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ORIGINAL ARTICLE

Positive Correlation Between the Level of Interferon-Gamma and the Severity of Periodontitis

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Abstract

Objective: To examine the IFN- γ levels in patients with periodontitis and determine the difference in the levels of IFN-γ with the severity of the disease. **Material and Methods:** The study design was cross-sectional, and the sample consisted of 31 patients, aged between 18 and 64 years. Plaque index (PlI), calculus index (CI), and papillary bleeding index (PBI) were measured. Pocket depth (PD), recession, and clinical attachment loss (CAL) (mm) were measured at six sites per teeth. For mild/moderate periodontitis, pocket depth ≥4 mm in 1–3 sites was required, while the essential criteria for severe periodontitis were pocket depth ≥5 mm, clinical attachment loss >3 mm in more than 3 sites ≥2 quadrants. The IFN-γ levels were measured by performing enzyme-linked immunosorbent assay with the gingival crevicular fluid (GCF) samples. The measurements were made in two different sites, and the severity of periodontitis was categorized based on the pocket depth, attachment loss, and the remaining natural teeth. Kruskal-Wallis test, Mann-Whitney test, and Spearman's rank correlation coefficient were. **Results:** The levels of IFN-γ (pg/mL) were correlated with the severity of the periodontal status, with $p < 0.05$. Clinical parameters of periodontitis also correlated with the level of IFN-γ (pg/mL). **Conclusion:** Subjects with periodontitis presented greater levels of IFN-γ (pg/mL) in GCF than the periodontal healthy individuals. This result showed the role of IFN-γ in the inflammation.

Keywords: Periodontitis; Gingival Crevicular Fluid; Interferon-gamma.

Introduction

In Indonesia, periodontal diseases had a fairly high prevalence of 95.2% in the year 2015 [1]. The illness has a multifactorial etiology, with environmental, systemic, host, and pathogen-specific (bacteria and/or viruses) factors contributing to it. Pathogenic bacteria present in the subgingival biofilms are associated with immune response in the susceptible host, resulting in periodontal tissue damage. Microbial presence triggers the production of cytokines by the gingival epithelium, while the cytokine imbalance results in uncontrolled inflammation and leads to tooth loss in adult populations [2].

Periodontitis is a chronic pathological condition characterized by gingival inflammation and the destruction of tooth support structures. The development of chronic periodontitis can be attributed to the multifactorial interactions between the microbe and the host immune system. According to the new periodontal classification, the two main forms of the disease were chronic and aggressive periodontitis. The severity level ranges from 1 to 4, while the grading based on the progression rate was grade A (slow), B (moderate), and C (rapid) [3]. In these studies, chronic periodontitis was referred to as periodontitis, with initial or moderate periodontitis and severe periodontitis, where the extent and distribution were localized and generalized.

T-cell-mediated immune response, involving killer T-cells and T-helper (Th) cells, plays a role in tackling the disease [4]. Cells such as macrophages recognize the microorganism and trigger the expression of proinflammatory genes. This occurs through nuclear factor kB (NF-kB) transcriptional activation and/or the expression of antiviral mediators, such as interferons, via interferon regulatory factors. This process results in the release of proinflammatory cytokines, chemokines, and antimicrobial effectors [5].

Interferon-gamma (IFN-γ) is type-II interferon whose structure is different from that of type I and type III interferons, and it gives signals through specific receptors [6]. IFN-γis triggered by interleukin (IL)-12 and IL-18 cytokines released by the immune cells, including CD4+ Th1 cells, cytotoxic CD8+ cells, and natural killers, such as macrophages and dendritic cells (DCs) [7].

The main function of Th1 cytokines, that is, IL-2 and IFN-γ, is to increase cell-mediated response, whereas the IL2-Th2 cytokine suppresses the response [8]. Cytokine IFN-γ was considered the main activator phagocyte and associated with the production of inflammatory cytokines and chemokines, although it also systematically inhibit osteoclastogenesis and stimulates the formation of osteoclast in vivo [9].

In the affected tissues, local inflammatory progression determines the conditions of the disease. Cytokines and chemokines direct the migration of leucocytes to the periodontal tissue, where these cells become involved in the destruction of pathogenic bacteria by releasing mediators of local inflammatory responses [10]. Therefore, detecting increased levels of this mediator in the gingival crevicular fluid (GCF), saliva, and blood was considered to be a biomarker for some aspects of periodontal disease [11]. Increased cytokine levels in the saliva and GCF of individuals with periodontal disease are pathological markers [12]. GCF is a physiological fluid and an inflammatory

exudate. This secretion is derived from the vascular flexing in the gingival corium, close to the edges of the epithelium, in the space between the tooth and the gingiva. GCF changes from the normal clinical tissue to serum transudate during inflammation, when the disease becomes clinically apparent. Therefore, GCF can be used as a diagnostic and prognostic tool in periodontal disease [11].

A previous study on the serum levels of IFN-γ and IL-4 in individuals with chronic periodontitis revealed wide variations in the cytokine concentrations, with the levels being quite low in serum samples with chronic periodontitis, thereby making it impossible to describe the severity of the disease [12]. In a GCF study, the IL-17 levels were declined, while the IFN-γ and IL-10 levels remained unchanged after dental plaque clearance within 8 weeks, 16 weeks and 24 weeks [14], in line with another study that no difference in IFN-γ serum concentration at baseline and 6 months post-therapy [15]. In contrast, high levels of IL-1-α, IL-1β, TNF-α, IL-6, IFN-γ, IL-4, and IL-10 in the gingival tissue, strongly illustrate the severity of the periodontal disease in Chinese subjects [16]. This observation was in agreement with a work in which IFN-γ level in GCF and T-bet expression was elevated in individuals with chronic periodontitis. T-bet is a T-Box transcription factor, expressed in T cells and required for differentiation of IFN-γ secretion by CD4+ Th1 T cells [17,18]. Sixteen proinflammatory and four anti-inflammatory cytokines, including IFN-γ, were significantly elevated in GCF of chronic periodontitis patients, facilitating the protective role of IFN-γ and IL-10 [19].

Since previous studies have yielded conflicting results, this issue needs to be investigated further. The present research was conducted to measure the level of IFN-γ in individuals with and without periodontitis, while the correlation between the IFN-γ level and the disease was also examined. We suggest that there is an increase in IFN-γ level according to the severity of periodontitis.

Material and Methods

Study Design and Sample

The study design was cross-sectional, and the sample consisted of 31 patients, aged between 18 and 64 years, who visited the Dental and Oral Hospital in Universitas Yarsi from March to May 2018 and who matched the inclusion criteria.

The inclusion criteria were: (1) male and female Indonesian patients aged 18-64 years; (2) absence of immunodeficiency disorder and autoimmune disease; (3) no prior periodontal therapy and no antibiotics or non-steroidal anti-inflammatory drugs used for a period of 3 months prior to the study; and (4) the presence of at least 14 natural teeth. The exclusion criteria were pregnant/lactating women and dental conditions necessitating antibiotics.

Clinical Parameters and GCF Collection

The status of oral hygiene and gingival inflammation was assessed by using the Plaque index (PlI) [20], calculus index (CI) [21], and papillary bleeding index (PBI) [22] in Ramfjord teeth. The clinical parameters of periodontitis were evaluated as the levels (in mm) of pocket depth (PD), recession, and clinical attachment loss (CAL). With the WHO pocket probe, the measurements included all teeth except for the third molar, six sites per tooth (distobuccal, midbuccal, mesiobuccal, mesiolingual, midlingual, and distolingual). A single qualified examiner conducted the clinical measurements.

The criteria for periodontal health were the presence of at least 26 natural teeth, ≤1 site with pocket depth ≥3 mm, general pocket depth ≤3 mm, no clinical attachment loss, and no teeth mobility. The periodontitis criterion was the presence of at least 14 natural teeth. For mild/moderate periodontitis, pocket depth ≥4 mm in 1–3 sites was required, while the essential criteria for severe periodontitis were pocket depth ≥5 mm, clinical attachment loss >3 mm in more than 3 sites ≥2 quadrants. The healthy periodontal was grouping with the mild periodontitis and compared to the severe periodontitis.

Phosphate-buffered saline (PBS) was prepared by dissolving 1 tablet in 100 mL of distilled water; subsequently, 1 mL of the solution was transferred into an Eppendorf tube and placed in a cooler box. GCF was obtained from patients by a paper point after supragingival plaque removal by using a cotton pellet. Then, the tooth being examined was isolated using a cotton roll and dried gently. The sample was collected using paper point ISO 30 (Roeko GmbH, Langenau, Germany) by inserting it into the mid buccal pocket carefully until resistance was felt, then maintaining it for 30 s. Blood or saliva contaminated paper points were discarded. Four paper points per site from two different quadrants were pooled in the Eppendorf tube containing PBS and transferred to the laboratory in a cooler box.

Enzyme-Linked Immunosorbent Assay

GCF samples were analyzed for the level of IFN-γ by using ELISA. The sample was vortexed, then centrifuged at 1000 g (5000 rpm) for about 15 min. The supernatant was taken, and the sample was stored at -20°C for further analysis.

ELISA (MyBioSource, San Diego, CA) was performed for the quantitative detection of IFN-γ, according to the manufacturer's recommendation. For this procedure, 100 µL of detection antibody was added to the wells, mixed gently, covered with strips of membrane plates, and incubated at 37°C for 60 min. The wells were washed four times with the wash buffer. The plate frame was held firmly, inverted, and tapped onto absorbent papers or paper towels to remove any remaining buffer. Each of the chromogen solutions A and B (50 µL each) were added. The wells were protected from light using a well cover and aluminum foil, followed by incubation at 37°C for 15 min.

Later, the reaction was stopped by adding 50 µL of stop solution to each well. The color changed from blue to yellow. The optical density was read at 450 nm using a microplate reader (Vmax®, Molecular Devices, LLC, San Jose, CA, USA) with the SoftMax Pro Software (Molecular

Devices, LLC, San Jose, CA, USA). The concentration of each sample was calculated using a standard curve (7.8–1000 pg/mL). Values lower than the detection limit (<4.1 pg/mL in our assay) were considered undetectable.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics, version 23 (IBM Corp., Armonk, New York, USA). Normality was checked with a Shapiro-Wilk test; as the data distribution was not homogeneous, the analysis was performed using non-parametric tests. Kruskal-Wallis test was employed for comparison between the periodontal status and the levels of IFN-γ (pg/mL). If a significant difference was found, the variations between the groups were assessed using a Mann-Whitney test. The Spearman's rank correlation coefficient was used to analyze the correlation between IFN-γ level (pg/mL) and the periodontal status, clinical disease parameter, and risk factor; $p<0.05$ was considered statistically significant.

Ethical Aspects

The study was approved by the Ethics Committee of the Faculty of Dentistry, Universitas Yarsi (No. 207/KEP-UY/BIA/IX/2017) and the research was performed in accordance with the recommendations of the Helsinki Convention, and that positive opinion of the local Bioethics Committee has been sought and obtained. The subjects were instructed about the purpose and procedure of the study, and informed consent was obtained from each patient.

Results

The results of this study were obtained 62 samples. The demographic data, including distribution of the gender, smoking status, and periodontal status, are presented in Table 1.

Table 1. Sample distribution according to demographic and clinical characteristics.

Variables	Value
Age (Years) (Mean ± SD)	35.66 ± 13.56
Periodontal Status	
Healthy Periodontal and Mild Periodontitis	32 (32.88 ± 12.0)
Severe Periodontitis	30 (38.63 ± 14.6)
Gender	
Male	22 (35.5)
Female	40 (64.5)
Smoking Status	
No Smoking	51 (82.3%)
Smoking/ Previous Smokers	11 (17.7%)
Plaque Index (Mean ± SD)	1.56 ± 0.78
Calculus Index (Mean ± SD)	1.92 ± 0.71
Papilla Bleeding Index (mm) (Mean ± SD)	1.97 ± 1.05
Pocket Depth (mm) (Mean ± SD)	2.2 ± 0.84
Recession (mm) (Mean ± SD)	0.19 ± 0.44
Clinical Attachment Loss (mm) (Mean ± SD)	1.30 ± 1.09
Number Natural Teeth (mm) (Mean ± SD)	25.66 ± 3.57
Level of IFN-γ (pg/ml) (Mean ± SD)	729.33 ± 598.63

The level of IFN-γ in healthy periodontal and mild periodontitiswas448.267± 450.45, and severe periodontitis was 1029.13 ± 597.27 pg/mL, respectively (p<0.001) (Figure 1).

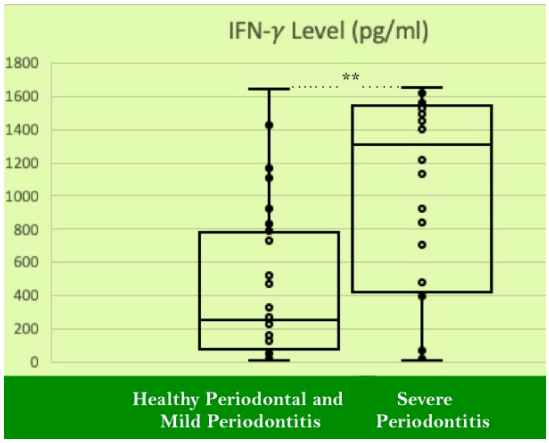

Figure 1. GCF cytokine concentrations of IFN-γ (pg/ml) in healthy periodontal and mild periodontitis and severe periodontitis. Boxplots present the median, minimum, maximum values. Differences among the groups (Mann-Whitney; **p<0.001).

The correlation between factors, such as age and gender, smoking status with IFN-γ (pg/) levels were given in Table 2. Positive moderate correlations were found in PlI, PBI, PD, and periodontal status, while CI, CAL showed a weak correlation with the IFN-γ (pg/mL) levels. The other factors were not significantly correlated with the level of IFN-γ.

Table 2. Correlation between the demographic, clinical parameter of periodontitis, and the level of IFN-γ.

Variable IFN-γ	Level of IFN-γ pg/ml	
	r	p-value
[a]Age	0.185	0.149
[a]Gender	0.122	0.343
[a]Smoking Status	0.136	0.293
[a]Plaque Index	0.449	0.000**
[a]Calculus Index	0.381	0.002*
[a]Papilla Bleeding Index	0.514	0.000**
[b]Pocket Depth	0.469	0.000**
[a]Recession	0.248	0.052
[a]Clinical Attachment Loss	0.343	0.006*
[a]Number of Natural Teeth	0.091	0.481
[a]Periodontal Status	0.451	0.000**

[a]Spearman Correlation test (p<0.05); [b]Pearson Correlation test (p<0.05).

Discussion

The present findings showed statistically significant results between the healthy periodontal and periodontitis that support reports of previous investigations that had detected IFN-γ (pg/mL) with higher frequency in periodontitis patients than in healthy periodontal, but the sample was taken from serum [10]. This study was also in line with other studies that reported an increased level of

IFN-γ, along with IL-10 [23], and elevated IFN-γ concentration and T-bet expression in periodontal tissue and depicted increased severity of the disease as the active sites revealed higher concentrations than inactive sites; hence, it was speculated that cytokine is involved in the inflammatory response [17].

A significant correlation was observed between the clinical parameters of periodontitis and the level of IFN-γ, as in a previous report [10]. In a clinical study, the level IFN-γ in the GCF of patients with periodontitis before and after the treatment yielded different results. There was no difference in the IFN-γ levels before and after plaque removal, although all clinical parameters showed improvement [14,15] but another study presented a decreased amount of IFN-γ [24] and an increased ratio of IL-4 to IFN-γ after treatment, which indicates higher levels of IFN-γ during active periodontitis and decreased levels after the inflammation subsides [23]. The higher level of IFN-γ in periodontitis was explained by a previous study on the basis of the activation of CD4+ cells and enhanced expression of IFN-γ and IL-2 in aggressive periodontitis when compared with periodontally healthy individuals [25]. The levels of IFN-γ in our study were not related to age and sex; this result agrees with that of certain previous studies [17,26] and contradicts others that documented a correlation between IFN-γ and age [10].

IFN-γ is only one of the proinflammatory mediators that have an influence on the severity of periodontitis. The levels of 10 influential cytokines in the GCF have been studied simultaneously to assess their effects on periodontitis. The results stated that IL-1α, IL-1β, and IL-17A levels in the GCF were the best biomarkers for chronic periodontitis by combining IFN-γ and IL-10 [19]. In research involving many of these cytokines, age, smoking status, and periodontal severity markers (PD, PBI, BPL [bacterial plaque level], and CAL) had significant effects, while sex and remaining teeth had no effect. This difference might be attributed to the fact that the number of subjects in our study was smaller and less diverse, and that 20 paper points representing all the teeth were collected in one examination while in this study, four paper points representing each site was obtained from only two different quadrants per patient.

The previous study about the role IFN-γ gene showed the inhibition of osteoclastogenesis by Neopterin, chemical group, which synthesized upon stimulation IFN-γ by human macrophages as an important factor in GCF and response of inflammation in periodontitis [27].

The smoking status in this research showed no significant association, as seen in an earlier study, between the IFN-γ (pg/mL) levels of smokers and non-smokers [28], while in another study, smoking was very important and showed significant result [19].

A meta-analysis indicated the role of IFN-γ and its variations in GCF; however, it did not provide a conclusive result as some studies showed high levels of IFN-γ in the healthy sites, while others pointed to an increased frequency in the periodontitis site [7]. This result was important in helping us understand that further study is needed to elucidate the exact roles of IFN-γ in GCF.

The present study faced some limitations, as the samples were small and other anti-inflammatory cytokines were not included. Hence, the present study could be considered as a preliminary study that needs to be expanded upon in order to provide a comprehensive result.

Conclusion

Subjects with periodontitis presented greater levels of IFN-γ (pg/mL) in GCF than the periodontal healthy individuals. This result showed the role of IFN-γ in the inflammation.

Authors' Contributions: CM performed the experiments, analysis, and interpretation of the results, and wrote the manuscript, SLCM critically revised the manuscript, SP managed funding and data analysis, TPS contributed to data analysis and interpretation, and EIA designed the study and contributed to the final manuscript revision.

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Conflict of Interest: The authors declare no conflicts of interest.

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