Gene expression of inflammatory immune factors and clinical parameters in diabetes and nondiabetes patients with periodontal disease

Expressão genética de fatores imunológicos inflamatórios e parâmetros clínicos em pacientes com diabetes e não diabéticos com doença periodontal

Expresión genética de factores inmunes inflamatorios y parámetros clínicos en pacientes diabéticos y no diabéticos con enfermedad periodontal

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Abstract
Objective: This study evaluated clinical, glucose, and immunological parameters in patients with type 2 diabetes mellitus (DM) compared to those without systemic alterations (NDM), both with generalized chronic periodontitis. Methodology: Twenty-one patients were selected with indications of tooth loss. Surgeries were performed using the Widman flap modified to obtain a gingival collar at 1 mm from the gingival margin. Before the surgical procedure, the following clinical parameters were evaluated: pocket probing depth (PPD), gingival recession (GR), clinical attachment level (CAL), and bleeding on probing (BOP). Fasting glucose levels (FGL) and glycosylated hemoglobin Hba1c (HbA1c) were also assessed. During the surgery, gingival tissue samples were collected and frozen for later laboratorial analysis. The samples were processed to obtain mRNA, cDNA and determine the gene expression of the immune parameters IL-1β, IL-6, TNF-α, IL-10 and NF-kB by real-time polymerase chain reaction (RT-PCR). Data were analyzed statistically considering p<0.05. Results: The clinical and glucose parameters BOP, FGL, and HbA1c were statistically higher in the DM group. RNAm levels of IL-1β, TNF-α, and NF-kB were higher in the DM group (p<0.05). Conclusion: The presence of diabetes and hyperglycemic status increase the levels of pro-inflammatory immune factors and severity of the periodontal disease.

Keywords: Diabetes mellitus; Periodontal disease; Cytokines; Real-Time Polymerase Chain Reaction.

Resumo
Objetivo: Este estudo avaliou parâmetros clínicos, glicêmicos e imunológicos em pacientes com diabetes mellitus tipo 2 (DM) comparados a aqueles sem alterações sistêmicas (NDM), ambos com periodontite crônica generalizada. Metodologia: Vinte e um pacientes foram selecionados com indicação de perda dentária. As cirurgias foram
realizadas con retalho de Widman modificado para obtenção de colar gengival a 1 mm da margem gengival. Antes do procedimento cirúrgico, os seguintes parâmetros clínicos foram avaliados: profundidade de sondagem de bolsa (PPD), recessão gengival (GR), nível de inserção clínica (CAL) e sangramento à sondagem (BOP). Níveis de glicose em jejum (FGL) e hemoglobina glicosilada Hba1c (HbA1c) também foram avaliados. Durante a cirurgia, amostras de tecido gengival foram coletadas e congeladas para posterior análise laboratorial. As amostras foram processadas para obtenção de mRNA, cDNA e determinada a expressão gênica dos parâmetros inmunológicos IL-1β, IL-6, TNF-α, IL-10 e NF-kB por reação em cadeia da polimerase em tempo real (RT-PCR) Os dados foram analisados estatisticamente considerando p <0,05. 

Resultados: Os parâmetros clínicos e glicêmicos BOP, FGL e HbA1c foram estatisticamente maiores no grupo DM. Os níveis de RNAm de IL-1β, TNF-α e NF-kB foram maiores no grupo DM (p <0,05). 

Conclusão: A presença de diabetes e o estado hiperglicêmico aumentam os níveis de fatores imunes pró-inflamatórios e a gravidade da doença periodontal. 

Palavras-chave: Diabetes mellitus; Doença Periodontal; Citocinas; Reação da Polimerase em Cadeia em Tempo Real.

Resumen

Objetivo: Este estudio evaluó parámetros clínicos, glucémicos e inmunológicos en pacientes con diabetes mellitus (DM) tipo 2 en comparision con aquellos sin alteraciones sistémicas (NDM), ambos con periodontitis crónica generalizada. 

Metodología: Se seleccionaron 21 pacientes con indicación de pérdida dentaria. Las cirugías se realizaron utilizando el colgajo de Widman modificado para obtener un collar gengival a 1 mm del margen gengival. Previo al procedimiento quirúrgico, se evaluaron los siguientes parámetros clínicos: profundidad de sondaje de la bolsa (PPD), recesión gengival (GR), nivel de inserción clínica (CAL) y sangrado al sondaje (BOP). También se evaluaron los niveles de glucosa en ayunas (FGL) y la hemoglobina Hba1c glicosilada (HbA1c). Durante la cirugía, se recolectaron muestras de tejido gengival y se congelaron para su posterior análisis de laboratorio. Las muestras se procesaron para obtener ARNm, ADNc y se determinó la expresión génica de los parámetros inmunológicos IL-1β, IL-6, TNF-α, IL-10 y NF-kB mediante Reacción en Cadena en Tiempo Real de la Polimerasa (RT-PCR). Los datos fueron analizados estadísticamente considerando p <0,05. 

Resultados: Los parámetros clínicos y de glucosa BOP, FGL y HbA1c fueron estadísticamente superiores en el grupo DM. Los niveles de RNAm de IL-1β, TNF-α y NF-kB fueron mayores en el grupo DM (p <0,05). 

Conclusión: La presencia de diabetes y el estado hiperglicemia aumentan los niveles de factores inmunes proinflamatorios y la gravedad de la enfermedad periodontal. 

Palabras clave: Diabetes mellitus; Enfermedad periodontal; Citocinas; Reacción de la Polimerasa en Cadenas en Tiempo Real.

1. Introduction

The periodontal disease (PD) is a chronic infectious condition characterized by the destruction of protective tissues (gingiva) and tooth support, periodontal ligament, cementum, and alveolar bone (Herring & Shan 2006; Negroto et al., 2013). This process occurs in response to the bacterial antigens of the dental biofilm that accumulate along the gingival margin (Herring & Shan 2006). The interactions between bacteria and host determine the nature of the disease, which has been divided into gingivitis and periodontitis.

Gingivitis is the initial manifestation of the illness and is considered an inflammatory condition, limited to the gingival margin. It is characterized clinically by edema, hyperemia, and bleeding (Ayilavarapu et al., 2014; Jindal et al. 2015). Untreated gingivitis may progress to periodontitis which is influenced by microbiota and individual defense response (Tanner et al., 1998; Weinspach et al., 2013). Periodontitis is the result of an inflammatory immune response of the host caused by polymicrobial dysbiosis, characterized by infiltration of the gingival tissues by neutrophils, monocytes, macrophages and lymphocytes and the generation of high concentrations of mediators, including cytokines, chemokines, arachidonic acid metabolites and proteolytic enzymes. The nature and extent of this host immune response are important determinants of the susceptibility and progression of periodontitis (Tomas et al., 2017).

Diabetes is a chronic disease that affects the metabolism of carbohydrates, proteins and lipids and is characterized by hyperglycemia due to deficiency in the secretion or action of insulin, or both (Grover et al., 2013). Symptoms of hyperglycemia include polyuria, polydipsia, polyphagia, weight loss, and blurred vision. Chronic hyperglycemia is associated with increased development of and susceptibility to infections (American Diabetes Association, 2013).

Numerous factors are involved for diabetic patients to present a greater severity of periodontal disease, such as
vascular abnormalities, neutrophil dysfunction, non-enzymatic glycosylation, altered collagen metabolism and altered monocytic response (Buzinini et al., 2014). Modifications in the host's immune response may exert a wide influence on the higher prevalence and severity of periodontal disease in diabetic patients. The function of immune cells, including neutrophils, macrophages and monocytes, is altered in diabetes (Santos et al., 2010) because there is a reduction in the chemotactic and phagocytic functions of PMN and a depression of humoral response, which causes diabetic patients to present a reduction in the ability to fight infections, including periodontal infection (Tan et al., 2006). Although the role of neutrophils is decreased in diabetes, monocytes/macrophages are over-regulated in relation to bacterial antigens, resulting in an increased production of cytokines and proinflammatory mediators (Santos et al., 2010).

Based on this, the aim of this study was to evaluate the effect of diabetes mellitus on clinical parameters and the gene expression of the inflammatory markers IL-1β, IL-10, IL-6, NF-κB and TNF-α in the gingival tissue of patients with generalized chronic periodontal disease.

2. Methodology

Study population

Twenty-one patients (14 non-diabetics – NDM and 7 diabetics – DM), aged 42 to 64 years, were selected to participate in this study. Samples size was based on previous pilot studies evaluating inflammatory markers in patients with periodontitis and diabetes (Longo et al., 2015; Mesia et al., 2016; Franco et al., 2017). All subjects were recruited from the Department of Periodontology, School of Dentistry, Fluminense Federal University – UFF, Nova Friburgo, Rio de Janeiro State, Brazil, for a period between 2013 and 2017. The study protocol (CAAE 33417414.0.0000.5626) was approved by the Ethics Committee of the Fluminense Federal University – UFF. Prior to participation, the study purpose and procedures were fully explained to all patients, who eventually signed a written informed consent form pursuant to the Declaration of Helsinki. All subjects underwent anamnesis and clinical and periodontal examination. Gingival biopsies were collected from a tooth indicated for exodontia attributable to advanced periodontitis to obtain representative areas of inflammation (Duarte et al., 2007; Cesar-Neto et al., 2006; Benatti et al., 2008). Inclusion criteria were patients with severe periodontitis (Stage III), generalized distribution and moderate rate of progression (Grade B), according to 2018 new classification scheme for periodontal and peri-implant diseases and conditions (Caton et al., 1999). Exclusion criteria included any systemic disorder that would require antibiotic prophylaxis or affect the periodontitis condition, except for diabetes; in this case, patients who received scaling and root planing and/or used systemic or subgingival antimicrobials or anti-inflammatory medication within 6 months prior to baseline examination were excluded (Cesar-Neto et al., 2006). All patients were non-smoking and female patients were not pregnant, lactating or using any method of birth control (Duarte et al., 2007; Cesar-Neto et al., 2006; Benatti et al., 2008).

Experimental design

Fasting glucose levels (FGL) and glycated α-hemoglobin (HbA1c) were obtained from patients evaluating recent clinical laboratories exams. An experienced periodontist evaluated the clinical parameters for previous surgical procedure. Each selected tooth was measured according to periodontal parameters: plaque index (PI) (presence/absence), bleeding on probing (BOP) (presence/absence), pocket probing depth (PPD), gingival recession (GR) and clinical attachment level (CAL) using a periodontal probe PCP15 (PCP-UNC15, Hu-Friedy, Chicago, IL). All results were recorded in the six sites (mesio-buccal, buccal, disto-buccal, mesio-lingual, lingual, disto-lingual). Gingival biopsies were obtained from two sites diagnosed with periodontitis by using the Widman flap modified to obtain a gingival collagen at 1 mm from the gingival margin, tissues
were rinsed with cold sterile saline solution, and stored in a tube containing RNAlater® (Ambion Inc., Austin, TX, USA) at -80°C (Duarte et al., 2007; Cesar-Neto et al., 2006; Benatti et al., 2008). Gingival biopsies were divided into two different groups according to the systemic status as follows – Control group (NDM) and Diabetes group (DM). The control group was formed by subjects who were systemically healthy and clinically diagnosed with generalized chronic periodontitis with probing depth ≥ 5 mm and bleeding on probing (n=14) (Caton et al., 1999). In parallel, the diabetes group was formed by 2 type diabetic individuals characterized either by daily metformin consumption (blood glucose levels >100 mg/dl) and clinically diagnosis of chronic periodontitis (Duarte et al., 2007; Benatti et al., 2008; Duarte et al., 2009).

Primer’s design, RNA extraction and qPCR assays

For checking the mRNA levels of IL-1β, IL-10, IL-6, NF-κB and TNF-α, a total RNA from gingival biopsies was extracted using the TRIZOL reagent (Gibco BRL, Life Technologies, Rockville, MD, USA) according to the manufacturer’s recommendations. RNA samples were resuspended in diethylpyrocarbonate-treated water and stored at -80°C (Duarte et al., 2007; Benatti et al., 2008; Duarte et al., 2009). The RNA concentration was determined from the optical density (Duarte et al., 2009) using a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, EUA) followed by reaction using the first-strand cDNA synthesis kit (Roche Diagnostic Co., Indianapolis, IN, USA) following the manufacturer’s recommendations. Primers were acquired by Idt (Síntese Biotecnologia, Rio de Janeiro, RJ, Brazil). Experiments were run in triplicate with comparable results. The amplification profile and primer sequences used in the present study were described by Duarte et al. (Duarte et al., 2009).

The quantitative polymerase chain reaction (PCR) was performed in the Eco Real Time PCR system – Illumina (Illumina inc. San Diego, CA, USA) using the SYBR Green kit (Applied Biosystems, Thermo Fisher scientific Inc., Rio de Janeiro, RJ) (Duarte et al., 2007; Cesar-Neto et al., 2006). For each run, water was the negative control. The reaction product was quantified with the Relative Quantification tool (LightCycler_Software 4; Roche Diagnostics GmbH), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference gene (Cesar-Neto et al., 2006).

Statistical analysis

Statistical tests were accomplished using the Statistix software (Analytical Software, Tallahassee, USA, Version 8, 2003). The Shapiro Wilk test was used to check normality of variables. The Mann-Whitney tests was used to compare hematological and clinical parameters (Age, PI, BOP, PPD, GR, CAL, FGL and HbA1c), and t Student test to compare mRNA levels between NDM and DM groups. Statistical significance for all variables was defined at the 5% level.

3. Results

Hematological and clinical observations

All hematological parameters were higher in the DM group, however, statistical difference between the groups was observed only for FGL and HbA1c (p<0.05). Clinical parameters were similar in both DM and NDM groups, except to bleeding on probing (BOP) which that was higher in the DM group (p<0.05) [Table 1].
Table 1: Clinical and Hematological parameters for non-diabetics (NDM) and diabetics (DM) groups. Data were expressed in medians and quartiles.

| Parameter          | NDM (n=14)       | DM (n=7)      | p value |
|--------------------|------------------|--------------|---------|
| Age (years)        | 52.5 (42-60.75)  | 61 (48-64)   | 0.1982  |
| PI (%)             | 83 (0-100)       | 100 (25-100) | 0.1236  |
| BOP (%)            | 50 (4-100)       | 100 (100-100) | 0.0461  |
| PPD (mm)           | 5 (5-5.75)       | 5 (5-5.75)   | 0.8009  |
| GR (mm)            | 0 (0-2)          | 1.5 (0-3)    | 0.6899  |
| CAL (mm)           | 6 (5-8.75)       | 7 (5.25-8)   | 0.7463  |
| FGL (mg/dl)        | 82 (76.5-87.25)  | 141 (131-182) * | 0.0000 |
| HbA1c              | 4.75 (4.5-5.0)   | 6.9 (6.2-8.1) * | 0.0000 |

PI - Plaque Index; BOP - Bleeding on probe; PPD - pocket probing depth; GR - Gingival recession; CAL - Clinical attachment level, FGL - Fasting Glucose Levels, HbA1c - glycated hemoglobin.

* Statistical difference between the NDM and DM groups (Mann-Whitney test, p < 0.05)

Source: Authors.

Gene expression analysis

Data analysis demonstrated that mRNA levels for IL-1β, TNF-α and NF-kB were significantly higher in the diabetes type 2 group compared with non-diabetes group (p=0.045, 0.046, 0.050, respectively). In addition, no differences were observed for mRNA levels of IL-6 and IL-10 [Figure 1].

Figure 1: Mean and standard deviation of mRNA levels (mRNA/GAPDH) of IL-1 β, IL-6, IL-10, NF-kB and TNF-α in the gingival tissues from sites with periodontitis of the non-diabetics (NDM) and diabetics (DM) groups. Different letters indicate significant statistical differences determined by t Student test (p < 0.05).

Source: Authors.

4. Discussion

This study evaluated the gingival tissue gene expression of cytokines in the periodontal tissue of patients with
moderate to advanced periodontal disease, with or without diabetes mellitus. In general, the results demonstrated that there were statistically significant differences in the IL-1β, TNF-α and NF-κB expression and in the bleeding on probing to DM group.

Hematological tests also confirmed the intergroups difference; the DM group had higher FGL and HbA1c results. Considering that diabetes is associated with a greater extent and severity of PD (Bastos et al., 2012; González-Moles & Ramos-García, 2021), for deregulating the humoral and cellular response to the microbial challenges, it may thus influence the response in the gene expression of cytokines in periodontal tissue (Geerlings & Hoepelman, 1999). Biological mechanisms for this association include the persistence of inflammatory infiltrate, increased periodontal bone loss, because of the decreased bone formation, and increased resorption in diabetic patients (Herring & Shan, 2006; Negrato et al., 2013; Ayilavarapu et al., 2014; Genco et al., 2020). In addition to the fact that hyperglycemia predisposes the patient to a hyperinflammatory state, modulating not only macrophages activities but also an increase in oxidative stress, it thus triggering the release of inflammatory cytokines in diabetic patients (Bastos et al., 2012).

The clinical parameters of this study presented higher values for BOP to the diabetic group. However, PI, PPD, GR and CAL were not different between the groups, according to previous studies (Tan et al., 2006; Lalla et al., 2006; Duarte et al., 2007). Karjalainen e Knuuttila (Karjalainen & Knuuttila, 1996) found a lower incidence of gingivitis in well controlled diabetic patients than in poorly controlled ones. The poorly controlled diabetics exhibited much more severe inflammation. Kakade et al. (Kakade et al., 2014) reported higher levels of bleeding probing, depth of probing, and clinical attachment level to the DM group. Taylor et al. (Taylor et al., 1998) showed significantly greater progression of alveolar bone loss in diabetic type 2 subjects after 2 years follow-up. Preshaw et al. (Preshaw et al., 2012) reported a higher clinical attachment levels to diabetic children. Novaes et al. (Novaes et al., 1997) described greater bone loss in diabetics.

Regarding genetic expression of inflammatory immune factors, Southerland et al. (Southerland et al., 2000) state that increased Advanced Glycation End Products (AGE), because of the hyperglycemic state associated with an inflammatory host response, is the cause of the exacerbation of diabetes systemic complications and severity of periodontal disease (Deng et al., 2021). The AGE-RAGE (RAGE - receptors for AGE) interactions result in the production of inflammatory mediators such as IL-1β, TNF-α and IL-6, and enzymes like MMPs which are responsible for bone resorption (Preshaw & Taylor, 2011). In the present study, although IL-1β, TNF-α and IL-6 were higher in the DM group, statistical difference between the groups (DMx NDM) were found only for IL-1β and TNF-α. These results are according to Cutler et al. (Cutler et al., 1999), Garcia-Hernández et al. (Garcia-Hernandez et al., 2012) and Mesia et al. (Mesia et al., 2016) that found similar increase in the IL-1β expression in diabetic patients with periodontitis. The higher levels of IL-1β are associated with the severity of periodontitis (Salvi et al., 1997; Tan et al., 2020). Tan et al. (Tan et al., 2006), Bastos et al. (Bastos et al., 2012), Negrato et al. (Negrato et al., 2013), Gomes et al. (Gomes et al., 2016) also related high levels of TNF-α in diabetics. TNF-α was shown to induce insulin resistance and a potential link between the progression of periodontal disease and worsening of the diabetic state (Chang & Lim, 2012). Considering IL-6, our results were similar to Cole et al. (Cole et al., 2008) and Duarte et al. (Duarte et al., 2007).

Effective immunological responses against pathogens depend on the balance between pro- and anti-inflammatory reactions and IL-10 is essential in controlling or preventing the production of important inflammatory mediators such as TNF-α and IL-6 to control this balance. IL-10 has shown to be widely expressed in inflamed periodontal tissues and it is associated with decreased severity of periodontitis (Carey et al., 2012). In the present study, there was no statistical difference in the IL-10 levels between the groups. This result was expected because both group of patients were diagnosed with chronic periodontitis and presented similar periodontal conditions.

NF-κB is a family of ubiquitous transcription factors which is produced by immune stimulated by various activators such as TNF-α, IL-1, lipopolysaccharide (LPS) and others to induce the expression of target gene such as cytokines, MMP,
RANKL etc., related to bone destruction and progression of periodontal disease. Risk factors of periodontal disease such as diabetes, smoking, and obesity activate NF-κB leading to persistent inflammation (Caamaño & Hunter, 2002). In this study, NF-κB levels were superior in DM patients compared to NDM. Vernal et al. (Vernal et al., 2004) correlated the increased expression of NF-κB with the presence of the binding receptor (RANKL), in patients with periodontal disease. From another point of view, Zheng et al. (Zheng et al., 2018) suggested that diabetes induces a change in the periodontal ligament fibroblast gene expression and it may increase neutrophil recruitment and bone resorption, which can be explained by means of the activation of glycaemia-induced binding NF-κB to RANKL. Arabaci, et al. (Arabaci et al., 2010) associated periodontitis with diabetes and reported an increase in NF-κB activation.

It is important to point out the limitations of this study, such as small sample size and the limited number of genes evaluated. Besides, large cytokine levels can be different in the studies due to the variety of techniques that can be used in the detection of mediators and other factors such as time from biopsy to analysis, number of samples, severity of periodontitis and diabetic controls (Duarte et al., 2012).

5. Conclusion
Based on these preliminary findings, this study suggested that DM type 2 and hyperglycemic status can influence the immune response exacerbating the inflammatory state in patients with chronic periodontal disease.

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