IL-18 and Anti-*P. gingivalis* Antibodies in Patients with Diabetes and Chronic Periodontitis

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Abstract: Periodontitis is an important complication in patients with diabetes mellitus. Chronic periodontitis is caused mostly by *Porphyromonas gingivalis* in Mexico, and by proinflammatory cytokines like IL-1, IL-6, TNF α , and IL-18. The aim of our study was to quantify IL-18 and to detect antibodies against *P. gingivalis* in gingival crevicular fluid of type 2 diabetes mellitus (T2DM) patients with chronic periodontal disease (PD). We studied a total of 40 patients divided in four groups: group 1 T2DM patients with PD, group 2 T2DM patients without PD, group 3 non-diabetic patients with PD, and a control group of healthy individuals. IL-18 was quantified using a commercial ELISA kit. Detection of IgG and IgM antibodies in gingival crevicular fluid was carried out through ELISA using *P. gingivalis* semi-purified proteins. Our results showed T2DM patients with chronic PD had low levels of IL-18, whereas non-diabetic patients with chronic PD had the highest. IgM antibodies were detected in diabetic and non-diabetic with chronic PD. IgG antibodies were detected in patients with T2DM and chronic PD, non-diabetic patients with chronic PD, and healthy individuals. In conclusion, IL-18 was detected in low concentrations in gingival crevicular fluid of patients with T2DM and chronic PD. This could be due to a dysregulation of immune-inflammatory responses secondary to diabetes, or by the presence of IL-18 binding protein in gingival fluid. Low levels of IgG antibodies in T2DM patients with PD could be due to the formation of immune complexes, which would explain the greater periodontal damage these patients present.

Keywords: Antibodies, *Porphyromonas gingivalis*, Cytokines, Type 2 Diabetes, Periodontitis

1. Introduction

Periodontal Disease (PD) is an inflammatory disease of the tooth-supporting tissues caused by specific microorganisms that produce progressive destruction of the periodontal ligament and alveolar bone with formation of pockets and loss of attachment, or both [1]. Diabetes mellitus (DM) encompasses a heterogeneous group of disorders with the common characteristic of hyperglycemia that results from defect in insulin secretion, action or both, and Type 2 diabetes mellitus (T2DM) constitutes 90 to 95% of all cases of diabetes [2]. The classic complications of DM include

retinopathy, nephropathy, neuropathy, macrovascular disease, altered wound healing, and PD has been described as the sixth principal complication in DM [3]. The association between DM and PD has been recognized in the dental literature for several years and it is well known as a bidirectional relationship or a “two-way relationship” [4].

Different factors may explain the effects of diabetes on PD, including pro-inflammatory events, changes in the subgingival microbiota, or the accumulation of advanced glycation end products. These factors provide the basis for

diabetes being an important risk factor for periodontitis [5].

From the perspective of the oral cavity, while several cytokines have been extensively studied in relation to gingival inflammation and found in gingival-crevicular fluid, interleukin-18 (IL-18) has received only scant attention. IL-18 was first described in 1989 as an endotoxin-induced serum factor that itself induces the production of IFN- γ [6]. Today IL-18 is recognized as a cytokine that is able to play major roles in different types of reactions. High levels of IL-18 have been found in autoimmune diseases, inflammatory tissue damage, and systemic inflammatory diseases. Oral epithelial cells are thought to act as a physical barrier against the entry of pathogenic organisms and produce different pro-inflammatory cytokines, which potentially participate in the initiation and development of oral chronic inflammation such as periodontitis [7]. IL-18 could be involved in the pathogenesis of T2DM because hyperglycemia causes elevated blood concentrations of pro-inflammatory cytokines. Several studies have found elevated plasma concentrations of IL-18 in type 2 diabetic patients [8, 9]. Since it has a variety of immunostimulatory properties, IL-18 may play a role in the pathogenesis of metabolic diseases such as diabetes. On the other hand, *Porphyromonas gingivalis* is an oral Gram-negative anaerobic bacterium greatly related to PD. There are several studies that have associated the severity of PD with an elevated proportion of *P. gingivalis* in the subgingival flora [10-12].

Following the first exposure to a foreign antigen, like *P. gingivalis*, a lag phase occurs in which no antibody is produced, but activated B cells are differentiating into plasma cells. The first antibody produced is mainly IgM in relatively low quantities and, over time, antibody levels decline to the point where it may be undetectable. If a second dose of the same antigen is given days or even years later, an accelerated second or anamnestic immune response occurs due to the presence of memory cells. The amount of IgG antibody produced rises to a high level and tends to remain high for a longer time [13]. The production of antibodies in T2DM is not well studied but a non-enzymatic glycosylation reaction of serum IgM and IgG is present, and the glycosylation of these proteins could be causing the deficient defensive mechanism. An increase of IgG and IgM has been reported in patients with uncontrolled diabetes [14-16].

The aim of this study was to quantify IL-18 levels and to determine the presence of IgM and IgG antibodies against proteins from *P. gingivalis* in gingival crevicular fluid of T2DM patients with chronic PD prior to periodontal treatment to determine their role in periodontal damage.

2. Material and Methods

2.1. Patient Population

Samples were obtained from 40 Mexican healthy subjects or patients that arrived to the Clinic of Periodontics of the Dental School of UANL. Four groups were formed: group 1 consisted of T2DM patients with chronic PD (n=10); group 2

had T2DM patients without PD (n=10); group 3, non-diabetic patients with chronic PD (n=10); and group 4 was the control group formed by healthy subjects (n=10). This study included female and male T2DM patients with and without PD, and healthy individuals with and without PD, without any associated comorbidities, except hypertension, aged 30 to 70 years old, non-smokers, without any previous periodontal or antibiotic treatment in the previous 3 months. Females included were not taking any hormonal treatment and were not pregnant. A complete clinical history was obtained from each patient and different parameters were evaluated such as crevice depth, erythema, pain, bleeding, tooth mobility, and keratinization of the gingiva, as well as the Gingival (Löe y Sillness) [17] and Plaque (Quigley y Hein, modified by Turesky) Indexes [18]. Glycated hemoglobin (HbA1c) tests were performed in T2DM patients to assess the control of their disease in the past 3-4 months. The research was submitted and accepted by the ethics committee and all patients signed a letter of informed consent according to the World Medical Association Declaration of Helsinki.

2.2. Sample Collections

Gingival-crevicular fluid samples were collected from periodontal pockets in patients with chronic periodontitis and from gingival sulcus in periodontally healthy patients. Four samples were collected from each patient by placing periopaper strips in the sulcus or pocket for 30 seconds. The oral and interproximal surfaces of teeth were cleaned free of supragingival plaque, dried gently with air, and kept dry with gauzes. Once samples were obtained, they were placed on sensors of Periotron® 6000 and the reading was correlated to the total volume of gingival fluid obtained. Samples of filter periopaper strips were kept in Eppendorf tubes at -20°C for later immunological analysis. For determination of the cytokine IL-18 and anti *P. gingivalis* antibodies, samples in filter periopaper strips were obtained with PBS 0.1M at pH 7.2 according to the volume measured by the Periotron® 6000.

2.3. Enzyme-Linked Immunoabsorbent Assay (ELISA) for Measurement of IL-18

IL-18 concentration was measured in gingival crevicular fluid samples by sandwich ELISA using a commercial kit according to the manufacturer's instructions (Biosource International, USA).

2.4. Porphyromonas Gingivalis Culture

Porphyromonas gingivalis ATCC 33277 was grown on blood agar plates for 5-6 days in an anaerobic chamber with an atmosphere containing 80% N₂, 10% H₂, and 10% CO₂. Then the strain was inoculated on thioglycolate medium and after 48 h of incubation bacteria were harvested by centrifugation at 10,000 x g for 15 min at 4°C, where supernatant and biomass were obtained.

2.5. Semi-purified Culture Filter Proteins from *Porphyromonas Gingivalis*

Semi-purified culture filter proteins were obtained by precipitation using ammonium sulfate (v/v) at 4°C. The precipitate was first diluted in PBS and then dialyzed against distilled water during 72 hours until absence of salts. The protein concentration was determined by the Bradford method [19] sterilized by filtration through a 0.2 µm pore size filter (Millipore), lyophilized, and stored at -20°C until use.

2.6. Detection of IgM and IgG Antibodies

Semi-purified culture filter proteins (0.5 µg/well in 200 µL acetates buffer pH 5.0) from *Porphyromonas gingivalis* ATCC 33277 were fixed on 96-well polystyrene plates overnight at 4°C. The unspecific binding was blocked by 5% skim milk (Difco) dissolved in PBS at 37°C for 1.5 h. Then, 100 µL of gingival crevicular fluid from patients and controls were added to the plate and incubated for 1 h at room temperature with shaking at 300 rpm. The bound antibodies were visualized using horseradish peroxidase-coupled goat anti-human IgG (γ-chain specific, Chemicon, 1:3000) or anti-human IgM (µ-chain specific, Chemicon, 1:5000) as secondary antibodies and incubated at 37°C for 1 h. The chromogen substrate solution was composed of ortho-phenylenediamine and hydrogen peroxide (Sigma); 1 N sulfuric acid was used as a stop reagent. The A₄₉₂ was read with a semiautomatic ELISA plate reader (iMark™ Microplate Absorbance Reader - Bio-Rad).

2.7. Statistical Analysis

The data was analyzed using the *Kruskal-Wallis* test with SPSS version 10.0 software. $P < 0.05$ was considered significant

3. Results

3.1. Study Population

Patients between 30-70 years old were studied. Of all patients, 80% were women and 20% were men (Fig. 1 and Table 1). Of all T2DM patients, 40% were receiving anti-hypertensive agents and 80% were receiving oral hypoglycemic agents.

Figure 1

Figure 1. Distribution of patients.

Table 1. Distribution of patients by age and sex. The age among all groups was very similar (51 on average) and females predominated.

Patients	Age	Male	Female
Diabetic patients with PD	53.02 ± 9.9 years	2	8
Diabetic patients without PD	50.90 ± 11.26 years	0	10
Non-diabetic patients with PD	51.67 ± 6.24 years	3	7
Healthy subjects	48.48 ± 11.65 years	3	7

In T2DM patients with chronic PD, the plaque and gingival index were 2 and 1, respectively; in T2DM patients without PD they were 1.01 and 0.22; for non-diabetic patients with chronic PD they were 2.03 and 0.74; and for the control group they were 0.40 and 0.24. Glycated hemoglobin (HbA1c) for T2DM patients with PD was 9.11% and for T2DM patients without PD was 7.37% (Table 2).

Table 2. In T2DM patients with PD, gingival indexes were deeper than in non-diabetic patients with PD, T2DM patients without PD, and the control group. Gingival index in T2DM without PD and in the control group was similar. Plaque index was higher in patients with PD, both diabetic and non-diabetic. However, T2DM without PD had a higher plaque index than the control group. Glycated hemoglobin (HbA1c) for T2DM patients with PD was higher than T2DM patients without PD. HbA1c was not evaluated in non-diabetic patients and the control group.

Patients	Plaque Index	Gingival Index	Glycated Hemoglobin
Diabetic patients with PD	2.17 ± 0.54	1.27 ± 0.62	9.11%
Diabetic patients without PD	1.28 ± 0.64	0.18 ± 0.21	7.37%
Non-diabetic patients with PD	2.06 ± 0.36	0.84 ± 0.36	N/A
Healthy subjects	0.65 ± 0.66	0.25 ± 0.16	N/A

Statistical significant differences between plaque and gingival indexes of all groups are shown in Table 3.

Table 3. No significant differences were found in gingival and plaque indexes between T2DM patients with PD and non-diabetic patients with PD, and between healthy individuals and diabetic patients without PD. Significant differences in both indexes were found in the remaining groups related.

Group comparison	Plaque index	Gingival Index
DP with PD vs DP without PD	P value: 0.0058 Significant	P value: 0.0001 Significant
DP with PD vs NDP with PD	P value: 0.6192 Not Significant	P value: 0.0913 Not Significant
DP with PD vs Healthy Individuals	P value: <0.0001 Significant	P value: 0.0002 Significant
DP without PD vs NDP with PD	P value: 0.0057 Significant	P value: 0.0002 Significant
DP without PD vs Healthy Individuals	P value: 0.0582 Not Significant	P value: 0.4744 Not Significant
NDP with PD vs Healthy Individuals in gingival and plaque index	P value: <0.0001 Significant	P value: 0.0004 Significant
DP= Diabetic Patients PD= Periodontal Disease NDP= No Diabetic Patients		

3.2. IL-18 in Gingival Crevicular Fluid

T2DM patients with chronic PD had lower levels of IL-18 than healthy subjects (7.0 ± 5.18 pg/mL versus 22.6 ± 3.36 pg/mL, $p < 0.001$) and non-diabetic patients with PD ($p < 0.001$). T2DM patients without PD had levels of 17.1 ± 7.28 pg/mL and non-diabetic patients with chronic PD had levels

of 36.7 ± 9.05 pg/mL ($p < 0.001$). Similar to T2DM patients with chronic PD, T2DM patients without PD had lower IL-18 production compared to healthy subjects ($p < 0.01$) or non-

diabetic patients with chronic PD ($p < 0.001$). On the other hand, non-diabetic patients with PD had higher levels of IL-18 than healthy subjects ($p < 0.001$) (Figure 2).

Figure 2. T2DM patients with PD had lower levels of IL-18 than healthy subjects ($P < 0.0001$) and the results obtained between T2DM patients without PD and healthy individuals ($P < 0.001$) were similar. On the other hand, non-diabetic patients with PD showed higher levels of IL-18 than healthy subjects ($P < 0.001$).

3.3. Detection of IgM and IgG antibodies

IgM was detected in 50% of T2DM patients with chronic PD, while it was detected in 40% of non-diabetic patients with chronic PD, where cut off was 0.055 optic density (O.D.) in reference with T2DM patients with chronic PD and the control group. IgG antibodies were detected in 30% of T2DM patients with chronic PD and in 100% of non-diabetic

patients with chronic PD, while 60% of healthy subjects had IgG antibodies (Figure 3). In T2DM patients without PD, IgM and IgG anti *P. gingivalis* were not detected. Negative control serum was obtained from the Pediatric Clinic of the University Hospital “Dr. José Eleuterio González” UANL to establish optical density cut off.

Figure 3. Comparative results of IgM and IgG antibodies against *P. gingivalis* show that only patients with PD had IgM antibodies in their gingival crevicular fluid. IgG levels were found only in T2DM patients with PD, non-diabetic patients with PD, and healthy individuals, and not in diabetic patients without PD.

4. Discussion

The justification for the utilization of gingival crevicular fluid is that it is an ideal sample for monitoring the changes occurring in the progression of PD since its collection is done by a non-invasive procedure. This fluid can transudate (in normal and basal conditions) or exudate (in inflammatory process) and contains a great amount of biochemical factors that offer a potential use as diagnostic or prognostic biomarkers of the biological status of the periodontium in health and disease. The fact that gingival crevicular fluid can contain biochemical mediators and/or metabolic products, such as antibodies, enzymes, inflammatory mediators, and products from tissue breakdown [20], makes it a great sample to use as a new diagnostic technique applicable in the clinical practice.

It is known that PD is the sixth complication of diabetes mellitus and that there is a bidirectional relationship between both. Taking this into account and the fact that IL-18 has been associated with a major predisposition to cardiovascular events [21] and nephropathy in T2DM patients [22], to induction of synthesis of TNF- α , IL-1 β , IL-8, and intracellular adhesion molecule-1 (ICAM-1), to neutrophil activation, and osteoclast activation, and that several studies have found elevated plasma levels of IL-18 in diabetic patients [23, 24] it was thought that elevated levels of IL-18 could be found in gingival crevicular fluid samples of diabetic patients.

There are studies that have measured IL-18 in gingival crevicular fluid in non-diabetic patients, and they have demonstrated that patients with chronic PD have IL-18 levels 7 to 10 times higher than healthy persons [25, 26]. In our study, IL-18 was only 62% higher in patients with chronic PD than in healthy persons. This lesser difference could be explained by the fact that our patients had lower gingival indexes than the patients in those studies. Gingival index is a reflection of the severity of the lesion, thus, we hypothesize that our patients with chronic PD had lower levels of IL-18 because their periodontal disease was less severe.

On the other hand, IL-18 levels were not elevated in T2DM patients with chronic PD. This could be the result of a lower *in situ* production and/or excretion of IL-18, and the periodontal damage could be produced by alterations in the host inflammatory response secondary to diabetes, which may lead to further dysregulation of immune-inflammatory responses in the periodontium causing increased periodontal destruction, but not by IL-18 action [27].

Another aspect that should be studied is the presence of IL-18 binding protein (IL-18BP) in gingival fluid. IL-18BP is a member of the immunoglobulin superfamily and acts as a negative regulator of IL-18 action. This 38- kDa soluble protein displays some sequence homology with the IL-18 Receptor (IL-18R) [28]. In addition, *in vitro* secretion of IL-18 and its natural IL-18 binding protein (IL-18BP) can be induced by *P. gingivalis* LPS in cultures of the human monocytic cell line [29].

Thus, an explanation could be that low levels of IL-18 may be due to the presence of IL-18R in gingival epithelia. It has been reported that the expression of IL-18R on renal and epithelial cell lines can be induced in response to TNF alpha and other proinflammatory cytokines [30]. Another important aspect which may explain the low levels of IL-18 in diabetic patients with PD, is the neutrophils' function in the inflammatory process. Due to a lower number of neutrophils (which produce IL-18) resulting from a defect in chemotaxis, there is less IL-18 production [31, 32]. However, in order to confirm these assumptions, we would need to find studies on the detection of IL-18BP or IL-18R in gingival fluid and gingival epithelia, respectively, and research if there is a difference in number of local neutrophils in gingival tissue or fluid in T2DM patients versus non-diabetic patients with periodontal disease.

We can conclude that the diabetic state influences the secretion of IL-18 and that the mechanisms involved in the development of PD in this study, can probably be due to other pro-inflammatory cytokines such as IL-1, TNF α , or IL-6, or due to the local effect of IL-18 bound on its receptor on blood vessels of gingiva producing vasodilation. This fact is supported by several studies that have found IL-18 in biopsies of gingival tissues in patients with PD. In a study realized by Johnson *et al* obtained gingival tissue biopsies and observed of IL-18 within gingiva [33]. On the other hand, Tardif *et al* found that IL-18 was not expressed or secreted by gingival or dermal fibroblasts with or without stimulation of lipopolysaccharide from *P. gingivalis* or *E. coli* [34]. On the other hand, in non-diabetic patients with chronic PD, IL-18 levels were the highest, meaning that this cytokine could be involved in the inflammatory periodontal process.

P. gingivalis is one of the pathogens most commonly found in PD in T2DM patients and in non-diabetic patients [35, 36] Ojima *et al* observed that *P. gingivalis* was the unique pathogen significantly related in the deterioration of PD [37]. Onoue *et al* found that plasma levels of IgG against *P. gingivalis* LPS were higher in patients with periodontitis than in healthy persons; while IgM levels were relatively lower and there were no significant difference between patients and healthy persons [38]. In our study, we decided to determine the levels of IgM and IgG antibodies against *P. gingivalis* in gingival crevicular fluid of diabetic and non-diabetic patients with and without chronic PD. Our results suggest that two events are occurring; first, there is a good IgM antibody response against this microorganism in patients with PD. Second, the lower levels of IgG encountered in T2DM patients with PD compared to non-diabetic patients with PD could be the result of the formation of immune complexes with soluble antigens of *P. gingivalis*. There are several studies that strongly suggest that immune complexes are involved in the inflammatory process behind periodontitis [39, 40], and this may be a potential explanation for the diminished levels of IgG in T2DM patients with chronic PD and for their higher periodontal affectation (deeper periodontal pockets, tooth mobility, etc.). On the other hand,

periodontally healthy individuals can present antibodies against *P. gingivalis* and perhaps prevent the periodontal damage by these bacteria and the high levels of IgG would then be due to immunological memory.

Gingival index among diabetics with PD and non-diabetics with PD was not significantly different. This suggests that diabetes did not influence in the sulcus depth. Dental Plaque was present among healthy and diabetic subjects with no PD. (Table 3)

We did not find information about the effect of antihypertensive or hypoglycemic agents on IL-18 and antibody production

5. Conclusion

This study showed that T2DM patients with PD have lower levels of IL-18 and lower levels of IgG antibodies against *P. gingivalis* in their gingival crevicular fluid compared to the non-diabetic patients. These results contradict other studies that have found high levels of IL-18 in plasma of diabetic patients, demonstrating that *in situ* levels of certain cytokines are not always comparable to the levels in plasma. Furthermore, this difference could be the result of an immune deficiency secondary to the diabetic state. The low percentage of IgG present in the crevicular fluid of diabetic patients with PD could mean that immune complexes are being formed, and this could explain the greater affection seen in these patients.

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