ResearchArticle

ESTIMATION OF SALIVARY LEVELS OF TOLL-LIKE RECEPTOR 4 IN PATIENTS WITH CHRONIC GINGIVITIS AND CHRONIC PERIODONTITIS

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ARTICLE INFO	ABSTRACT
	Background: Toll-like receptor 4 (TLR4), the most extensively studied Pattern recognition receptor plays a vital role in maintaining periodontal health and hence overexaggerted or chronic TLR4 signaling during inflammation could lead to the destruction of periodontal tissues. Because of the potential association between periodontal disease and host immune response, the aim of the present study was to analyse the salivary levels of TLR4 in patients with chronic periodontitis, chronic gingivitis compared to periodontally healthy individuals. **Materials and methods:** 14 patients with chronic periodontitis, 14 patients with chronic gingivitis and 14 periodontally healthy subjects were selected and the clinical parameters such as plaque index, gingival index, probing pocket depth and clinical attachment level were recorded. TLR4 levels in the saliva of 42 individuals were assessed by Enzyme linked immunosorbant assay (ELISA). **Results:** The healthy group exhibited significantly lower values in all clinical measurements (P <0.001) compared to other groups. Salivary TLR4 levels were found to be higher in patients with chronic periodontitis but the difference in levels was not statistically significant compared to other groups. **Conclusion:** TLR4 expression was demonstrated in saliva of all the three groups with an increased level in the chronic periodontitis group compared to others but the difference in levels were not statistically significant. Further studies are expected in the future to draw definitive conclusions regarding the role of TLR4 in periodontal diseases and diagnostic usefulness of TLR4 in progression of periodontitis.

INTRODUCTION

Periodontal disease is an all encompassing term relating to the inflammatory disorders of the periodontium which includes gingivitis and periodontitis, the two most common diseases affecting the periodontium that comprises gingiva, periodontal ligament, alveolar bone and cementum.[1,2] Periodontal pathogens in the dental biofilm is the major etiological factor in the initiation of periodontal diseases, the fundamental mechanisms that lead to the development of periodontitis are closely related to the dynamics of the host immune and inflammatory responses to periodontal pathogens present in the dental biofilm which plays a major role in determining the extent and severity of tissue destruction. Differential host responses are thought to contribute to various susceptibilities that play an important role in determining the progression of inflammatory lesion.[3]

Recently, Toll-like receptors (TLRs), a subgroup of the signaling family of pattern recognition receptors (PRRs), that sense the pathogen associated molecular patterns (PAMPs) were identified in the periodontal tissues.[4,5] Bacterial cell components can stimulate host cells via TLRs to produce proinflammatory cytokines, such as interleukin-1beta (IL-1β) and tumor necrosis factor-α (TNF-α). Thus, TLRs mediate the first line of defense against infections and also provide secondary stimulus to the adaptive immune response.[5,6] Several studies have highlighted the role of TLRs in the initiation and progression of periodontal inflammation. TLRs form an important and potentially controllable checkpoint for a limited number of PAMPs derived from a large number of different bacterial species.[6] TLR4, the most extensively studied member of TLR family, is a principal signaling receptor for bacterial lipopolysaccharide (LPS) which is important in the activation of the innate immune system.[7,8,9]

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TLR4 in human gingival epithelial cells and gingival tissues have been investigated in previous studies and elevated expressions of TLR4 have been suggested as an important feature of chronic periodontitis.[10-15] However, there are relatively very few studies that have analysed the salivary TLR4 levels in patients with periodontal diseases.[10,16] saliva has become an emerging tool for the diagnostic assessment of various oral and systemic diseases, particularly periodontal diseases.[17,19] Because of the association of gingivitis with periodontitis and the unspecified biologic processes that contribute to the transition between these two inflammatory conditions, early diagnostic aids at the biochemical level are preferable.[18]

Little is known about the levels of TLR4 in saliva of patients with periodontal diseases. Therefore, this study was undertaken to investigate the corresponding levels of TLR4 in saliva of patients with chronic gingivitis, chronic periodontitis compared with periodontally healthy subjects.

MATERIALS AND METHODS

Study population

A total of 42 patients were recruited from the outpatient ward, Department of Periodontology, K.S.R Institute of Dental Science and Research, Tiruchengode, Namakkal district, Tamil Nadu. The study protocol was analyzed and approved by the Institutional Ethical Review Board. The procedures, possible risks/discomforts and benefits were fully explained to the participants. Written and verbal informed consent was obtained from the subjects participating in the study.The study included a total of 42 male and female subjects of age between 18-50 years and were grouped as follows: Group I with 14 chronic periodontitis patients with presence of more than 30% of sites with clinical attachment loss ≥3mm and probing depth ≥4mm and radiographic evidence of alveolar bone loss on atleast two teeth per quadrant excluding the third molars, Group II - 14 chronic gingivitis patients with no sites of probing depth ≥4mm or clinical attachment loss ≥1mm and bleeding on probing at > 20% of sites and Group III with 14 periodontally healthy subjects wih no sites with probing depth ≥4mm or clinical attachment loss ≥1mm and bleeding on probing at <20% of sites.Smokers and pan chewers, pregnant and lactating women, patientswho have undergone periodontal treatment within a period of 1 year, patients who had used systemic antibiotics within the last 6 months, patients with systemic diseases were excluded.

Saliva sampling
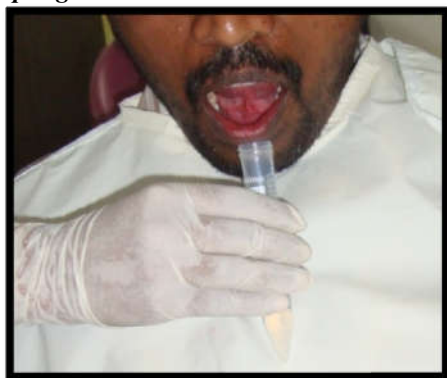
Saliva collection by passive drooling method

Saliva samples were collected, processed and stored from all the patients who were selected based on the above said inclusion and exclusion criteria. Study participants were instructed to allow saliva to pool in the mouth. With head tilted forward, research participants should drool down and collect 1 ml saliva in the polypropylene tubes before clinical measurements and any periodontal intervention in the morning after an overnight fast. The collected 1 ml unstimulated whole saliva samples by passive drooling method were clarified by centrifugation (800 g) for 10 minutes at 4ºC and aliquoted into 500 µl amounts with water. The samples were immediately frozen and stored at -40ºC until the sample collection period was completed and thawed immediately before assays.[10,16]

Clinical Measurements

Baseline clinical examination was performed for all the individuals and the clinical parameters were recorded. Clinical parameters like Plaque Index - Loe's modification (1967)[19], Gingival Index - Loe's modification (1967)[19], Probing pocket depth (PPD)[3,] Clinical attachment level (CAL)[3] recorded.

Measurement of TLR2 and TLR4 in Saliva Samples

The TLR4 levels in the saliva samples were measured using Enzyme Linked Immunosorbent Assay (ELISA). The assay employed the quantitative Sandwich ELISA technique. The RayBio Human TLR4 ELISA kit was employed to analyse the salivary TLR4 levels following the Manufacturers' guidelines.

Elisa reader and Elisa plate

Statistical Analyses

TLR4 in saliva has been assessed using Q-Q plot and kolmogorov-smirnov test and non-parametric kruskalwallis test has been used to compare the three groups. If the results are significant, Post-hoc (Multiple comparison) test for Kruskalwallis test has been applied. To assess the relationship between the clinical parameters and TLR4 values, spearman's rank correlation test has been used. SPSS software version 15 has been used for the statistical calculations.

RESULTS

Clinical Analyses

Clinical variables and mean values of clinical measurements are outlined in Table 1.The significant p-value reveals that the mean age of the three groups has been statistically different. To know which of the groups are statistically different, multiple comparison test for Kruskalwallis test has been applied. The

result indicates that the age of periodontitis patients has been higher than gingivitis and healthy subjects and further the mean age of gingivitis and healthy subjects has been similar. The non-significant p-value infers that sex wise distribution has been similar for the three groups (Table.2).

Table 4 Spearman's rank correlation coefficient between selected clinical parameters and salivaryTLR4 levels

| Variables	alivary TLR4
Age	.136
PI	.089
GI	.100
PPD	.102

Table 1 Clinical Periodontal Measurement in study groups

Clinical variables	Periodontitis (Mean)	Gingivitis (Mean)	Healthy (Mean)	Kruskal-wallis test value	P-value	Multiple comparison test result for Kruskalwallis test
Age(years)	37.21	21.71	21.85	25.267	<0.001	Periodontitis >Gingivitis, Healthy
Plaque index	1.77	1.30	0.0079	31.303	<0.001	Periodontitis >Gingivitis >Healthy
Gingival index	2.03	1.73	0.10	35.102	<0.001	Periodontitis > Gingivitis >Healthy
PPD	3. 86	2.13	1.89	31.545	<0.001	Periodontitis >Gingivitis >Healthy
CAL	3.44	-----	------	------	------	------

Table 2 Sex wise distribution of the subjects by group wise

Gender		Periodontitis	Gingivitis	Healthy	Chi-square test value	P-value
Male	No.	6	3	9	5.250	0.072
	%	42.9	21.4	64.3		
Female	No.	8	11	5		
	%	57.1	78.6	35.7		

The siginificant p-value of the Kruskalwallis test for plaque index infers that plaque level has been different for the three groups of respondents. Multiple comparison test for Kruskalwallis test indicates that all the three groups are statistically different and patients with chronic periodontitis have higher plaque index than gingivitis and healthy subjects.
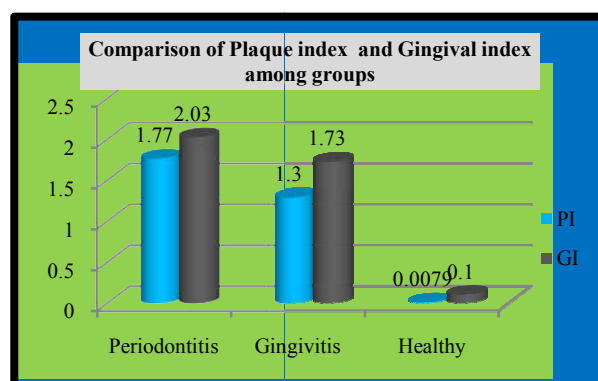

**Graph 1** Comparison of plaque and gingival index among groups

Table 3 Descriptive statistics of salivary TLR4 by group wise

Descriptive statistics	Periodontitis	Gingivitis	Healthy	Kruskal-wallis test value	P-value	Multiple comparison test result for Kruskal-wallis test
Mean	2.99	1.38	1.06	.994	0.608	--------
SD	4.10	1.67	3.51			
Minimum	-.390	-.870	-7.290			
Maximum	14.800	4.710	7.460			

Further gingivitis group has higher plaque index than healthy subjects.The siginificant p-value of the gingival index infers that gingival inflammation level has been different for the three groups of respondents. To get more details, multiple comparison test for Kruskalwallis test has been applied. The result indicates that all the three groups are statistically different. The periodontitis respondents are having higher gingival index than gingivitis and healthy subjects. Further gingivitis group has higher gingival index than healthy subjects. The significant p-value of the Kruskalwallis test infers that PPD has been different for the three groups. Further, the multiple comparison test result indicates that the periodontitis patients are having higher PPD than gingivitis and healthy subjects and also gingivitis are having higher PPD than healthy subjects as expected. The main objective of the study is to find out whether the TLR4 level has been different for the periodontitis patients compared to other groups. The average TLR4 level for the periodontitis group has been 2.99, the mean TLR4 level in saliva for gingivitis group has been 1.38 and the TLR4 level in saliva for healthy respondents was 1.06.
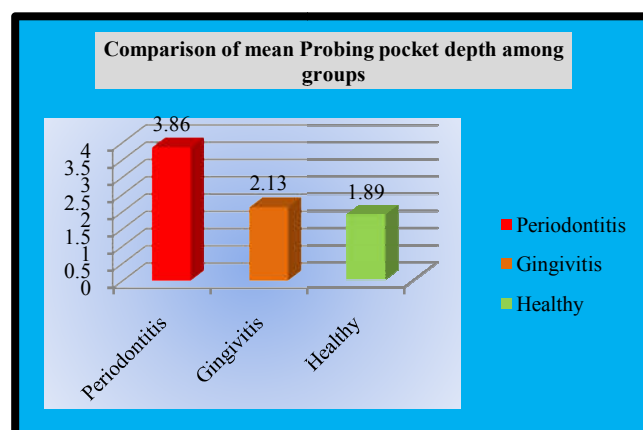
Graph 2 Comparison of mean PPD among groups
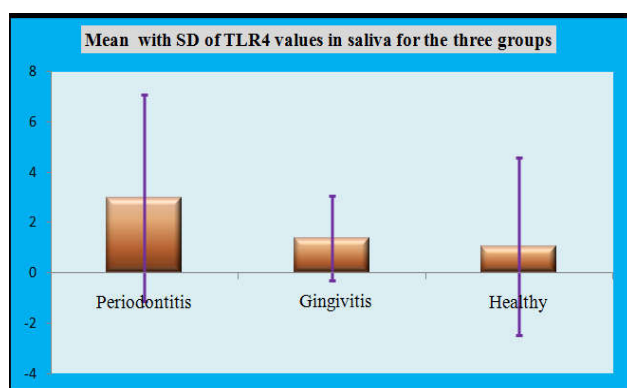
Graph 3 Mean with SD of TLR4 values in saliva for the three groups

DISCUSSION

A complex interplay between the bacterial pathogens in the dental plaque biofilm and the host immune inflammatory events plays a critical role in the development of periodontal diseases.[3] Human gingiva is exposed to a broad range of gram positive and gram negative bacteria. A change in the subgingival ecology by microbial succession from predominantly gram positive to gram negative rods in the biofilm is associated with transition from health to disease in the periodontium.[15] Specific gram negative anaerobic bacteria in subgingival pockets and their products are linked with initiation and exacerbation of periodontitis.[14,15] The host's immunoinflammatory response is cardinal in determining individuals susceptibility to periodontitisand TLRs plays a central role in innate immunity.[3] TLR4, the most extensively studied PRR of the TLR superfamily which is also described as immunity's eye or bug detectors has been identified as principal signaling receptor for gram negative bacterial cell wall components and is essential in maintaining periodontal health. TLRs have been widely implicated in periodontal diseases especially in chronic periodontitis.[21-23] However, overexaggerated or chronic TLR4 signaling could lead to the breakdown of periodontal tissues.[8,9,24]

Previous studies have reported the expression of TLR4 in gingival tissues.[13,] Certain studies have displayed a stronger expression of TLR4 in inflamed tissues than healthy tissues.[25] No difference in TLR4 expression was demonstrated in healthy and inflamed tissues by Beklenet al (2008)[13] and lower levels of TLR4 expression was displayed in inflamed tissues by Promsudthiet al (2014).[25] Several studies also have displayedstronger relationship between TLR4 expression and inflammation severity in periodontal tissues.[11,,13,25,26] Few studies have also reported significantly elevated expression of TLR4 in the tissues of patients with chronic periodontitis compared with periodontally healthy individuals [11-15] and downregulated TLR4 expression was also displayed in chronic periodontitis patients.[27]

Buduneliet al (2011)[16] and Banuet al (2015)[10]analysed the salivary and plasma levels of TLR4 by enzyme linked immunosorbant assays and reported increased TLR4 levels in both plasma and saliva of patients with periodontitis when compared with periodontally healthy subjects.[10,22] Sarah et al (2006)[12] has reported significantly elevated TLR4 expression in tissues of patients with gingivitis compared with healthy individuals. There are no studies regarding TLR4 levels in saliva and plasma of patients with chronic gingivitis[28]and there

are relatively very few studies that have analysed the salivary and plasma levels of TLR4 in patients with periodontitis.[10,16]

Because of the potential association between periodontal disease and host immune response, the aim of the present study is to analyse and compare the salivary and plasma levels of TLR 4 in patients with chronic periodontitis, chronic gingivitis and periodontally healthy individuals by sandwich enzyme linked immunosorbant assay using Raybio human TLR4 ELISA kit. The minimum sensitivity of this kit for Human TLR4 was determined to be 0.4 ng/ml.

Unstimulatedwhole Saliva has been used as diagnostic fluid as it is a easy, non invasive, and reliable. Unstimulated saliva is preferred due to variations of analytes in stimulated saliva. The plasma levels of TLR4 have also been analysed in these groups as they will reflect the systemic status of the individual.[10,16] Dependent variables like salivary and plasma TLR4 concentrations were assessed by comparing the average sample optical density readings to the concentrations from the assay standard curve.

The mean age of the periodontitis, gingivitis and healthy patients were 37.2, 21.71 and 21.85 years respectively and the age of the periodontitis patients has been higher than gingivitis and healthy subjects. The mean age of the gingivitis and healthy subjects has been similar (Table no.1). Sex wise distribution has been similar for the periodontitis, gingivitis and healthy patients with a distribution percentage of 42.9, 21.4 and 64.3 for males and 57.1, 78.6 and 35.7 for females respectively. There was no significant difference in sex distribution between the study groups (p value -0.072) (Table 2).

The results of descriptive statistics of plaque index by groupwise had displayed mean plaque index of 1.77, 1.30 and 0.0079 in periodontitis, gingivitis and healthy groups respectively. The patients in chronic periodontitis group have higher plaque index than gingivitis and healthy subjects with a significant p value < 0.001 (Table 1). Further, Gingivitis patients have higher plaque index than healthy subjects. This is in concordance with the studies by Buduneliet al (2011)[16] and Banuet al (2015)[10] who had also demonstrated significantly higher plaque index in patients with periodontitis with p value < 0.001. The mean gingival index of the periodontitis, gingivitis and healthy groups were 2.03, 1.73 and 0.10 respectively. The patients in chronic periodontitis group have higher gingival index than gingivitis patients and healthy subjects with a significant p value < 0.001(Table no.1). Further, gingivitis patients have higher gingival index than healthy subjects.

In our study, the mean PPD of the periodontitis, gingivitis and healthy groups were 3.86, 2.13 and 1.89 respectively. The patients in chronic periodontitis group have higher PPD than gingivitis patients and healthy subjects with a significant p value < 0.001 (Table no.1). Further, gingivitis patients have higher probing pocket depth than healthy subjects. The results are in concordance with the studies by Buduneliet al (2011)[16] and Banuet al (2015).[10] The healthy group exhibited significantly lower values in all clinical measurements (P <0.001) than the gingivitis patients and also the gingivitis patients exhibited significantly lower values in all clinical measurements (P<0.001) as expected. The significant

difference in clinical measurements between chronic periodontitis and healthy groups is in accordance with the previous studies by Buduneli*et al* (2011)[16] and Banu*et al* (2015).[10]

Buduneli*et al* (2011)[16] in his cross sectional study have analysed the salivary and plasma TLR4 levels in chronic periodontitis patients and healthy subjects and concluded that inflammation increases the expression of TLRs which leads to an increased detection of TLRs in saliva and plasma.

The results of our study found that there was no significant difference in the salivary TLR4 levels among the three groups (Table 3). Salivary mean TLR4 concentrations in chronic periodontitis patients was 2.99 ng/ml which is more than the levels of chronic gingivitis group (1.38 ng/ml) and healthy group(1.06 ng/ml) (Table no.3). However there was no statistically significant difference in the salivary TLR4 levels between the groups which is not in concordance with the studies by Buduneli*et al* (2011)[16] and Banu*et al* (2015)[10]. No difference in TLR4 expression was also demonstrated in healthy and inflamed tissues byBeklen*et al*(2008)[13].Correlation analyses between the selected clinical parameters and analyte concentrations (TLR4 levels in saliva) was performed to find out the relationship between them (Table no.4) No significant correlations was demonstrated.

Differences in geography and demographics, ethnic differences, genetic factors and usage of different ELISA kits may potentially be the cause of a different result of our study. Finally, 'normal' values of salivary and plasma TLR4 in periodontal health and disease requires further investigations to define a true normal/abnormal value. The precise role of TLR4 in sensing the LPS of periodontopathogens is also not yet elucidated. Additional studies in the future will clarify the range of TLR4 concentration in biologic fluids in relation with periodontal disease.

SUMMARY AND CONCLUSION

TLR4 expression was demonstrated in saliva of all the three groups with an increased level in the chronic periodontitis group compared to others but the difference in levels were not statistically significant. Within the limitations of the study and based on the findings obtained from our study, we summarize that the salivary TLR4 levels were found to be higher in patients with chronic periodontitis but the difference in levels was not statistically significant compared to other groups.

However our understanding of the functions of TLR4 is at a very early stage and there remains many questions to be answered. Only limited studies have been conducted on salivary levels of TLR4 in patients with periodontal diseases. Further studies are expected in the future to elucidate the "normal and abnormal" values of salivary TLR4 in periodontal disease and health. Larger-scale, stringently controlled, interventional studies in different populations are required to better address this issue and to draw definitive conclusions regarding the role of TLR4 in periodontal diseases and diagnostic usefulness of TLR4 in progression of periodontitis.

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