Research Article

JRAD Journal of Research and Advancement in Dentistry

J Res Adv Dent 2019;10:1s:16-23.

Levels of Interleukin - 21 Levels in Gingival Crevicular Fluid in Patients with Chronic and Aggressive periodontitis: A Comparative Clinico-Biochemical Study

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ABSTRACT

Background: To investigate the levels of IL-21 in gingival crevicular fluid of patients with chronic periodontitis and aggressive periodontitis.

Materials and Methods: Gingival crevicular fluid samples were collected from chronic periodontitis (n=10), aggressive periodontitis (n=10) and controls (n=10). Clinical parameters like Plaque index, Gingival index, Gingival bleeding index, probing depth and Relative attachment level were recorded at baseline. Patients with chronic periodontitis and aggressive periodontitis received non-surgical periodontal treatment (Scaling and Root planing). IL-21 was quantified through an Enzyme-linked Immunosorbent Assay. Data was expressed using the $x^{2,}$ student t and Mann- Whitney U tests.

Results: The baseline levels of IL-21 in GCF was significant in aggressive periodontitis ($p<0.05$) and highly significant in chronic periodontitis patients ($p<0.001$) when compared to controls.

Conclusion: With increase in severity of the periodontal destruction in chronic periodontitis a substantial increase in the concentration of IL-21 was observed ($p<0.05$) and in aggressive periodontitis there was significant increase but not to the degree compared with destruction. The level of IL-21 were well correlated with clinical parameters of periodontal tissue destruction in chronic periodontitis. This shows the host susceptibility factors for aggressive periodontitis, including family aggregation, single nucleotide polymorphisms, polymorphonuclear neutrophils, antibodies to bacteria, smoking, stress.

Keywords: Aggressive periodontitis, chronic periodontitis, cytokine, gingival crevicular fluid.

INTRODUCTION

Periodontal disease is a poly-microbial disease that affects tooth-supporting tissues. Integral part of periodontal disease involves destruction of periodontal tissues that results from stimulation of the bacterial challenge to host immune-inflammatory response[1]. At the "International Workshop for a Classification of Periodontal Diseases and Conditions" in 1999, the classification of periodontal diseases was revised[2]. The various types of periodontitis were divided into three main categories (chronic, aggressive, and necrotizing periodontitis) as well as into a periodontal a manifestation of systemic diseases. Aggressive periodontitis is characterized by a rapid loss of

Received: May. 12, 2019**: Accepted**: July. 17, 2019
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pISSN 2278-0076
eISSN 2321-9270

clinical attachment and alveolar bone and normally affects young adults[3-5]. In aggressive periodontitis subjects is inconsistent with the severity and progression of the periodontal destruction, elevated proportions of Aggregatibacter actinomycetemcomitans or Porphyromonas gingivalis, hyper-responsive macrophage phenotypes and phagocyte abnormalities.[4] These infections are subdivided into localized and generalized cases, according to the extent of the periodontal destruction. Diagnosis of aggressive periodontitis requires exclusion of the presence of systemic diseases that may severely impair host defenses and lead to premature tooth loss[4,5]. As a result of cellular activation, inflammatory mediators include cytokines, chemokines etc. collectively contribute to tissue destruction and bone resorption[6]. Cytokines can be grouped as Th1, Th2, Th17 and T regulatory (Treg) based on their expression pattern and effects on target cells or tissues[7]. Classically, periodontal disease has been explained by Th1/Th2 pathway[8]. Th1 cells are more frequently detected in the early stage of periodontal lesions suggesting that Th1 cells are associated with a protective response against bacterial infection whereas Th2 cells predominate in the later and advanced period of the disease, having a role in the destruction and progression of periodontal lesions[9]. Alternative type of host response to pathogenic bacteria has been described by the IL-23/IL-17 pathway[10]. This alternative pathway occurs when bacteria induce synthesis of IL-23/17 rather than IL-12, which plays a pivotal role in initiation and maintenance of inflammatory response. IL-23/17 pathway as compared to classical Th1/Th2 pathway is a much fine tuned cellular immune response that has a multitude of functions that bridges the innate and adaptive arm of the immune response. Th17 cytokines (IL-17, IL-21, IL-22, IL-6, TNF-a, etc.) participate in periodontal disease, but whether their dominant role is host-protective or destructive is questionable . IL-21 levels have not been elucidated at different inflammatory status in periodontitis.

IL-21 is a type-I cytokine, structurally it appears similar to IL-2, IL-4, and IL-15 proteins. IL-21 is principally composed by activated T cells, but it directs a vast spectrum of myeloid and lymphoid cells of the immune system, which facilitate IL-21 to modulate the acquired and innate immunity[10]. IL-21 compete in the immunity against tumor cells[12] and chronic viral infections (Nui et al. 2010), however, enormous accomplishment of IL-21 has been correlated with the advancement of immune inflammatory diseases in various organs[13]. IL-21 levels are raised in dermatological conditions like skin biopsies of patients with systemic lupus erythematosus, psoriasis, and atopic dermatitis. In addition, IL-21 interpretation corresponds with the presence of Th17 cells in synovial fluid and peripheral blood of rheumatoid arthritis patients[11]. There is no study present in literature comparing the levels of IL-21 in different types of periodontitis, hence the present study is designed.

Therefore from the above observation, the aim of present study is to evaluate the role of GCF IL-21 level in health and disease further correlate with the clinical parameters of periodontal tissue destruction.

MATERIAL AND METHODS

Thirty patients (19 males and 11 females, aged 20-60 years) were consecutively enrolled over a two year period from the outpatient department of periodontology, Sarjug Dental College, Darbhanga. Ethical clearance for the study was obtained from the institutional ethical committee. The study was conducted in accordance with the ethical principles described in the Declaration of Helsinki 2008. Procedure of the study was explained and informed written consent was obtained from all the participants before their inclusion in the study. Among them, 20 patients having diseased periodontium were grouped into 10 chronic periodontitis and 10 aggressive periodontitis patients whereas 10 healthy individuals were included as control. Inclusion criteria were: patients having more than or equal to 14 functional teeth, systemically healthy patients who had not received any form of surgical and non surgical periodontal therapy or received antibiotics or non-steroidal anti-inflammatory therapy within the past 6 months of the study. Chronic periodontitis was defined as having probing depth (PD) less than or equal to 4 mm, relative attachment loss (RAL) less than or equal to 3mm and more than to 25% sites with gingival bleeding present (BOP). Diagnosis of patients with aggressive periodontitis according to the criteria of the American Academy of Periodontology International Classification of 1999 (Table 1). Additional inclusion criteria were as

follows: 1) The patient's was under 40 years old, 2) The patients had familial aggregation of periodontal disease. Patients who volunteered with no evidence of periodontal disease determined by the absence of increased PD or AL were considered as healthy control group.

Clinical Measurement

Clinical parameters were measured and evaluated for all the teeth excluding 3rd molars, on all the six sites for each tooth (mesio-buccal, disto-buccal, disto-lingual, mid-lingual and mesiolingual). These parameters consisted of pocket depth (PD) assessments of gingival bleeding i.e. gingival index (GI)[14] (Loe 1963), dichotomous measurement of supragingival plaque accumulation i.e plaque index (PI)[15] (Silness 1964) and bleeding on probing (BOP)[16] to the base of the crevice.

GCF samples collection and analysis:

A calibrated appliance[1] was used to quantify the GCF sample volume and reading were converted to actual volume (μL) by reference to a standard curve, prepared using the periotron reading of volume of fluid (μL) distilled water in perio-col strips. A blank gingival fluid collection (perio-col) strip was placed between the periotron fluid Meta-sensors and the instrument was adjusted to display a reading of zero. A microlitre syringe was used to accurately deliver 0.25-1.25 μL fluid (distilled water) to perio-col strip. The strips were immediately placed between the periotron sensors. The periotron score volume displayed the known volume of fluid recorded. This step was repeated three more time with 0.25 μL of test fluid and the average score recorded. The above step was repeated using volume of 0.5, 0.75, 1.0, 1.25 μL, and in every instance the mean periotron value calculated and recorded. Once all the score were obtained, a standard curve was computed with known fluid volume (X-axis) and periotron score (Y-axis). In all three groups, GCF samples were collected at baseline. Site selection was based on the highest score recorded for single site in the oral cavity. A single site in each subject that showed increased inflammatory manifestations (chronic gingivitis) and the highest RAL level (aggressive periodontitis) was selected for gingival crevicular fluid collection.

[1] Periotron 8000 Proflow Inc., Amityville, NY, USA

In our study, we selected a single site in each subject to avoid pooling of samples from multiple sites, as periodontitis is a site specific disease. Prior to the collection of GCF samples supragingival plaque was removed with cotton pellets avoiding contact with marginal gingiva. Standard paperstrips[2] were carefully inserted to a depth of approximately 2mm, into the sulcus/pocket for 30 seconds for collection of GCF. Blood contaminated strips were discarded and alternative site was used to obtain replacement samples. In chronic periodontitis patients, alternative sample were collected with the next highest GI scores and in agressive periodontitis patients the deepest PPD was selected for the alternative sample site. In a similar way GCF volumes (from health, chronic periodontitis and aggressive perodontitis) from study patients were obtained automatically with periotron score. The interpolation from standard caliberation graph gave volume of fluid. After measurement of volume, the strips from the selected sites were placed immediately into individual microcentrifuge tubes[3] containing 200 μL of phosphate buffer solution. The samples were stored at -80 °C until further analysis.

ELISA

The levels of IL-21 in the GCF were determined using ELISA[4] according to the manufactures instruction. To elute the proteins, the tube containing the periopaper strips were vortexed and homogenized for 30 seconds and then centrifuged at 12,500 rpm at 4°C for 5 minutes. The kit used monoclonal antibody MT 21.3 biotin and human recombinant IL-21 standard (captured antibody). Samples were run in triplicate to authenticate the sensitivity of ELISA and all the samples were found to be within the detection limit of ELISA. An ELISA reader (spectramax 190 Ab sorbance microplate reader; molecular devices; Sunnyvale, CA, USA) with 450 nm as elementary wavelength was used to measure the absorbance of the substrate. Conversion of the absorbance readings obtained, into definite volume (pg/mL) were performed using standard reference curve. The protein concentration at each site (pg/mL) were

[2] PerioPaper, ProFlow, Amityville, NY
[3] Eppendorf, Hamburg, Germany
[4] Quantine human IL-21 immunoassay, MABTECH SWEDEN

Table 1: Diagnostic criteria based on the 1999 AAP classification of periodontal diseases.

Diagnostic criteria based on the 1999 AAP classification of periodontal diseases.
Localized aggressive periodontitis : Rapid attachment and bone loss in otherwise healthy patients First molar-incisor presentation with no more than two other teeth affected At least two permanent teeth affected where at least 1 is a first molar Lifetime cumulative attachment loss (LCAL) ≥ 4 mm at the affected sites
Generalized aggressive periodontitis
Rapid attachment and bone loss in otherwise healthy patients
Generalized interproximal attachment loss affecting at least three teeth other than first molars and incisors LCAL ≥ 4 mm at the affected sites

AAP: American Academy of Periodontology.

Table 2: Clinical Characteristics of Test and Control Group.

Charateristics	Chronic periodontitis (n=10)	Aggressive periodontitis (n=10)	Healthy volunteers (n-10)
Age (mean year)	45.4±4.42	33.0±2.58	26.2±2.12
Female %	56	38	52
PI %	2.34±0.35	1.71±0.46	0.31±0.06
GI%	2.34±0.35	1.91±0.46	0.31±0.04
GBI%	40.1±6.95	37.6±3.52	8.30±0.98
PD (mm)	6.75±1.13	6.50±0.52	1.60±0.51
RAL(mm)	11.42±1.44	10.32±1.84	---

n=no.of patients; PI=plaque index; GI=gingival index; GBI= gingival bleeding index;
PD=probing depth; RAL= relative attachment level.

Table 3: Periodontal Clinical Parameters of Chronic periodontitis and Aggressive periodontitis.

Clinical parameters	Chronic periodontitis	Aggressive periodontitis
Plaque index (PI)	1.81±0.59	1.51±0.43*
Gingival index (GI)	1.91±0.46	1.04±0.53*
Gingival bleeding index (GBI)	37.63±3.57	26.6±2.04
Probing depth (PD)	2.50±0.52	3.05±0.45*

Highly statistically significant i.e. p< 0.001; NS-non significant.

Table 4: Mean IL-21 levels in Chronic Periodontitis, aggressive periodontitis and healthy patients.

Characteristics	Chronic Periodontitis	Aggressive periodontitis	Healthy
IL-21	66621.667±11411.219	47865.700±1288.868	2245.900±771.718

determined by dividing the total amount of IL-21 (pg) by gingival crevicular fluid volume (μL) and subsequently the (pg/μL) values were converted into (pg/mL).

Statistical analysis:

The power of study and sample size calculation was determined on the basis of change in GCF IL-21 level. Current estimates as observational study which included 50 patients in each test group and 50 in control group with total sample size of 150 patients. Type II error level of β = 0.20 (80% power) and type I error level of α=0.05 (5% probability) was calculated. The distribution of the samples with respective values of PI, GI, BOP, PD, RAL and IL-21 levels of pre-operatively was analyzed statistically. Data was entered in

Microsoft excel and analysed using SPSS (statistical package for social science, ver.10.05) package. Proportions were compared using Chi-square test (χ^2) test of significance. Normality of data was tested using Shapiro-Wilk test. One way analysis of variance (ANOVA) was used to test the difference between the groups. Comparison of the biochemical and clinical parameters were performed using Kruskal-Wallis non-parametric test. $P<0.05$ was considered statistically significant.

RESULTS

Clinical parameters of patients with chronic periodontitis, aggressive periodontitis and healthy volunteers included in this study are summarised in table 2. The healthy control group exhibited significantly lower values in all clinical periodontal measurements. There was statistically difference in age and sex between the groups. Highly significant ($p<0.05$) difference with a higher percentage of sites with plaque, gingival index (GI) and gingival bleeding index (GBI) as well as PD were observed in periodontitis when compared to control group.

IL- 21 levels in gingival tissues from periodontal sites of chronic periodontitis and aggressive periodontitis patients were found to be significantly higher than in gingival tissues from periodontal sites of healthy individuals. Before non-surgical periodontal therapy the levels of IL- 21 in aggressive periodontitis patients was 11.5 fold more than the control group and 28 folds higher in chronic periodontitis patients when compared to healthy individuals. (Table 4)

DISCUSSION

IL-21 is a proinflammatory cytokines expressed by activated Th1 and Th17 cells (proinflammatory lineage) functions via receptor IL-21R, a type I cytokine receptor[17]. IL-21 expressed at site of inflammation in varying quantities are mainly secreted by activated CD_4^+ T cells and natural killer T cells (NKT). The activities of IL-21 have effects on both lymphoid and myeloid lineages[18]. IL-21 is a helper cytokine that orchestrate a potent innate response. It has effect on leukocyte subsets, such as antigen presenting dendritic cells and phagocytic macrophages to a lesser extent compared to T cells, NK cells and B cells. IL-21 regulates IgE production and eosinophillic requirements.[19] IL-21 activates the JAK/signal transducer and activator of transcription (STAT) pathway[20]. Two other major pathways have been associated with IL-21 signalling: mitogen activated protein kinase (MAPK) and phosphoinositol -3 kinase (PI-3K), thus interlinked to cellular functions.[21]

IL-21 plays a pivotal role in the pathogenesis of various inflammatory systemic diseases like inflammatory bowel disease, rheumatoid arthritis and colitis.[13,14] Th1/Th2/Th17 paradigm has offered an explanation of periodontal pathogenesis on the principle of periodontal disease activity dictated by a complex interplay between the host immune system and the periodontal pathogens. Thus IL-21 plays a paramount role in inflammation and its overproduction leads to complication of the inflammatory response intensifying tissue damage. Since rheumatoid arthritis and periodontitis has been associated with one another increasing evidence has led to the hypothesis that dysregulation of IL-21 signalling pathway during microbial infection may be a determinant periodontal disease activity[22]. This has given way for several studies to elucidate the role of IL-21 cytokine in the pathogenesis of periodontal disease in subjects with & without systemic involvement. There are few observational studies[23-26] and one interventional study regarding the role of IL-21 cytokine in periodontal disease progression.[27] However, to date no similar study on the role of IL-21 in periodontal health & disease has been performed and we have considered investigating the role of IL-21. The discovery of Th17 related cytokine IL-21 in patient with chronic periodontitis has however given way to a new research area for the pathogenesis of many inflammatory diseases where IL-21 plays a paramount role in inflammation.[27]

In the our study, IL-21 levels in chronic periodontitis was found to be 28 folds more than the healthy controls and 18 folds more than in aggressive periodontitis. We have shown the expression of IL-21 levels in chronic periodontitis, aggressive periodontitis and have correlated its level with clinical parameters and compared with healthy controls.

These results were similar to the findings that GCF IL-21 levels were over expressed in patients with untreated chronic periodontitis suggesting their

role in tissue destruction depicting chronic periodontal disease.[24] Non-surgical periodontal treatment is the most basic and effective method for majority of periodontal patients. This is the first study to compare the levels of the expression of IL-21 on chronic and aggressive periodontitis patients. We found IL-21 levels in chronic periodontitis was drastically higher than aggressive periodontitis suggesting its major role as destructive cytokine in periodontal disease progression. Results showed positive co-relation of IL-21 with the clinical parameters (PI, GI, GBI, PD, and RAL) in chronic periodontitis group.

There are very few observational studies of IL-21 levels in saliva of patients with chronic Periodontitis.[25,26] This is the first study of its type comparing the levels of IL-21 in chronic and aggressive periodontitis. The result of the study correlates with the study by Li et al. where IL-21 level was observed increased in rats infected with Aggregatibacter actinomycetemcomitans (Aa)[28]. Bone resorption was evident and early rise of IL-2, IL-9, IL-21 and tumor necrosis factor was observed.

Hence within the limitations of our study it can be summarized that with the variations in periodontal inflammation, there is a significant increase in the local concentration of IL-21 in gingival crevicular fluid. It can be postulated that greater the extent of periodontal destruction, the higher the gingival crevicular fluid IL-21 concentration that signifies the proinflammatory role of IL-21. The stimulus and function-specificity of the defect in neutrophils from patients indicates the existence of a dysregulation of the signal transduction pathway distal to fMLP receptor and proximal to NADPH oxidase activation.[29]

CONCLUSION

Human IL-21 is present in gingival crevicular fluid in periodontal health, chronic periodontitis and aggressive periodontitis. A significant increase in the concentration of IL-21 in gingival crevicular fluid is observed with an increase in the amount of periodontal destruction. However further longitudinal study, elucidating the molecular mechanisms of IL-21 in the inflammatory process in periodontal tissues, with larger sample size would be required to validate the role of IL-21 as a biomarker for periodontal destruction. IL-21 in GCF was found multifold times high in periodontal inflammation compared to periodontal health. This study signifies the role of IL-21 as a biomarker for periodontal destruction and crucial role of adaptive immunity in bone resorption.

CONFLICTS OF INTEREST

The authors declare they have no potential conflict of interests regarding this article.

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