Estimation of Toll-like receptor 9 in gingival tissues of patients with chronic periodontitis with or without hyperlipidemia and its association with the presence of Porphyromonas gingivalis

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Abstract:
Background: Recent evidence suggests the interactions between bacterial DNA and nucleic acid receptors to play a role in inflammatory tissue destruction. The current study aims to evaluate the expression of Toll-like receptor 9 (TLR9) in periodontal disease associated with or without hyperlipidemia and to associate it with the presence of Porphyromonas gingivalis. Materials and Methods: Thirty participants in the age range of 25–50 years were randomly recruited and divided into three groups, i.e., healthy (Group I), chronic periodontitis without hyperlipidemia (Group II), and chronic periodontitis with hyperlipidemia (Group III). The gingival tissue samples were analyzed for TLR9 using immunohistochemistry, and plaque samples were analyzed for P. gingivalis using polymerase chain reaction. Results: The TLR9-positive cell ratio in gingival connective tissue for Group II and Group III was 0.95 ± 0.03 and 0.94 ± 0.03, respectively, which was significantly higher than that of Group I, with P < 0.001 (0.88 ± 0.04). These groups also demonstrated significantly higher presence of P. gingivalis as compared to Group I with P < 0.001. There was a positive association between TLR9 in gingival connective tissue and presence of P. gingivalis. Conclusion: The results of this study reveal a potential role of TLR9 in chronic periodontitis, in association with P. gingivalis. Furthermore, these variables do not show an appreciable change in hyperlipidemics suggesting a weak relation between TLR9 and lipid levels. Key words: Chronic periodontitis, hyperlipidemia, immunohistochemistry, polymerase chain reaction, Porphyromonas gingivalis, Toll-like receptor 9

INTRODUCTION
Periodontitis is a multifactorial, biofilm-associated inflammatory disease of the periodontium. It represents atypical host-driven inflammatory response initiated by bacterial insult, most commonly involving Porphyromonas gingivalis. In turn, the activated immune system induces a disproportionate host response, eventually causing breakdown of supporting tooth structures. Thus, the host defense system acts against infectious agents initially by inducing innate responses through pattern recognition receptors (PRRs). These receptors are mainly involved in recognizing highly conserved structures on the surface of the bacteria, called the pathogen-associated molecular patterns.

It has now clearly been established that innate immune responses are chiefly brought about through crucial PRRs called Toll-like receptors (TLRs). On microbial invasion, these receptors through the intracellular signaling pathways induce innate immune responses critical for the induction of adaptive immunity. Numerous reports have recently postulated nucleic acid sensing to play a crucial role in these responses. This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms. For reprints contact: reprints@medknow.com

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significant role in inflammation-induced tissue destruction seen in various pathological conditions also comprising periodontal disease.\cite{14,18}

Nucleic acid sensing involves TLR9, which is a major receptor for bacterial DNA containing unmethylated cytosine–phosphate–guanine motifs.\cite{19} Recent data suggest TLR9 to be one of the most upregulated PRRs in chronic periodontitis.\cite{20} TLR9 is unique since it can communicate with other TLRs such as TLR2 and TLR4. Hence, the DNA of a major periodontopathic pathogen, \textit{P. gingivalis}, is proposed to contribute to its virulence in periodontitis through expression of inflammatory cytokines through the TLR9 signaling pathway.\cite{21,14,15} Moreover, it is due to these very cytokines and mediators that the periodontium acts as a potential reservoir for the rest of the body.

Interestingly, such interactions are also proposed to play a significant role in inflammation-induced tissue destruction in a myriad of diseases, including sepsis,\cite{22} systemic lupus erythematosus,\cite{23} rheumatoid arthritis,\cite{24} and periodontal disease. In fact, they may be responsible for a two-way relationship seen between conditions such as hyperlipidemia and periodontal disease.\cite{16,17} This relation seems probable since these diseases share similar inflammatory responses to periodontal pathogens such as \textit{P. gingivalis}. Moreover, the inflammatory mediators thus produced are said to play a dual role by altering lipid metabolism\cite{16} and promoting the periodontal tissue breakdown.\cite{17}

Thus, given the current level of evidence, it becomes imperative to find the causal link and further examine the relationship between these diseases. As a recent article has indicated a role of TLR9 in hyperlipidemia,\cite{18} this article intends to analyze the levels of TLR9 and its association with \textit{P. gingivalis}, as an early indicator of inflammation in patients with chronic periodontitis with or without hyperlipidemia which is further compared with periodontally healthy controls.

**MATERIALS AND METHODS**

**Study population**

A cross-sectional study design was proposed for the research. The study was carried out from November 2014 to September 2015. The research protocol was approved by the Institutional Ethical Committee and Review Board (BDC/Exam/393/2013-14), and written consent was obtained from each patient. The sample size was estimated by fixing the probability of Type I error (α) at 5% and Type II error (β) at 20%. Thus, the power of the study was 80%. Effect size was set at 0.9. The data required for sample size were obtained from previously published article.\cite{26}

A total of 30 participants (15 females and 15 males; between the age group of 25–50 years) were selected for the study and divided into three groups, i.e., healthy (Group I), chronic periodontitis without hyperlipidemia (Group II), and chronic periodontitis with hyperlipidemia (Group III). Each group consisted of 10 patients. Clinical measurements included the presence of plaque, periodontal pocket depth, bleeding on probing, and clinical attachment level.

In this study, inclusion criteria comprised participants having at least twenty natural teeth. Participants exhibiting at least eight periodontal pockets of ≥3 mm, ≥80% of the proximal sites with bleeding on probing, clinical attachment loss of ≥3 mm and ≥50%, and alveolar bone loss in ≥2 quadrants were diagnosed with chronic periodontitis. In addition, assessment of hyperlipidemia was done as follows: for triglyceride levels ≥150 mg/dL, low-density lipoprotein levels ≥130 mg/dL, or HDL levels ≤40 mg/dL.

A participant was assigned to the periodontally healthy reference group in the absence of periodontal pockets of >3 mm at 90% of the measured sites with no radiographic sign of alveolar bone loss. Individuals having a current or previous history of systemic disorder such as cardiovascular disease or diabetes mellitus or those having undergone recent interventions in the form of periodontal therapy were excluded from the study. In addition, history of smoking, pregnancy, or any form of hormonal/antibiotic therapy necessitated exclusion from the research.

**Periodontal evaluation**

The periodontal evaluation was performed by a single-blinded, calibrated, and well-trained examiner. The pocket depth and clinical attachment level were measured using William’s graduated probe, on six sites per tooth, and the mean was calculated. Bleeding on probing and presence of plaque were marked as dichotomous variables.

**Collection of samples**

The subgingival plaque samples were obtained before the biopsy, from the area of deepest probing depth adjacent to the location of biopsy with sterile Gracey curettes. Samples were then placed in Eppendorf sterile tubes containing 1 ml of reduced transport fluid media.\cite{19}

Gingival tissue samples were collected from the ten periodontally healthy controls during tooth extractions. These extractions were indicated for disimpaction or orthodontic treatment. In patients with chronic periodontitis (Group II and Group III participants), gingival samples were collected during modified Widman flap procedures after scaling and root planing. Here, the marginal gingiva obtained was carefully released using a curette and immediately stored in 10% buffered formalin solution.

**Tissue processing**

Gingival tissue sections of 3.5–4 μm thickness were deparaffinized. Heat-induced epitope retrieval method using trisodium citrate buffer in a water bath set to 96°C for 10 min was served to break the methylene bridges and expose the antigenic sites for immunohistochemical analysis. The resultant sections were washed in phosphate-buffered saline followed by treatment with 3% PolyExcel H$_2$O, block. Sections were then incubated at 4°C overnight with a primary antibody, rabbit antihuman TLR9 (rabbit IgG, Cell Signaling Technology, Massachusetts), following which incubation with target binder and secondary antibody (PolyExcel PolyHRP, PathnSitu biotechnologies, Bengaluru) was done. Signal was developed with PolyExcel Stunn DAB chromogen (PathnSitu biotechnologies, Bengaluru). Hematoxylin was used as a counterstain. The
slides were lastly dehydrated and finally mounted with (distyrene, plasticizer, and xylene).\[20\]

**DNA extraction and polymerase chain reaction**

Genomic DNA was obtained by the centrifugation of plaque sample with TE buffer and lysis buffer I followed by lysis buffer II. DNA was amplified in a series of forty cycles, each cycle comprising denaturation, annealing, and extension phases in the thermal cycler (Applied Biosystems, USA).\[21\] The following sets of DNA primers were used,

\[\begin{align*}
P. gingivalis & \text{ (forward and reverse primer):} \\
1 & \text{AGG CAG CTT GCC ATA CTG CG} \\
2 & \text{ACT GTT AGC AAC TAC CGA TGT.}
\end{align*}\]

Horizontal gel electrophoresis system was employed for detection of DNA components followed by visualization under ultraviolet light (transilluminator). The horizontal gel electrophoresis system required pre-mixing of the DNA sample to a dye (bromophenol blue). The basepair (bp) size of fragment of the ladder used was previously known, against which the size of experimental band (fragment) was compared\[21\] for the presence of \( P. gingivalis.\)

**Evaluation of immunostaining**

The expression of TLR9 in epithelium was evaluated by analyzing three layers: basal, intermediate, and superficial. These results were evaluated and graded by two independent researchers who were blinded to the disease status of the individuals. Immunohistochemical staining was graded according to percentage of positive cells and staining intensity. For number of positive cells, scores were given as 0 – ≤10% positive, 1 – 11%–25% positive, 2 – 26%–50% positive, 3 – 51%–75% positive, and 4 – ≥76% positive. Staining intensity was given a score according to the following criteria: light yellow was scored 1, yellow color was scored 2, and brown was given a score of 3. The above-mentioned two scores were multiplied to obtain final scores which ranged between 0 and 12. The median score was calculated. Scores ≥6 were demarcated as high expression group. Moreover, those with scores less than median were defined as low expression group.\[20\]

The immune expression of TLR9 in gingival connective tissue was evaluated in three areas selected subepithelially. The ratio of TLR9-positive cells to total number of cells was calculated in each of the 250 \( \mu \text{m}^2 \) field, and a mean ratio of the values from three different areas was obtained.

### Table 1: Intergroup comparison of the expression levels of Toll-like receptor 9 in epithelium and connective tissue

|                     | Number of samples | Mean±SD | Group I versus Group II | Group I versus Group III | Group II versus Group III |
|---------------------|-------------------|---------|-------------------------|--------------------------|--------------------------|
| TLR9 expression     |                   |         |                         |                          |                          |
| in gingival epithelium | Group I           | 10      | 7.40±3.16               | 0.64 (NS)                | 0.28 (NS)                | 0.61 (NS)                |
|                     | Group II          | 10      | 8.00±3.71               |                          |                          |
|                     | Group III         | 10      | 9.00±2.44               |                          |                          |
| TLR9 expression     |                   |         |                         |                          |                          |
| in connective tissue | Group I           | 10      | 0.88±0.04               | 0.001**                  | 0.001**                  | 0.56 (NS)                |
|                     | Group II          | 10      | 0.94±0.02               |                          |                          |
|                     | Group III         | 10      | 0.94±0.02               |                          |                          |

\( P \) – Probability value (\( P \) value)

**Statistical analysis**

The statistical analysis of these values was performed using SPSS Statistics version 20 software (IBM corporation, Armonk, New York, United states). TLR9 levels in gingival epithelium and connective tissue and clinical parameters such as probing depth and CAL were compared among the three study groups using Kruskal–Wallis test followed by pairwise comparison using Mann–Whitney U-test. Fisher’s exact value test was done for the intergroup comparisons for parameters having a dichotomous outcome such as presence of \( P. gingivalis \) and presence of plaque and bleeding on probing. Mann–Whitney U independent sample test was used to test association between presence and absence of \( P. gingivalis \) with TLR9 levels in gingiva.

\( P \leq 0.05 \) was considered statistically significant, \( P < 0.001 \) was considered statistically highly significant, and \( P > 0.05 \) was considered statistically nonsignificant.

**RESULTS**

The TLR9 expression in epithelium did not vary significantly among the three groups with \( P > 0.05 \) [Table 1 and Figure 1]. Thus, comparable TLR9 activity is demonstrated in the epithelium of all groups [Figure 1a–c]. The mean difference in the expression levels of TLR9 in the gingival
connective tissue between Group I and Group II and Group I and Group III was highly significant with $P < 0.001$ [Figure 1d,e and f]. However, the mean difference was statistically nonsignificant for Group II and Group III with $P > 0.05$ [Table 1]. Hence, comparable staining for TLR9 was present in the connective tissue of Group II and III [Figure 1e and f].

A significantly increased number of participants in the periodontitis groups (Group II and Group III) showed the presence of *P. gingivalis* with $P < 0.05$ [Table 2].

There was no association between TLR9 levels in the epithelium and the presence of *P. gingivalis* with $P > 0.05$ [Table 3]. However, there was a positive association between TLR9 in gingival connective tissue and presence of *P. gingivalis* with $P < 0.05$, except in Group III [Table 3].

A highly significant increase in the presence of plaque and bleeding on probing was noted in the periodontitis groups with $P < 0.001$ [Table 4]. Clinical attachment levels and probing depth showed a nonsignificant difference in values between Group II and Group III. The lipid levels were greater for Group III as compared to the other groups. Furthermore, healthy individuals belonging to Group I were a younger cohort in comparison to Group II and Group III [Table 4].

DISCUSSION

Chronic periodontitis is an infectious disease of the supporting tooth structures or the periodontium. The immune response mediated through TLRs is a major contributory factor in tissue destruction of the periodontium.[25] TLRs yield an immediate response against microbial inflammation. In doing so, they limit the very progression of infectious insult. Hence, the gingival epithelium being directly in contact with oral environment is equipped with at least some of the recognized TLRs to maintain the normal microbe-host balance.[25] Of the 11 TLRs identified, TLR9 is a major intracellular endosomal receptor for hypomethylated or unmethylated cytosine–phosphate–guanine motifs within bacterial and viral DNA.[25] The interactions between DNA and TLR9 can be appreciated in host response-driven pathologies similar to periodontal disease.[25]

After an extensive literature search and a paucity of information regarding the same, we conducted a study to analyze the levels of TLR9 and its microbial response to *P. gingivalis*, as an early indicator of inflammation in chronic periodontitis with or without hyperlipidemia which was further compared with periodontally healthy controls.

In our study, when the different groups were compared for gingival TLR9 levels, the mean values of the receptor in the gingival epithelium were not significantly different for the three groups with $P = 0.53$. We partly attribute this result to

Table 2: Intergroup comparisons of the dichotomous variables (*Porphyromonas gingivalis*) using Fisher’s exact value test

| Presence or absence | Groups (%) | Total (%) | $P^*$ |
|---------------------|------------|-----------|-------|
|                     | I          | II        | III   |       |
| *Porphyromonas gingivalis* | 0          | 7 (70.0)  | 3 (30.0) | 1 (10.0) | 11 (36.7) | 0.027* |
|                     | 1          | 3 (30.0)  | 7 (70.0) | 9 (90.0) | 19 (63.3) |

*Fisher’s exact test, *$P*$<0.05 statistically significant, $P>0.05$ NS. NS – Nonsignificant; $P$ – Probability value ($P$ value)

Table 3: Association between *Porphyromonas gingivalis* and Toll-like receptor 9 expression in gingiva

| Presence of *Porphyromonas gingivalis* | Number of samples | Mean±SD | Association ($P^*$) |
|---------------------------------------|-------------------|---------|---------------------|
| TLR9 in epithelium                    |                   |         |                     |
| Group I                               | 0                  | 7       | 7.42±3.50           | 0.33 (NS) |
|                                       | 1                  | 3       | 7.33±2.88           |          |
| Group II                              | 0                  | 3       | 6.33±2.51           | 0.63 (NS) |
|                                       | 1                  | 7       | 8.71±4.07           |          |
| Group III                             | 0                  | 1       | 12.00               | 0.19 (NS) |
|                                       | 1                  | 9       | 8.66±2.34           |          |
| TLR9 in connective tissue             |                   |         |                     |
| Group I                               | 0                  | 7       | 0.87±0.05           | 0.04*    |
|                                       | 1                  | 3       | 0.90±0.02           |          |
| Group II                              | 0                  | 3       | 0.95±0.01           | 0.02*    |
|                                       | 1                  | 7       | 0.94±0.03           |          |
| Group III                             | 0                  | 1       | 0.90                | 0.11 (NS) |
|                                       | 1                  | 9       | 0.94±0.02           |          |

*Mann-Whitney U-test, *$P*$<0.05 statistically significant, $P>0.05$ NS. TLR9 – Toll-like receptor 9; NS – Nonsignificant; SD – Standard deviation; $P$ – Probability value ($P$ value)

Table 4: Intergroup comparison of various age, sex, triglyceride levels, and the clinical parameters

| Clinical parameters | Number of samples | Triglyceride levels (mean) | Number of males/females | Mean age±SD | Mean PD±SD | Mean CAL±SD | Presence of plaque (%) | Presence of BOP (%) |
|---------------------|-------------------|---------------------------|-------------------------|-------------|------------|-------------|------------------------|---------------------|
| Group I             | 10                | 101.8                     | 4/6                     | 31.27±5.14  | 3.00±0     | 1 (10.0)    | 3 (30)                 |
| Group II            | 10                | 103.9                     | 5/5                     | 36.40±5.85  | 6.30±1.49  | 8 (80.0)    | 9 (90)                 |
| Group III           | 10                | 194.9                     | 6/4                     | 36.78±6.30  | 5.70±0.82  | 10 (100)    | 10 (100)               |

SD – Standard deviation; CAL – Clinical attachment loss; BOP – Bleeding on probing; PD – Probing depth
the constitutive expression of TLR9 in healthy controls. As previously discussed, TLR9 mainly attempts to defend the host against microbial attack and limits the progress of insult. Since healthy gingiva also experiences some amount of microbial insult, a nominal value of these receptors is normally expected in this group.

A negative result is also seen due to initial therapy being done for these patients before the collection of biopsy sample, which decreases the inflammation and hence the receptor levels in Group II and III. This explanation is substantiated by a previous study\textsuperscript{[24]} where the author further argues that the profile of innate receptor expression at treated sites may not be a true reflection of disease. This, particularly in the epithelium, masks the perceived intensified response in the chronic periodontitis tissues.

In the current study, when the three groups were compared for TLR9 in the connective tissue, a highly significant difference was seen in the values with $P < 0.001$. Previously, authors\textsuperscript{[8,24]} have similarly reported a difference in the TLR9 receptor expression between chronic periodontitis and healthy tissues as mainly localized to the connective tissue. This was postulated to be perhaps because nucleic acid receptors are mainly expressed in gingival fibroblasts, monocytes/macrophages, and dendritic cells found chiefly in connective tissue.

No significant difference in the levels of TLR9 was found in the gingival epithelium or connective tissue of patients with chronic periodontitis and hyperlipidemia when compared to the patients having chronic periodontitis only. The findings of the current study are in consensus with previous studies\textsuperscript{[25]} which demonstrated that of 9 TLRs, the expression of only TLR1, TLR2, and TLR4 was markedly enhanced in human atherosclerotic plaques. This perhaps indicates a lesser role of TLR9 in causing hyperlipidemia. However, given the drawback of a small sample size here, studies involving larger sample population need to be conducted to confirm the same.

Our study indicates a significant difference in the presence of \textit{P. gingivalis} among the different groups with $P = 0.018$. This result is in consensus with previous studies\textsuperscript{[9]} and demonstrates the role of \textit{P. gingivalis} in chronic periodontitis. Moreover, positive correlations were observed for \textit{P. gingivalis} and TLR9 ratio in connective tissue.\textsuperscript{[26]} Hence, given the current evidence, we can conclude that DNA from periodontopathogenic bacteria stimulates host response through TLR9.

Our study also demonstrates a negative correlation between TLR9 levels in gingival epithelium and \textit{P. gingivalis} with $P > 0.05$. This is again explained by the constitutive expression of TLR9 in epithelium. There is a constant mild microbial insult seen even in healthy gingival tissue leading to its upregulation which is further intensified since epithelium happens to be the first-line barrier of defense in the mouth.

**CONCLUSION**

Hence, within the limitations of this clinico-biochemical and microbiological study, we observe periodontal destruction to be associated with increased TLR9 levels and increased presence of \textit{P. gingivalis} in gingiva. There also appears to be a significant correlation between the periodontopathic bacteria and the levels of TLR9. However, TLR9 has a weak role to play in hyperlipidemic patients.

Further longitudinal prospective studies involving larger sample population are needed to confirm these findings and also to better understand the correlation of \textit{P. gingivalis} with gingival levels of TLR9 in periodontal health and disease.

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**Conflicts of interest**

There are no conflicts of interest.

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