

As a Biomarker: Comparing the level of IL-35 in Biological Fluids with Age and Sex in Periodontitis Patients

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Abstract: Background: The Periodontitis is one of the most common diseases that affect dental health. It also poses a threat to human life when associated with other diseases, and since interleukin-35 is a newly type of cytokines belong to interleukin-12 family which is suggested to contribute together with some cytokines to the process of inflammation, so this study was designed to investigate the importance of interleukin-35 and evaluate its levels in gingival crevicular (GCF), saliva and serum of periodontitis patients compared to sex and age, in order to revealing the possibility of its future use as a biomarker and diagnostic tool for the disease. **Materials and Methods :** 60 participants, 40 periodontitis patient and 20 healthy person for control, by using Enzyme-linked immunosorbent assay (ELISA) kits were measured the levels concentration of interleukin 35. **Results:** The results obtained that there are significant differences between GCF and saliva concentration of IL-35 (147.3, 50.84)pg/ml in patients compare with healthy group (79.54, 18.99) pg/ml respectively, also there are no significant between female and male of IL-35 levels, When comparing the levels of interleukin 35 according to age groups, it was found that there was an increment in its levels in the serum among the elderly within the age group (55-65)years. **Conclusion:** The IL-35 appears to play a part in periodontal disease pathogenesis and it is possible to conclude that the IL-35 concentration may be considered as diagnostic biomarker in GCF or saliva for chronic periodontitis.

Keywords: Gingival Crevicular Fluid, Interleukin- 35, Periodontitis, Saliva, Serum.

Introduction

Periodontitis represent a chronic inflammatory which mainly caused by the pathogenic bacteria that due to an interaction between microbial antigen and an unbalanced host immune response^(1,2). Its one of the health problems affecting people all over the world, the rates of which have increased significantly in recent decades, and much evidence has indicated that it is closely related to systemic diseases³⁾. Periodontitis causes many risks and potential complications when treated, especially in patients with cardiovascular diseases who receive anticoagulant treatment⁽⁴⁾.

Some studies have shown that cytokines produced by cells play an important in the dysbiosis represented by the interplay between the imbalance within microbial community and the dysregulated immune response of the host, thus destroying periodontal tissues as gingival ligament and the alveolar bones⁽⁵⁾. cytokines produced by a number of immune cells responsible for inflammation, led by T helper cells types Th1, Th2 and macrophages, there is a balance in their production by these two cells⁽⁶⁾. Interleukin-35 is produced from Treg cell population Know have the Maximum suppressive effect⁽⁷⁾ and this interleukin is one of the effective inhibitory interleukin belonging to the IL-12 family that consists of four interleukins IL-23,IL-12, IL-27, and IL-35 (from

the most proinflammatory to the most inhibitory, respectively)⁸⁾. Considering this, members of the IL-12 family might be suitable candidates for clinical cardiovascular disease prevention, intervention, and therapy⁽⁹⁾. In a study conducted by¹⁰⁾ they revealed that the percentage of nTreg cells in periodontal tissue biopsies is higher than gingivitis, and they also pointed out that there is a possible role in regulating periodontitis.

The levels of inflammatory mediators in the body fluids are a good indicator of the effectiveness of inflammation in many cases, and considering the importance of this in gingival diseases, therefore, it is necessary taking into account the immunological and biochemical indicators in GCF to assess the future developments of the disease and extent of tissue damage, so, Given the lack of available literature on interleukin-35 and to investigate its role as a biomarker of periodontal infection, so this study aim to highlight its levels in both Saliva, GCF and serum in a sample of Iraqi patients and It can be suggested as a biomarker for periodontitis in order to improved our knowledge of oral health.

Material and Methods

The present study was conducted on periodontitis outpatients visiting counseling clinics at Faculties of Dentistry, the universities of Kufa and Al-Kafeel in Najaf, Iraq during the June to November 2023. The protocol of this study was elucidated to all participants and Written informed consent was gotten before clinical examination and biofluid sampling. A total of 40 periodontitis patients (13 male and 27 female), 20 healthy volunteers (11 male and 9 female) ; This two study groups had an average of (22-65)years. All individuals immune disorders such as diabetes mellitus, pregnant or lactating women, smokers, as well as those who have taken antibiotics or any treatment for periodontitis during the past three months have been excluded.

GCF and Saliva sampling

Filter paper strips (Periopaper, Oraflow Inc. , NY,USA) were employed for collection of Gingival Crevicular Fluid (GCF). In a very cautious and tentative way, two filter periopaper strips were inserted into gingival sulcus / cervice until light friction was felt, it were kept in place for thirty seconds and taken care to prevent any mechanical injury, so when contaminated any strips with blood, they were eliminated, after the strips absorbed GCF, it were transferred immediately in to micro centrifuge tubes containing 200 µl phosphate buffer saline and frozen at -80°C, until the assay was performed. On the day GCF sampling, Whole saliva samples were collected by expectorating in to sterile tubes and clarified by centrifuging at 800 x g for 10 minutes, all tubes were processed at -80°C until the time of experiment⁽¹¹⁾.

Five ml of intravenous blood was withdrawn from the patients and healthy participants in the study, the serum was separated and distributed in the Eppendorf tubes and kept at freezing -20°C to latter assess the level of Interleukin.

Assays of IL-35 level in biological fluids:

IL-35 levels were measured in serum, saliva and GCF by sandwich ELISA (Elabscience, USA)).Optical densities were read at 450 nm spectrophotometrically and analyzed using software program. IL-35 levels were read off from a standard curve according to the manufacturer's instructions.

Statistical analysis

The statistical analysis were performed using the statistical software (SPSS version24), all statistical interferences were set with p-value 0.05. student's t-independent test for all variables that followed the normality of the distribution and the non parametric for variable that didn't follow normality of the distribution were included Kruskal- Wallis test (multiples comparisons), Mann- Whitney test and Chi – square test. Spearman' s rank correlation was carried out in order to associate among levels of the IL-35 and other clinical parameters.

Results

Characteristics of study population

The number of participants in this study was 40 patients with periodontitis (27 female and 13 male) and 20 healthy volunteers (11 male and 9 female), According to the **Table 1**. Which shows descriptive statistics of age and gender for patients and control groups, there is no significant difference ($P > 0.05$) between patients and control groups regarding distribution of age, gender.

Table 1. Descriptive statistics of age and gender for patients and control groups.

Parameters	Subgroup	Patients (No.=40) Freq.(%)	Control (N0. =20) Freq.(%)	Chi Test P value(Sig.)
Age	22-32	11(27.5)	7(35.0)	1.87, 0.59 (NS)
	33-43	8(20.0)	6(30.0)	
	44-54	13(32.5)	5(25.0)	
	55-65	8(20.0)	2(10.0)	
Sex	Male	13(32.5)	11(55.0)	2.81, 0.08(NS)
	Female	27(76.5)	9(45.0)	

NS: Non-significant at P value > 0.05

Interleukin-35 levels in GCF, Saliva and serum

The results shown in Table 2. demonstrated that there are differences in IL-35 levels from serum, saliva and GCF between patients and control groups. This study has not revealed a significant difference ($P > 0.05$) in IL-35 level from serum between patients and control groups. Regarding to IL-35 in GCF there is highly significant increment ($P \leq 0.05$) between patients and control. But IL-35 in saliva, there is significantly increasing ($P \leq 0.05$) in patient group as compared to control group.

Table 2. Differences in IL-35 levels from serum, saliva and GCF between patients and control groups

Concentration of IL-35 (pg/ml) in	Statistics	Patients (N=40)	Control (N=20)	P value (Sig.)
Serum	Median (IQR)	18.43 (13.1-40.6)	17.27 (12.2- 39.3)	0.54 NS
Saliva	Median (IQR)	50.84 (19.6-50.8)	18.99 (12.8- 37.6)	0.012 S
GCF	Median (IQR)	147.93 (87.6-147.9)	79.54 (22.3- 77.6)	0.010 HS

GCF: Gingival Crevicular Fluid; SD: Standard Deviation; NS: Non-significant at P value > 0.05 ;
S: Significant at P value ≤ 0.05

Table 3. reveals the differences in IL-35 levels from serum, saliva and GCF between patients' subgroups classified according to gender. According to this table, there is no significant difference ($P > 0.05$) in IL-35 level from serum, saliva and GCF between male and female.

Table 3. Differences in IL-35 levels serum, saliva and from GCF between male and female in patients' group

Concentration of IL-35(pg/ml) in	Statistics	Male (N=13)	Female (N=27)	Mann- Whitney U	P value (sig)
Serum	Median (IQR)	17.98 (12.4-38.2)	19.25 (14.1-111.6)	157	0.608 NS
Saliva	Median (IQR)	70.44 (43.3-375.6)	45.58 (15.6-146.4)	120	0.113 NS
GCF	Median (IQR)	120.79 (97.6-157.9)	174.73 (92.5-117.3)	152	0.512 NS

NS: Non-significant at P value > 0.05

Table 4. shows the differences in IL-35 levels serum, saliva and from GCF between patients' subgroups classified according to age. This study has not revealed a significant difference ($P > 0.05$) in IL-35 level from serum and GCF between patients and control groups. Regarding IL-35 in serum, there is significant increase ($P < 0.05$) in patient with ages between (55-65) years as compared to other age subgroups.

Table 4. Differences in IL-35 levels from serum, saliva and GCF between age subgroups of patients

Conc. Of IL-35(pg/ml) in	Statistics	22-32 (N=10)	33-43 (N=8)	44-54 (N=13)	55-65 (N=9)	Kruskal-wallis H	P value (sig.)
Serum	Median (IQR)	13.65 (12.7-79.3)	18.00 (12.1-59.3)	15.31 (12.2-79.3)	96.63 (61.3-217.6)	7.81	0.05 S
Saliva	Median (IQR)	50.85 (12.7-68.6)	73.19 (62.3-114.6)	58.38 (12.3-166.5)	44.95 (12.4-267.6)	0.30	0.96 NS
GCF	Median (IQR)	118.86 (79.2-209.6)	132.06 (89.3-219.6)	177.85 (89.1-189.6)	141.20 (63.3-115.6)	0.89	0.83 NS

SD: Standard Deviation; NS: Non-significant at P value > 0.05 ; S : Significant at P value < 0.05

Discussion

Periodontitis and dental caries are highly worldwide prevalent; untreated dental caries is the most common illness impacting people globally ¹². It is characterized by irreversible loss of alveolar bone and connective tissue in the gingiva which eventually leads to tooth loss. The microbial pathogens produce several proteolytic enzymes as well as trigger immune responses; These responses result in either the successful removal of pathogens or the destruction of the periodontal tissue.

Driven by growing evidence that periodontal disease has an overall impact on the health status of patients, in this study, we sought to understand how IL-35 contributes to the pathobiology of periodontal disease. The results obtained showed that there no significant difference between IL-35 levels in patients serum compared to the healthy group, and this may be due to the short period of time in which interleukin remains in the bloodstream, while there is a significant increment in IL-35 levels in Patients saliva, as well as highly significant increment in GCF of patients. Also ^{13,14} deduced that IL-35 elevated levels in saliva and serum of patients with periodontitis relative to healthy controls suggest a pivotal role for this protein in the pathophysiology of the disease.

The current results may be belong to the role of IL-35 as anti-inflammatory in immune response to the infection in the Gingival Crevicular Fluid, its depicted that the IL-35 has anti-inflammatory properties while its levels remain constant in the tooth pocket of healthy people. The gingival crevicular fluid is a good medium for the growth of various bacteria, especially anaerobic bacteria. The presence of *Turicella otitidis* bacteria has recently been observed for the first time in patients with periodontitis ¹⁵. the anti-inflammatory role is due to research suggesting that IL-35 regulates the development of periodontitis by preventing the production of inflammatory cytokines (IL-6 and IL-8) ¹⁶. during non-surgical periodontal therapy, was found the IL-35 concentration in GCF increase gradually and considerably from baseline ⁽¹⁷⁾. The results obtained in this study support the results of the study conducted by **18**), in which she found a positive association between increased levels of interleukin 17 in serum and saliva and cell growth factor.

As for the increase in the level of IL-35 in patients over the age of 55 years, this is likely due to a decrease in the immune response in the elderly and deficiency of some Vitamin, as it was found ^{19,20} that the expressing of IL-35 by Treg cells is related to their ability Immunosuppressive and It may be caused by a deficiency of vitamin D in periodontitis, which negatively affects the results of treatment. In addition, Increasing IL-35 levels protect against periodontal disease by preserving immune system homeostasis and reducing inflammatory response ²¹. L-35 and IL-37 are produced

by a subpopulation of plasma cells which may directly prevent the production of osteoclasts, hence preventing the loss of alveolar bone and regulating the pathogenesis of periodontitis 22).

Our present results also emphasizes the findings of a recent study on IL-35 that there is an increase in its level in the saliva of patients at age more than 50 years²³⁾²³. In a recent study conducted by²⁴⁾ proved that the IL-35 has a role in inhibiting alveolar bone resorption in mice with periodontitis and is also closely related to the adjustment of the balance of T17/Treg cells. In vitro study by ²⁵⁾ concluded that IL-35 has osteoclastogenesis activity Which was synergistically stimulated by IL-35 and RANKL. Furthermore, a recent study demonstrated that IL35-coated extracellular vesicles could interact with bystander lymphocytes expressing IL-35 receptors to promote "infectious tolerance" and cross-mark non-Treg cells as IL-35 "producers." This finding highlights an unusual process through which IL-35 may mediate repression and reproduce tolerance ²⁶⁾ so, the use of IL-35 as a treatment is one of the promising therapeutic methods that can be adding to the immune suppression arsenal may use in transplantation ²⁷⁾

The current results showed that there were no significant differences between the levels of interleukin-35 in females and males, and this is consistent with what was reached by ²⁸⁾ concluded that protein expression of Interleukin-35 was not significant between males and females. In recent times, there has been a growing body of evidence demonstrating gender-based variability in periodontal disease In women, estrogen hormones play an immune and protective role against periodontitis before menopause, but they may appear after menopause, a condition equal to that of men ²⁹⁾. On the other hand, a recent study conducted by ²⁹⁾ revealed that the level of IL-35 had decreased in a non-significant manner in both males and females with viral hepatitis.

Conclusion

High levels of interleukin-35 in saliva and gingival crevicular fluid for periodontal patients compared to healthy people, and it seems to have a vital role as anti-inflammatory, especially in the area of the tooth pocket, which makes the probability strong in imposing it a biomarker of periodontitis.

Conflict of interest

The authors have no conflicts of interest to declare.

Author contributions

S.J.A. and A.J.M. conceived the presented idea and designed the study. S.A.H. collected samples. S.A.H. and S.J.A. conducted experiments. A.J.M. and S.J.A. performed calculation and verifying analytical methods. All Authors wrote the paper contributed to the final manuscript

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Informed consent

The consent of healthy Volunteers and patients was obtained when collecting samples.

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