderate chronic Periodontitis- Generalized Bleeding on probing, probing pocket depth >3 mm and clinical attachment loss of 1-4 mm at 30% of sites or more. [8]

Group 3 (10 subjects) Severe chronic Periodontitis – Generalized bleeding on Probing, probing pocket depth >3 mm and clinical attachment loss of $\geq$ 5 mm at 30% of sites or more.[8]

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The following clinical parameters were recorded
1. Plaque index (silness and loe 1964)[6]
2. Gingival bleeding index (Ainamo and Bay 1975)[7]
3. Probing pocket depth [8]
4. Clinical attachment loss [8]

The following Bio chemical parameters were analyzed
1. Salivary MIP-1 α level (pg/ml)
2. GCF MIP-1 α level (pg/ml)

Collection of saliva and GCF

GCF and saliva were collected on subsequent day of clinical periodontal examination to avoid contamination with blood. The saliva and GCF samples were collected between 9-11 am to standardize circadian variations.

5 ml of unstipulated whole saliva was collected by draining or spitting method [9] into a sterile test tube and the samples were placed immediately on ice and aliquoted prior freezing at −80°c. Samples were thawed and analyzed within 6 months of collection.

The GCF samples were collected from the buccal crevice of multiple sites in upper quadrants (pooled samples) by intra crevicular method [10.11] using filter paper strips ® for 30 seconds. The filter paper strips containing crevicular fluid was then transferred to a sterile eppendorf tube containing 2ml phosphate buffered saline and kept at −70°c until analyzed. Prior to analysis the GCF sample tubes were homogenized for 30 seconds and centrifuged for 5 minutes.

Macrophage inflammatory protein-1α analysis

Both the saliva and GCF samples were analyzed using Human macrophage inflammatory protein-1α ELISA kit # Analysis was carried out according to the manufacturers instructions. This assay employs an antibody specific for human MIP-1α coated on a 96 well plate.

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III. STATISTICAL ANALYSIS

The data obtained were subjected to statistical analysis using the software programme SYSTAT 12
1. One way ANOVA was used to analyse the mean and standard deviation of Salivary and GCF MIP 1α levels groupwise
2. Scheffe's post hoc multiple comparison test was done to compare the mean And standard deviation of salivary and GCF MIP-1α levels by groupwise.
3. Pearson's correlation co efficient test was used to determine the relationship between clinical parameters with salivary and GCF MIP-1 α levels.
4. Pearson's correlation co efficient test was done to find out the relationship between salivary and GCF MIP-1α levels.

IV. RESULTS

The mean and standard deviation of salivary MIP-1α were found to be 24.4100 ± 5.95995 for Group 1 (healthy) whereas 61.2900 ± 8.57703 for group 2(mild to moderate periodontitis) and 89.1420 ± 8.33997 for group 3 (severe periodontitis). The difference between the groups was found to be statistically significant ($p^{<}0.001$) (Table 1)

Table 2 reveals scheffe's post hoc multiple comparison test applied to compare the salivary MIP-1α Levels between the groups. When groups 1 and 2 were compared there was a negative mean difference (−36.88000) between the groups which was significant ($p^{<}0.001$). There was a mean negative difference between groups 1 and 3 (−64.73200) which was significant ($p^{<}0.001$) and there was a men negative difference between groups 2 and 3 (−27.85200) which was significant
($p^{<}0.001$)

The mean and standard deviation of GCF MIP-1α were found to be 19.020 ± 5.3822 for group 1(healthy) whereas 111.270±15.7716 for group 2 (mild to moderate periodontitis) and 169.100 ±10.3474 for group 3 (severe periodontitis) . The difference between the groups was found to be statistically significant ($p<0.001$) (Table 3)

Table 4 reveals scheffe's post hoc multiple comparison test applied to compare the GCF MIP-1α levels between the groups. When groups 1 and 2 were compared there was a significant negative mean difference (−92.2500) ($p<0.001$) between the groups. Similarly there was a significant negative mean difference between groups 1 and 3 (−150.0800) ($p<0.001$) and between groups 2 and 3 (−57,8300) ($p<0.001$) which was significant.

The Pearson's correlation co efficiency test revealed that all periodontal clinical parameters positively correlated with salivary MIP-1 α levels (PI- r=0.847) ,
(GBI- r=0.917), (PD-r=0.913),(CAL- r=0.977) with statistical significance
($p<0.001$). Similarly all clinical parameters positively correlated with GCF MIP-1α levels (PI-r=0.880), (GBI-r=0.948),(PD-r=0.907),(CAL-r=0.983) with statistical significance ($p<0.001$) (Table 5)

The Pearson's correlation co efficient test to find out the relationship between salivary and GCF MIP-1 α levels revealed a positive correlation (r value=0.967) which was statistically significant ($p<0.001$) (Table 6)

Tables

Table 1 ; Mean & std deviation of salivary MIP-1 α levels (pg/ml) group wise

Salivary MIP-1α	Mean	Std Dev	One way ANOVA F value	P value
Group 1 (healthy)	24.4100	5.95995	177.061	<0.001(s)
Group 2(mild to moderate periodontitis	61.2900	8.57703		
Group 3 (severe Periodontitis)	89.1420	8.33997		

Table 2: Scheffe's post-hoc multiple comparison test results for salivary MIP-1α levels

Group	Group	Mean difference	P value
Group 1	Group 2	−36.88000	<0.001 (s)
Group 1	Group 3	−64.73200	<0.001 (s)
Group 2	Group 3	−27.85200	<0.001 (s)

Table 3 ; Mean & std deviation of GCF MIP-1α levels (pg/ml) group wise

GCF MIP-1α	Mean	Std Dev	One way ANOVA F value	P value
Group 1 (healthy)	19.020	5.3822	446.728	<0.001(s)
Group 2(mild to moderate periodontitis	111.270	15.7716		
Group 3 (severe Periodontitis)	169.100	10.3474		

Table 4: Scheffe's post-hoc multiple comparison test results for GCF MIP-1α levels

Group	Group	Mean difference	P value
Group 1	Group 2	− 92.2500	<0.001 (s)
Group 1	Group 3	−150.0800	<0.001 (s)
Group 2	Group 3	− 57.8300	<0.001 (s)

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Table 5 ; Pearson's correlation co efficient test for correlating salivary and GCF MIP-1α levels with periodontal clinical parameters

Clinical parameters	Salivary MIP-1α		GCF MIP-1α	
	'r' value	'p' value	'r' value	'p' value
Plaque index	0.847**	< 0.001(s)	0.880**	< 0.001(s)
Gingival bleeding index	0.917**	< 0.001(s)	0.948**	< 0.001(s)
Probing depth	0.913**	< 0.001(s)	0.907**	< 0.001(s)
Clinical attachment level	0.977**	< 0.001(s)	0.983**	< 0.001(s)

Table 6 ; Pearson's correlation co efficient test to compare salivary and GCF MIP-1 α levels

Salivary MIP-1 α	GCF MIP-1 α	
	'r' value	'p' value
	0.967	< 0.001(s)

V. DISCUSSION

Periodontitis results from the inflammatory response to bacterial challenge in the gingival crevicular area. Numerous cytokines and chemokines are produced in response to microbes and other antigens that play a central role in the pathogenesis of periodontal disease.

Chemokines are responsible for the recruitment and subsequent activation of particular leukocytes in to inflamed tissues and therefore play a central role in the final outcome of the immune response by determining which subsets of leukocytes form the inflammatory infiltrate.[12]

Macrophage inflammatory protein (MIP)-1 α is a member of the c-c subfamily of chemokines , a large super family of low molecular weight, inducible proteins that exhibit a variety of pro inflammatory activities, including leukocyte chemotaxis. In addition to pro inflammatory activities, it inhibits the proliferation of hematopoietic stem cells.[1]

Hence, our clinico-Biochemical study was aimed at investigation of salivary and GCF MIP-1 α levels in periodontal health and disease and also to correlate the severity of periodontitis with MIP-1 α levels. This cross sectional case control study was done to evaluate the inflammatory marker MIP-1α in whole unstipulated saliva and GCF from 20 patients with mild to moderate and severe forms of chronic periodontitis and 10 healthy controls.

In our study we excluded smokers to eliminate the confounding variable of smoking since in a previous study done by Tymkiw KD et al in 2011 there was a significant relationship between smoking and GCF MIP-1α levels when they compared the expression of 22 chemokines and cytokines in GCF of smokers and non smokers with periodontitis[13]

Many previous studies have shown that components of saliva and GCF can provide vital diagnostic information and serve as useful aid in assessing the progression of periodontal disease by analysing various inflammatory markers.[3,14] MIP-1 α is chemotactic for polymorphonuclear leukocytes (PMN'S) in acute inflammation and is stimulatory for monocytes in relation to osteoclastogenesis.[15]

In a study done by Al-sabbagh et al in 2012[1], regression analysis showed that MIP-1α offered high specificity (94%) and sensitivity (92.5%) for distinguishing periodontal disease from health. They concluded that MIP-1α was a superior salivary biomarker when compared to other markers of bone loss including osteoprotegrin (OPG), C-telopeptide type 1 collagen and αβ-terminal c-type 1 collagen telopeptide. Hence our investigation was aimed at assessing the level of salivary and GCF MIP-1α in patients with chronic periodontitis.

The results of our study revealed that the mean salivary MIP-1α levels were found to be higher in group 3 (severe periodontitis)(89.1420) and group 2(mild to moderate periodontitis) (61.2900) when compared with group 1(healthy controls)(24.4100). Within the periodontitis group, group 3 Exhibited higher salivary MIP-1α levels than group 2 which was significant (p<0.001). This could be attributed to increased CD 8 and B cells with increasing inflammation and also due to higher proportion of macrophages in tissues with chronic inflammation.[12]. Our results correspond with that of Al-sabbagh et al (2012)[1] who showed that the mean level of MIP-1α was 18 fold higher in the periodontal disease group than the control group.

Also in our study the mean MIP-1α levels in GCF were found to be higher in group 3(169.100) and group 2 (111.270) when compared with group 3(19.020)

Which was significant ($p^{<}0.001$). Within the test groups group 3 subjects exhibited higher GCF levels of MIP-1α than group 2 subjects which was significant ($p^{<}0.001$). These findings are in accordance with the results of previous study done by Shaddox LM et al (2011)[16] to delineate chemokines in gingival crevicular fluid and evaluate systemic levels of endotoxin associated with localized aggressive periodontitis. They found significantly higher levels of MIP-1α in GCF obtained from diseased sites than healthy sites.The increase in GCF levels of MIP-1α could be due to the response of gingival epithelial cells and fibroblasts to gram negative bacterial LPS by the transient expression of cytokines.

When the clinical parameters were correlated with salivary and GCF MIP-1α levels, there was a positive correlation between all clinical parameters like PI, GBI,PPD and CAL with salivary and GCF MIP-1α levels ($p^{<}0.001$). Our findings are in accordance with the results of previous longitudinal study by Fine DH et al (2009)[15] who demonstrated that among the 21 cytokines assessed, MIP-1α showed highest specificity (96.8%) and sensitivity(100%) and also correlated with increased probing depth.

To the best of our knowledge this was the first attempt to correlate salivary and GCF MIP-1α levels. The results suggest that there was a positive relationship between salivary and GCF MIP-1α levels. ($p^{<}0.001$) We did evaluate both saliva and GCF since the latter indicates site specific changes. The GCF was harvested from multiple sites and hence there was a strong positive correlation with salivary MIP-1α levels.

Our study, however had few limitations like not correlating other cytokines with MIP-1α levels and probably an assessment could have been made after intervention which can also be a future direction of the study.Within these limitations, our findings suggest that macrophage inflammatory protein-1 α can be used as an inflammatory marker to assess the progression of periodontitis. However, further longitudinal studies with larger sample size are needed to confirm the findings our study.

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MIP-1α levels

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