Indirect immunofluorescence technique to study expression of toll-like receptor 4 in chronic periodontitis

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ABSTRACT

Aim: The aim of this study was to analyze the expression level and localization of Toll-like receptor (TLR) 4 in gingival samples of healthy and chronic periodontitis subjects by indirect immunofluorescence technique (IFT).

Materials and Methods: In this study, gingival tissue samples were obtained from 25 healthy and 25 periodontitis individuals. The tissues were processed and the initial characterization was done by hematoxylin and eosin staining. The expression and localization of the TLR4 receptor were determined in the epithelial and connective layer cells of the gingival tissue using the indirect IFT. Immunofluorescence images were acquired and quantitative expression of TLRs was analyzed by calculating the percentage of cells showing positive results.

Results: We found that the healthy control group exhibited significantly lower values of TLR4 expression in comparison with the periodontitis patients. We also found that in patients with periodontitis the concentration of TLR4 was higher in the epithelium as compared to their expression in connective tissue cells.

Conclusions: These data suggested a definite involvement of TLR4 in initiating and progression of an inflammatory response in periodontitis.

Key words: Chronic periodontitis, gingival connective tissue, gingival epithelial cells, immunofluorescence techniques, toll-like receptor 4

Ten TLRs in humans have been described to date.[7] They are classified according to the types of ligands that they recognize. For example, TLR1 recognizes triacyl lipopeptides, TLR2 recognizes peptidoglycan, TLR3 recognizes viral double-stranded RNA, TLR4 recognizes lipopolysaccharides, TLR5 recognizes flagellin, TLR8 recognizes viral single-stranded RNA, and TLR9 recognizes bacterial DNA.[8] TLR2 and TLR4 that recognize PAMPs from bacteria are the most extensively studied members of the TLR family.[9‑12] TLR4 is a principal signaling receptor for Gram-negative bacterial lipopolysaccharides.[13‑17] Several studies have been performed in the past few years for detection of TLR4 receptors in the cells of the gingival tissue by
immunohistochemistry (IHC). However, there are not many reports on TLR4 detection using immunofluorescence techniques (IFTs). This would be the first report from India to utilize IFTs for detection of TLR4 in gingival tissue from periodontitis patients and healthy individuals.

**MATERIALS AND METHODS**

In this study, fifty subjects were selected from the population referred to dental clinics. Individuals recruited included 25 healthy subjects without periodontal disease and 25 patients with periodontal disease. In patients with chronic periodontitis, gingival samples were collected during routine periodontal operations, which include scaling and root planning. Samples from the 25 healthy controls were obtained during tooth extraction operations performed for fully impacted, retained wisdom teeth. Chronic periodontitis subjects were selected based on the criteria of the American Academy of Periodontology classification.[18]

The inclusion criteria for patients with chronic periodontitis included the presence of at least 20 natural teeth, a minimum of 6 periodontal pockets of ≥5 mm probing depths and clinical attachment loss of ≥3 mm around the affected teeth. The age range of patients with periodontitis was 20–60 years, male or female.

The inclusion criteria for healthy controls comprised the absence of periodontal diseases having at least 20 natural teeth, age range 20–60 years, male or female.

The exclusion criteria for both healthy subjects and patients with periodontitis included individuals with any systematic disease/condition, pregnant or lactating women, and individuals with a history of dental treatment or drug therapy in the past 3 months before the study.

Gingival samples were directly collected in cold 95% ethanol (Changshu Yangyuan Chemical, China). The tissues were trimmed to a thickness of 2–4 mm and left for further incubation for 18–24 h at 4°C in ethanol. Tissue samples were then processed by dehydrating in 4 changes of precooled absolute alcohol 1 h each, transferred to 3 changes of Xylene™ (Biolab Diagnostics, Mumbai, India) 1 h each at 4°C, embedded in paraffin (Fisher Scientific, Bangalore, India) for 4 consecutive baths, 2 h each at 56°C. Tissue sections of 5 µm thickness were cut on a microtome (LEICA RM2245) and mounted on 3-(aminopropyl) triethoxysilane (Sigma, Bangalore, India) coated slide.[19]

Initial characterization of tissues was done by performing hematoxylin (NICE, Kerala, India) and eosin (SDFCL, Mumbai, India) staining on all the specimens.[20]

An indirect IFTs was performed to detect TLRs on mounted sections of 5 µm thickness. Tissue sections were incubated for 45 min at room temperature with TLR4 primary antibody (Purified Anti-human, BioLegend, San Diego, USA); at least two slides per sample were tested. We used mouse monoclonal antibody against human TLR4, 1:50 dilution in sterile phosphate buffered saline (PBS). At the end of incubation, the slides were washed with PBS-T (50 ml PBS + 25 µl Tween 20® [Hi-Media, Mumbai, India]) for 5 min 2–3 times. Tissue sections were then incubated for 1 h in secondary antibody conjugated with fluorescein isothiocyanate (Goat Anti-mouse IgG, Imgenex, India), 1:200 dilution, blocked with 5% goat serum in PBS-T for 5–10 min then washed with PBS-T for 5 min 2–3 times and mounted in DPX mountant (Biolab Diagnostics, India).[21]

Immunofluorescence images were acquired using a fluorescence microscope (Olympus BX41), with photographic attachment. At least 3 representative images were captured and analyzed per slide.

**Ethics**

The study was approved by the local ethical committee at the Maratha Mandal’s N.G.H Institute of Dental Sciences and Research Centre, Belgaum, Karnataka, India. Written informed consent was obtained from all the study participants before acquiring sample tissues. The periodontal evaluation was performed by well-trained examiners.

**Statistics**

The prevalence of TLR in healthy group is 5–10% assuming maximum prevalence of 10%, to demonstrate prevalence of 50% in period group, using type I error of 0.05, type II error of 0.2 or power of 80% the sample size was calculated using the formula:

\[ n = \frac{2(Z_\alpha + Z_\beta)^2 pq}{(p_1 - p_2)^2} \]

Where \( Z_\alpha \) and \( Z_\beta \) are standard normal constants, \( p_1 \) - prevalence in healthy samples, \( p_2 \) - prevalence in periodontitis samples. \( p = \frac{p_1 + p_2}{2} \), \( q = 100 - p \), \( Z_{\alpha} = 0.05 = 1.96, Z_{\beta} = 0.2 = 0.84 \). Adding the values to the formula the sample size was found out to be 25.

The results are presented as a mean ± standard deviation, the concentration of TLR4 was compared between the chronic periodontitis and periodontally healthy groups using the \( t \)-test and the Mann–Whitney U-test and \( P < 0.05 \) was considered statistically significant.

**RESULTS**

A total of fifty gingival tissue specimens were studied, with an equal number from healthy individuals and patients with chronic periodontitis. Each tissue was studied by hematoxylin and eosin (H and E) staining for histopathological characteristics and indirect
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immunofluorescence for expression of TLR4 in epithelial cells and connective tissue cells. A semi-quantitative analysis was carried out based on the number of cells that took up the specific stain and the positivity was expressed regarding percentage.

Sections of tissues were stained with H and E to determine the histological characteristics. Healthy gingival tissues showed a typical keratinized stratified squamous epithelium with minimal inflammatory infiltrates. In patients with chronic periodontitis, keratinized stratified squamous epithelium showed changes such as edema and exocytosis mainly due to the effect of chronic inflammatory infiltrate found predominantly in lymphocytes and plasma cells [Figure 1].

TLR4 expression in healthy gingival tissues was slightly lower than in the tissues of patients with periodontitis. In patients with periodontitis TLR4 expression was slightly higher in epithelium as compared to their expression in connective tissue [Figure 1].

TLR4 expression in the epithelial cell of periodontitis patients was 57.3% (±21.16) when compared to their expression in epithelial cells of healthy individuals which was 19.4% (±8.82). This difference was significant with a $P < 0.001$ [Table 1].

TLR4 expression in the connective tissue cells of periodontitis patients was significantly higher with a positivity rate of 50.1% (±49.25) when compared to their expression in the connective cells of healthy individuals which was 15.9% (±4.31). This difference was significant with a $P < 0.001$ [Figure 2].

**DISCUSSION**

The link between periodontal disease and systemic diseases has been scientifically proven over the last two decades. The principle reason for this oralsystemic connection is the dissemination of locally produced pro-inflammatory mediators. TLRs play a crucial role in innate immunity against invading microorganisms in the oral cavity by recognizing specific patterns of microbial components, also called as PAMPs. Lipopolysaccharides are bacterial components which are detected particularly by TLR4. Thus, activating the TLR signaling pathway which triggers a multitude of antimicrobial and inflammatory responses.

Gingival epithelial cells and gingival fibroblasts express TLR4. DNA microarray analysis demonstrated that expression of TLR4 in human gingival tissue was higher in patients with periodontitis than in healthy individuals. Furthermore in a study done on a Han Chinese Population, the host genetic susceptibility to and/or severity of periodontitis was seen.

In these past few years, several workers have attempted to detect and quantify the surface expression of TLR4 in the oral tissues. Most of them have used IHC for this purpose, and only a few have resorted to IFTs. The method that has been adapted by various researchers to express the levels of TLR4 are also different: Some have considered the intensity of staining for quantifying the expression, whereas others have considered the percentage of cells showing positive results for a given location, for the purpose.

During this study, we did a survey on the work done by various researchers in evaluating the expression of TLR4 in the gingival tissues [Table 2]. We realized that quantitative

![Image](image_url)

**Figure 1:** Immunolocalization of toll-like receptor-4 in gingival tissue samples from healthy controls and patients with chronic periodontitis. Respective images of H and E stained tissue sections are shown in the upper panel.

![Image](image_url)

**Figure 2:** Analysis of expression of toll-like receptor-4 in gingival epithelial cells and gingival connective tissue cells of healthy controls and patients with chronic periodontitis.
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The result of this study showed, using fluorescent microscopy that TLR4 is expressed in the human gingival tissue. The results also confirmed and extended previous evidence, which showed the difference of expression of TLR4 in different cells of gingival tissues, of healthy individuals and periodontitis patients. It also proved that this expression is significantly exacerbated in patients with periodontitis.

Difference of expression was found in gingival tissue, TLR4 expression was significantly increased in the epithelial region than the connective tissue. The increase in expression of TLR4 in the epithelial region and the connective tissue may also be positively regulated with the severity of periodontitis, an extension of this study is required to prove this and so we are working on this study further with increased number of groups, larger sample size and with more TLR markers.

The data of this study are sufficient enough to prove the possible involvement of TLR4 in the pathogenesis of periodontitis and IFT proved to be a better, rapid, and more sensitive method than IHC for detection as well as quantification of TLR4 expression and localization. However, the set-up is comparatively expensive and needs trained personnel for evaluation of the stained slides. Similar studies with variations in groups, population and markers need to be taken up for performing a definite opinion about the periodontal health and diseases.

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Table 1: Comparison of toll-like receptors 4 in epithelium and connective tissue cells of healthy and chronic periodontitis

| TLRs   | Tissue                  | Group            | Mean±SD    | t-test     | P and Significant | Mann-Whitney U-test | P and Significant |
|--------|-------------------------|------------------|------------|------------|-------------------|---------------------|-------------------|
| TLR4   | Epithelial cells        | Healthy          | 19.4±5.82  | 8.655      | <0.001, Significant | 6.196               | <0.001, significant |
|        |                         | Chronic periodontitis | 57.3±21.16 |            |                   |                     |                   |
| Connective tissue cells | Healthy          | 15.9±4.31       |            | 8.659      | <0.001, Significant | 6.061               | <0.001, significant |
|        |                         | Chronic periodontitis | 50.1±19.25 |            |                   |                     |                   |

TLRs=Toll-like receptors, SD=Standard deviation

Table 2: Comparison of methods and evaluation of expression of toll-like receptors 4 from various studies and the present study

| Author          | Year | Method | Measured                     | TLR4 expression                      | Reference |
|-----------------|------|--------|------------------------------|--------------------------------------|-----------|
| Mori et al.     | 2003 | IHC    | Cell percentage              | Zone                                 | Zone 1    |
|                 |      |        |                              | Mild                                 | 0.12±0.43 |
|                 |      |        |                              | Moderate                             | 0.06±0.18 |
|                 |      |        |                              | Severe                               | 0.16±0.41 |
| Ren et al.      | 2004 | IHC    | Cell percentage              | Expression of LBP in human gingival tissue | Zone 2    |
|                 |      |        |                              |                                      | 0.05±0.28 |
|                 |      |        |                              |                                      | 0.12±0.39 |
|                 |      |        |                              |                                      | 0.1±0±0.33|
| Sugawara et al. | 2006 | IHC    | Intensity                    | TLR4 expression more in inflamed gingival tissues | Zone 3    |
|                 |      |        |                              |                                      | 0.14±0.41 |
|                 |      |        |                              |                                      | 0.16±0.43 |
| Uehara and Takada | 2007 | IHC    | Intensity                    | Clear expression of TLR4 in normal oral epithelium | Cell layer   |
|                 |      |        |                              |                                      | Healthy    |
|                 |      |        |                              |                                      | 56.1±4.9  |
|                 |      |        |                              |                                      | 78.1±2.1  |
|                 |      |        |                              |                                      | 77.8±3.7  |
| Beklen et al.   | 2008 | IHC    | Cell percentage              | Epithelial tissue                   | 70.0±4.8  |
|                 |      |        |                              |                                      | 82.0±2.7  |
| Rojo-Botello et al. | 2011 | IFT    | Fluorescence intensity       | Connective tissue                   | 61.1±9.6  |
|                 |      |        |                              |                                      | 70.0±4.8  |
| Present study   | 2013 | IFT    | Cell percentage              | Control group (AU)                  | 15-18     |
|                 |      |        |                              | Chronic periodontitis (AU)           | 27-30     |
|                 |      |        |                              | Control group (AU)                  | 11-14     |
|                 |      |        |                              | Chronic periodontitis (AU)           | 16-19     |
|                 |      |        |                              | Control group                        | 19.4% (±5.82) |
|                 |      |        |                              | Chronic periodontitis                | 57.3% (±21.16) |
|                 |      |        |                              | Control group                        | 15.9% (±4.31) |
|                 |      |        |                              | Chronic periodontitis                | 50.1% (±19.25) |

IFT=Immunofluorescence technique, IHC=Immunohistochemistry, TLR4=Toll-like receptor 4, LBP=Lipopolysaccharide-binding protein, AU=Arbitrary units
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Conflicts of interest
There are no conflicts of interest.

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