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Estimation of Salivary Levels of Interleukin-35 and Osteoprotegerin in Periodontitis - A Cross Sectional Study

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Abstract: ***Background: Periodontitis is a multifactorial disease primarily caused by microbial biofilm containing different colonies of microorganisms. Inflammation and bone loss are considered the hallmarks of periodontal disease (PD). The evolution of biomarkers for early detection of periodontal disease and to quantitate disease progression would be highly desirable. It helps to reveal a hidden lethal threat before the disease becomes complicated. Aim & Objective: The aim of the present study is to estimate and correlate the salivary levels of IL-35 and Osteoprotegerin with the periodontal disease severity. Materials and methods: A total of 44 individuals with the age of 25-60 years were included in the study. Group 1 (n=22) were periodontally and systemically healthy individuals and Group 2 (n=22) systemically healthy individuals with periodontitis. Clinical parameters evaluated were Plaque index (PI), Gingival bleeding index (GBI), Probing pocket depth (PPD), Clinical attachment levels (CAL). Un-stimulated salivary samples were collected and analyzed for IL-35 and Osteoprotegerin using sandwich ELISA method. Statistical analysis were performed. Results: The levels of IL-35 and OPG were detected in both the groups. Statistically significant differences were found between IL-35 and OPG salivary levels, and clinical parameters such as Plaque index (PI), Gingival bleeding index (GBI), Probing pocket depth (PPD), Clinical attachment levels (CAL). The salivary levels of IL-35 (13.519±2.020) and OPG (3.327±0.437) was significantly higher in group 2 patients and it was statistically significant with p value of 0.003 and 0.004 respectively. Conclusion: The results of our findings suggests that increase in the salivary levels of IL-35 and OPG shows a predictable association with the presence of inflammation. However, longitudinal studies should be carried out with larger population to validate the role of IL-35 and OPG in periodontitis which may be useful for screening and diagnosis.***

Keywords: Periodontitis, Osteoprotegerin, Interleukin-35, Inflammation, Cytokines.

1. Introduction

Periodontitis is a complex multifactorial disease resulting in the progressive destruction of tooth-supporting structures with formation of the periodontal pocket, gingival recession or both. Periodontal disease occurs due to interplay of bacterial infection and host response to bacterial challenge, and the disease is modified by environmental, acquired risk factors, and genetic susceptibility.

Disease pathogenesis involves the cascade of sequential signaling molecular events leading to host immunomodulatory responses triggered by bacterial toxic byproducts. These bacterial byproducts, in turn, activate various cytokines, chemokines, pro-inflammatory mediators, and macrophages, which are responsible for the progressive destruction of underlying gingival tissues which may subsequently lead to tooth loss.

Inflammation and bone loss are considered the hallmarks of periodontal disease (PD). Bone destruction is highly correlated with the periodontal inflammation and can be influenced by the interactions between pathogenic bacteria, the immune host response, and other infections.

Bacteria release chemical mediators responsible for the activation of innate immunity to release proinflammatory cytokines contributing to soft tissue changes such as inflammation. This further activates the acquired immune system resulting in the production of various cytokines and antibodies. Cytokines are peptide mediators, function in an autocrine or paracrine manner by binding to their definite receptors on specific cells. They involve in discrete functions like cell signaling, communication, cell proliferation, cell differentiation, immune responses, and inflammatory responses. They are produced by host immune-inflammatory cells such as T-helper (Th) cells and macrophages in response to the endotoxins produced by the pathogens.

IL-35 is a newer generation of signal molecule belonging to the IL-12 cytokine family, produced by T-regulatory cells (Treg), detected as the most effective cytokine at high inflammation sites, and also act as a potent activator of Treg cells. Recent studies have shown that IL-35 is an anti-

inflammatory cytokine that suppresses the immune response through the expansion of Treg and suppression of Th17 cell development. This is suggestive of a possible role of IL-35 in chronic inflammatory disorders such as periodontitis.

OPG is a soluble TNF receptor–like molecule, and it is the inhibitor of osteoclast differentiation. It binds to RANKL and blocks RANKL from interacting with RANK and neutralizes its activity by inhibiting the cell-to-cell signaling between osteoblast/bone stromal cells and osteoclast precursor cells, resulting in the inhibition of osteoclast formation. OPG a crucial molecule acts as negative regulators of osteoclastogenesis and bone resorption.

Together with OPG, IL-35 has a potent suppressor function in preventing the periodontal disease progression through their interactions with Treg cells.

There were studies that provide sufficient information on the individual levels of IL-35 and OPG in periodontitis, and their levels were found to be decreased during disease progression.

But no such study was done for the combined estimation of IL-35 and OPG. Since, IL-35, an anti-inflammatory cytokine suppresses bone destruction. Similar to IL-35, OPG also reduces osteoclastogenesis by binding to RANKL and blocks RANKL from interacting with RANK and neutralizes its activity by inhibiting the cell-to-cell signaling mechanism between osteoblast/bone stromal cells and osteoclast precursor cells, resulting in the inhibition of osteoclast maturation and differentiation.

The expression of IL-35 and OPG in periodontal disease was found to be decreased and they both tends to decrease the alveolar bone destruction in periodontitis.

So, this study was conducted to investigate the salivary levels of IL-35 and OPG in the pathogenesis of chronic periodontitis.

Educed with the above stated findings, we had an interest to investigate the salivary levels of IL-35 and OPG in the pathogenesis of periodontal disease.

Aim

- To determine the salivary levels of Interleukin-35 and Osteoprotegerin (OPG) in Periodontal health and disease.
- To correlate the salivary levels of IL-35 and OPG with the nature of disease activity.

2. Materials and Methods

This cross-sectional study was conducted with 44 subjects and they were divided equally into two different groups.
Group 1 (n=22)-Systemically and Periodontally healthy individuals.
Group 2 (n=22)-Generalised periodontitis individuals who were systemically healthy.

Plaque index (PI), Gingival index (GI), Probing pocket depth (PPD) and Clinical attachment level (CAL) were measured using a mouth mirror, dental explorer and William's graduated periodontal probe.

Unstimulated saliva samples were collected by spitting method from all the individuals prior to nonsurgical periodontal therapy. The salivary levels of IL-35 and Osteoprotegerin were analyzed by sandwich enzyme-linked immunosorbent assay using commercially available Human Salivary IL-35 and OPG (Shanghai Korain Biotech Co. Ltd. (BT Bioassay).

Inclusion criteria:
Subjects with age group of 25-60 years who are both systemically and periodontally healthy with no attachment loss and bone loss were grouped as Group 1 (Periodontally healthy). Subjects with at least 20 remaining natural teeth, bleeding on probing ≥10%, probing depth ≥3 mm, clinical attachment level ≥2 mm at more than two teeth were grouped as Group 2 (Periodontitis)

Exclusion Criteria:
Smokers and pan chewers / Tobacco users. Patients having systemic illness, H/O previous surgical and non-surgical periodontal therapy for past 6 months to 1 year. Patients who are under Orthodontic treatment Antibiotics, NSAIDS for past 6 months, Pregnant women and Lactating mothers were excluded from the study.

Clinical parameters such as Plaque index (PI) (Modified by Loe, 1967), Gingival Bleeding index (GBI) – Ainamo and Bay-1975, Probing pocket depth (PPD) and Clinical attachment level (CAL) were measured using a mouth mirror, dental explorer and graduated periodontal probe.

Saliva Sampling:
5 ml of unstimulated salivary samples were collected early in the morning prior to brushing and any other oral hygiene procedures. The participants were advised to rinse their mouth completely with water and then asked to expectorate unstimulated saliva into a disposable centrifuge tube which is made up of Polypropylene. The collected samples were immediately centrifuged for 10 minutes at a centrifugal rate of 800 xg. The supernatant thus obtained were transferred to a vial of 1.5ml and immediately stored at-70°C. The analysis of IL-35 and OPG was performed using ELISA (Biochemical assay).

Statistical Analysis
All analyses were done using SPSS version 22 (released 2013. Armonk, NY: IBM Corp.,). Descriptive Statistical methods such as mean and standard deviation were used to compare age and gender distribution among groups. The mean values of clinical parameters such as (PI, GBI, PD, CAL) and comparison of salivary OPG and IL-35 levels between the two groups were compared using the Mann Whitney test. The relationship between salivary IL-35 and clinical parameters as well as the OPG levels of the two groups were assessed using Spearman's Rank correlation test. p-values of less than 0.05 are considered to be statistically significant.

3. Results

The mean age of individuals in group I and II were 35.05 &

48.00 respectively. Among the study participants 50% were males 50% were females in Group 1 whereas 40.9% were males and 59.1% were female in Group 2. (Table 1)

Mean and standard deviation of Plaque index (PI), Gingival index (GI), Probing pocket depth (PPD) and Clinical attachment level (CAL) among the three groups were compared and it were found to be higher in group II followed by group I with statistical significance ($p < 0.001$) (Table 2)

The salivary levels of IL-35 (13.519±2.020) and OPG (3.327±0.437) was significantly higher in group 2 patients and it was statistically significant with p value of 0.003 and 0.004 respectively (Table 3)

Table 1: Distribution of age (years) and sex between the groups

Age and gender distribution among 2 groups						
Variable	Category	Group 1		Group 2		P-Value
		Mean	SD	Mean	SD	
Age	Mean	35.05	3.74	48.00	7.58	<0.001*a
	Range	30 – 42		36 – 60		
		n	%	n	%	
Sex	Males	11	50.0%	9	40.9%	0.55b
	Females	11	50.0%	13	59.1%	

Table 2: Comparison of Plaque Index (PI), Gingival Bleeding Index (GBI), Probing depth, and Clinical attachment level (CAL) between two groups

Comparison of mean clinical parameters between 2 groups using Mann Whitney Test						
Parameters	Group	N	Mean	SD	Mean Diff	p-value
PI	Group 1	22	0.27	0.184	- 1.309	<0.001*
	Group 2	22	1.57	0.359		
GBI	Group 1	22	6.1	1.231	- 64.650	<0.001*
	Group 2	22	70.7	6.670		
PD (mm)	Group 1	22	2.17	0.546	- 1.048	<0.001*
	Group 2	22	3.22	0.855		
CAL (mm)	Group 1	22	0.000	0.000	- 4.142	<0.001*
	Group 2	22	4.14	0.866		

Table 3: Comparison of mean salivary levels of OPG & IL-35 (in ng/ml) between two groups

Comparison of mean values of Salivary OPG & IL-35 levels (in ng/ml) between 2 groups using Mann Whitney Test						
Parameters	Group	N	Mean	SD	Mean Diff	p-value
OPG (ng/ml)	Group 1	22	2.647	0.779	- 0.680	0.004*
	Group 2	22	3.327	0.437		
IL-35 (ng/ml)	Group 1	22	11.050	2.926	- 2.469	0.003*
	Group 2	22	13.519	2.020		

4. Discussion

Periodontitis is a chronic inflammatory disease which is initiated by biofilm that forms on the tooth surface, which affects the gingiva and supporting tooth structures such as periodontal ligament, cementum, and alveolar bone. It is considered as the most common osteolytic diseases of jaw resulting in progressive destruction of tooth supporting structures.

Clinical diagnosis of periodontal disease demands a firm knowledge of recognition of various signs and symptoms in the periodontal structures which herald the departure of periodontal health.

In spite of the numerous advancements in understanding the pathogenesis of periodontal disease, still the disease can only be diagnosed once connective tissue breakdown and bone loss has initiated.

The relevant changes which are of clinical importance in basic periodontal diagnosis can be monitored using periodontal parameters such as bleeding on probing, probing depth, clinical attachment level, mobility, furcation involvement etc., which can be properly correlated with radiographic analysis.
Advances in Molecular and immunological analyses have enhanced the knowledge of various biomarkers in the diagnosis of periodontal disease.

The evolution of biomarkers for early detection of periodontal disease and to quantitate disease progression would be highly desirable. It helps to reveal their concealed level in patients, before the disease becomes complicated.

In addition, identification of salivary bio-mediators associated with disease will facilitates the development of novel therapies aimed at governing cytokine bioactivity, through anti-cytokine antibodies, antagonists or soluble receptors or by targeting the intra-cellular signalling pathways directly.

Cytokines are soluble mediators produced by one immune cell that act on another cell within the same milieu (autocrine or paracrine).

Although the initiating factors are the dental biofilm and its by-products in gingivitis and periodontitis, the pathogenesis and tissue destruction occurs by the development of exaggerated host immune response. The function of immune cells and non-immune cells in orchestrating periodontal disease pathogenesis is established by the functional diversity of cytokines. With the existence of Enzyme-linked immunosorbant assays, interleukin-1 β was the first cytokine to be specifically measured in the gingival tissue of patients with periodontal disease.

Based on the cytokine production, T-cells get activated and it is subdivided into subsets such as Th1, responsible for cellular and pro-inflammatory cytokine production whereas Th2 is responsible for humoral immunity with anti-inflammatory properties. Another group of T-cell subsets like Th17 and Treg cells presents antagonistic role such as effectors and suppressors.

It was evident from the study that B-cells have predominant role in production of RANKL contributing to alveolar bone destruction in periodontitis. (Crotti. T. Smith et al; 2003, Yakunhan et al; 2018, Cun-sheng Bi et al; 2019).

In our study, we estimated the salivary levels of Interleukin-35 and OPG in periodontal health and disease using Enzyme linked Immunosorbent assay (ELISA). A total of 44 subjects were included and they were grouped into Group 1 (n=22) as periodontally and systemically healthy subjects; Group 2 (n=22) as periodontitis subjects who were systemically healthy. Clinical parameters such as Plaque index (PI), Gingival bleeding index (GI), Probing pocket depth (PPD), Clinical Attachment Level (CAL) were recorded.

Unstimulated salivary sample was collected into a polypropylene tube from all the study participants and centrifuged at 800xg for 10 minutes. The supernatant obtained was stored at-80°C until ELISA was performed for estimating the Salivary levels of OPG and IL-35.

The mean age of the subjects in group 1& 2 was 35.05 and 48.00 respectively. The mean gender distribution among the study participants for group 1 was 50% male and female whereas for group 2 40.9% were male and 59.1% were female.

The results shows that mean age of the patients in group 2 (periodontitis) has been higher than the group 1 (healthy).

Role of IL-35 in periodontal disease is relatively an unexplored area. Kalburgi et al in 201339 evaluated the Expression of IL-35 mRNA in the Gingiva of Chronic Periodontitis and Aggressive Periodontitis Patients.

Clinical parameters such as Gingival index, OHI-S, PD, and CAL were recorded. The results of his study showed an increased tissue expression of IL-35 mRNA levels in chronic periodontitis subjects as compared to the aggressive periodontitis and was least expressed in the control group. The expression of IL-35 mRNA was stastistically significant with the following clinical parameters such as Gingival index, OHI-S, PD, and CAL.

In accordance with previous study results, the salivary levels of IL-35 was found to be elevated in periodontitis patients when compared with the healthy patients. A positive correlation of salivary IL-35 (13.519±2.020) was obtained with the clinical parameters such as Plaque index (PI) (1.575±0.359), Gingival bleeding index (GBI) (70.741±6.670), Probing depth (PD) (3.222±0.855), Clinical Attachment level (CAL) (4.142±0.866).

Thakare S Kaustubh et al in 201744 investigated the expression of the levels of IL-35 in gingival crevicular fluid (GCF) in patients with chronic gingivitis and chronic periodontitis. Clinical measurements like probing pocket depth, bleeding on probing, papillary bleeding index, and modified plaque index were recorded. The results showed that Clinical parameters were significantly higher in the chronic periodontitis group than the chronic gingivitis group. Whereas IL-35 levels in GCF were significantly higher in the chronic gingivitis group than the chronic periodontitis group. In contrast to our study, the clinical parameters were in accordance with the above study whereas the levels of IL-35 was in disagreement with the results of our study.

Saba Asif et al in 202250 investigated the Salivary RANKL and OPG levels in Periodontitis Patients. Periodontal parameters such as periodontal pocket depth (PPD), clinical attachment level (CAL), plaque score (PS), and gingival bleeding index (GBI) were recorded. The mean salivary RANKL and OPG was 0.23 ± 0.07 ng/mL and 1.78 ± 0.70 ng/mL respectively in moderate to severe periodontitis. The results of this aforementioned study was in accordance with the results of our study.

A positive correlation of salivary OPG (3.327±0.437) was obtained with the clinical parameters such as Plaque index (PI) (1.575±0.359), Gingival bleeding index (GBI) (70.741±6.670), Probing depth (PD) (3.222±0.855), Clinical Attachment level (CAL) (4.142±0.866).

It has been reported that Tregs cells act as a negative regulators of immune response through the suppression of pro-inflammatory cytokines production. OPG in turn regulates osteoclastic activity by covalently binding with RANKL thereby inhibiting the interaction of RANKL with RANK.

In our study, Salivary IL-35 and OPG levels were found to be elevated in periodontitis patients when compared with healthy subjects suggesting that both IL-35 and OPG acts as a regulators of immune system thereby showing increased expression in periodontal disease.

So, the results of our present study revealed a predictable association between the salivary levels of IL-35 and OPG with the disease severity. Stepwise multiple regression analysis showed a significant beta coefficient value for every 1mm of CAL even after the adjustments done for age, gender. This suggests that the variability salivary IL-35 and OPG will be able to explain by CAL by 25% and 31% suggesting that IL-35 and OPG can be considered as a predictable biomarker for periodontal disease.

Within the limitations of our present study,

- The study group included the subjects from out-patients of KSR dental college, Tiruchengode. A diverse range of patients from different places might possess a better representation of the sample.
- It is essential to increase the sample size
- The therapeutic effects of periodontal treatment can be correlated with the salivary levels of OPG and IL-35.
- The limitations of our cross-sectional study can be over-come by conducting series of longitudinal studies.

5. Conclusion

Salivary levels of IL-35 and OPG was statistically significant at p=0.003 and 0.004 in group II. The levels of salivary IL-35 and OPG was found to be increased in periodontitis group when compared with healthy individuals which indicates the prevalence of inflammation. Thus in the future, further longitudinal studies with therapeutic intervention need to be conducted to interpret IL-35 and OPG related protein as a prognostic biomarker in inflammatory periodontal disease.

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