PRISCILLA FARIAS NAIFF

EFEITOS DA PERIODONTITE SOBRE A RESPOSTA IMUNE DE INDIVÍDUOS COM OU SEM DIABETES MELLITUS DO TIPO 2.

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Tese apresentada como requisito parcial para obtenção do Título de Doutor em Ciências da Saúde pelo programa de Pós-gradução em Ciências da Saúde da Universidade de Brasíia.

Orientadora: Profª. Drª. Maria do Carmo Machado Guimarães
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Aprovada em 26 de novembro de 2018

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Aos Meus Pais
Roberto Daibes Naiff e Maricleide de Farias Naiff

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RESUMO

Uma vez que a periodontite consiste em uma doença inflamatória e infecciosa, a resposta imune contra periodontopatógenos pode resultar em uma mudança na função leucocitária comparada àquela observada em indivíduos periodontalmente saudáveis ou naqueles com Diabetes Mellitus do tipo 2 (DM2). Este trabalho teve como objetivo fazer uma breve revisão da literatura sobre a importância do tratamento periodontal mecânico em indivíduos com diabetes e periodontite. Também objetivou avaliar, de forma inédita, em amostras provenientes de sangue periférico, a produção de corpúsculos lipídicos por monócitos na periodontite associada ou não ao DM2. Foi ainda analisada a fagocitose por neutrófilos e monócitos. Além disso, foi verificada a produção de radicais peróxido pelos fagócitos. Também foram acessados os dados referentes ao hemograma e lipidograma completos, dosagem de proteína C reativa (PCR), glicemia em jejum e hemoglobina glicada. 58 participantes, 23 homens e 35 mulheres, com diabetes tipo 2 ou sistemicamente saudáveis foram divididos em quatro grupos: controle (16), periodontite (14), diabetes (11) e diabetes com periodontite (17). Os seguintes parâmetros clínicos periodontais foram avaliados: profundidade de sondagem, nível clínico de inserção, índice de placa visível e índice de sangramento à sondagem. Imediatamente após a obtenção das amostras, os corpúsculos lipídicos foram obtidos pelo método de marcação citoquímica com óleo vermelho O (*Oil-Red-O*). Foi realizado o teste de atividade fagocitária por aderência em lâmina de microscopia de luz e para obtenção do radical peróxido*,* o teste *nitroblue de tetrazolium* (NBT). A contagem total de leucócitos foi avaliada no hemograma. O lipidograma, a glicemia de jejum, a hemoglobina glicada (A1c) e os níveis de PCR também foram avaliados por análise bioquímica de amostras de sangue. Quando o grupo com periodontite foi comparado com indivíduos saudáveis, houve um aumento na frequência de monócitos com corpúsculos no citoplasma em amostras contendo leveduras opsonizadas ($p = 0,012$) ou não ($p = 0,003$), Kruskal-Wallis. Além disso, o índice corpuscular aumentou nos pacientes com diabetes e periodontite ($p < 0,001$ não sensibilizados; $p = 0,022$ sensibilizado; Kruskal-Wallis) comparado ao grupo diabetes sem periodontite. Em relação à fagocitose no grupo controle, observou-se redução significativa da atividade fagocitária dos neutrófilos nas amostras não opsonizadas em periodontite ($p = 0,008$, Kruskal-Wallis) e nas amostras opsonizadas essa redução foi observada em pacientes com diabetes e periodontite ($p = 0,029$, Kruskal-Wallis) quando foi utilizado um estímulo de levedura por fagócito na proporção de 20:1 (levedura:monócito). Nas amostras sensibilizadas, os monócitos mostraram uma redução na função fagocítica dos indivíduos com periodontite nas proporções 5:1 e 20:1, em relação aos controles ($p = 0,018$ e 000,7, respectivamente; Kruskal-Wallis). Também houve redução significativa da atividade fagocítica de monócitos em indivíduos com diabetes e doença periodontal em relação aos indivíduos saudáveis ($p = 0,0007$, sensibilizado; Kruskal-Wallis, proporção 20:1). Não houve diferença significativa na produção de superóxido entre os grupos avaliados. Os pacientes com as doenças associadas também apresentaram níveis mais elevados de PCR ($p < 0,001$, Kruskal-Wallis) em comparação aos pacientes com somente diabetes. Nossos resultados sugerem que a periodontite pode contribuir para uma suscetibilidade sistêmica ou diminuição das respostas imunes inatas com o prejuízo na atividade fagocítica e com maior frequência de monócitos contendo corpúsculos lipídicos. Estes fatos poderiam levar ao aumento do risco à aquisição de doenças sistêmicas em indivíduos saudáveis. Além disso, o aumento dos níveis de PCR encontrados em pacientes com diabetes e periodontite poderiam favorecer o surgimento de complicações nesta população.

Palavras chave: Periodontite crônica; Diabetes; Neutrófilos; Monócitos; Fagocitose; Radicais de oxigênio; Corpúsculos lipídicos.

ABSTRACT

Since periodontitis consists of an inflammatory and infectious disease, the immune response against periodontopathogens may result in a change in leukocyte function compared to that observed in periodontally healthy individuals or those with Type 2 Diabetes Mellitus (DM2). This study aimed to make a brief review of the literature on the importance of mechanical periodontal treatment in individuals with diabetes and periodontitis. It also aimed to evaluate, in an unprecedented way, in samples from peripheral blood, the production of lipid corpuscles by monocytes in periodontitis associated or not to DM2. Phagocytosis was also analyzed for neutrophils and monocytes. Besides, the production of peroxide radicals by phagocytes was verified. Data were also accessed for complete blood count and lipidogram, C-reactive protein dosage, fasting glycemia, and glycated hemoglobin. 58 participants, 23 men and 35 women with DM2 or systemically healthy were divided into four groups: control (16), periodontitis (14), diabetes (11) and diabetes with periodontitis (17). The following periodontal clinical parameters were evaluated: probing depth, clinical attachment level, visible plaque index and bleeding on probing. Immediately after obtaining the samples, the lipid corpuscles were obtained by the cytochemical labeling method with red oil O (Oil-Red-O). Phagocytic activity was performed by adherence in a light microscope slide and to obtain the peroxide radical, the tetrazolium nitroblue test (NBT) was also conducted. The total count of leukocytes was evaluated on the blood count. Lipidogram, fasting glycemia, glycated hemoglobin (A1c) and CRP levels were also evaluated by biochemical analysis of blood samples. When periodontitis' group was compared with healthy individuals, there was an increase in the frequency of monocytes with corpuscles in their cytoplasm in samples containing opsonized yeasts ($p = 0.012$) or not ($p = 0.003$), Kruskal-Wallis. Additionally, corpuscular index was increased in patients with diabetes and periodontitis ($p < 0.001$ non-sensitized; $p = 0.022$ sensitized; Kruskal-Wallis) compared to diabetes alone. Regarding phagocytosis of the control group, a significant reduction in the phagocytic activity of neutrophils in non-opsonized samples in periodontitis ($p = 0.008$, Kruskal-Wallis) was observed and, in the opsonized samples, this reduction occurred in patients with diabetes and periodontitis ($p = 0.029$, Kruskal-Wallis) when a 20:1 (yeast: monocyte) phagocyte yeast stimulus was used. In the sensitized samples, monocytes showed a reduction in the phagocytic function of the individuals with periodontitis in the proportions 5:1 and 20:1, concerning the controls ($p = 0.018$ and 000.7, respectively; Kruskal-Wallis). There was also a significant reduction in monocyte phagocytic activity in individuals with diabetes and periodontal disease compared to healthy subjects ($p = 0.0007$, sensitized; Kruskal-Wallis, 20: 1 ratio). There was no significant difference in the production of superoxide between the evaluated groups. Patients with both diseases also presented higher CRP levels ($p < 0.001$, Kruskal-Wallis) compared to patients with diabetes. Our results suggest that periodontitis may contribute to a systemic susceptibility or fragility of the innate's immune responses to the impairment of phagocytic activity and a higher frequency of monocytes with lipid bodies. These facts could lead to an increased risk for the acquisition of systemic diseases in healthy individuals. In addition, higher CRP levels found in patients with diabetes and periodontitis could favor the onset of complications in this population.

Key words: Periodontitis; Diabetes; Neutrophils; Monocytes; Fagocytosis; Reactive oxygen species; Lipid droplets.

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INTRODUÇÃO

1.1 DIABETES MELLITUS DO TIPO 2

O Diabetes mellitus (DM) do tipo 2 representa um importante problema de saúde pública em nível mundial. Trata-se de um distúrbio metabólico que resulta em defeitos da secreção de insulina ou de sua ação, levando à hiperglicemia crônica (1). A hiperglicemia crônica em pacientes com diabetes está associada a outras doenças ou complicações, como retinopatias, nefropatias, neuropatias periféricas e doenças cardiovasculares (1). O DM deriva de uma combinação da resposta compensatória inadequada à secreção e resistência à insulina, promovendo deficiência na liberação desse hormônio (2). A insulina é produzida e liberada pelas células β das ilhotas de Langerhans no pâncreas e é exclusivamente responsável pela redução da glicose no sangue (2,3).

1.2 PERIODONTITE

A periodontite consiste em uma doença inflamatória crônica multifatorial associada a uma disbiose do biofilme e caracteriza-se por destruição do periodonto de suporte. As principais manifestações clínicas incluem perda de inserção clínica, perda do osso alveolar, presença de bolsas periodontais e sangramento gengival (1,4). Constitui um grande risco para a mutilação dentária, disfunção mastigatória e é considerada a sexta complicação do DM (5). Micro-organismos e seus produtos oriundos das bolsas periodontais podem se disseminar intravascularmente por todo o corpo humano (1). Entretanto, pela ausência de sintomatologia dolorosa, na maioria das vezes, a periodontite geralmente não é percebida pelos seus portadores e nem pelos profissionais de saúde, embora possa estar associada a várias condições sistêmicas (1,6).

O gatilho para a periodontite origina-se com a disbiose e consequente colonização dos tecidos periodontais e proliferação por micro-organismos patogênicos como *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Tannerella forsythia, Prevotella intermedia, Prevotella nigrescens, Fusobacterium nucleatum,* dentre outras, que se encontram frequentemente associadas umas às outras (7).

Esses periodontopatógenos estimulam células do sistema imune como neutrófilos e monócitos, o que pode estimular uma resposta inflamatória local ou sistêmica exacerbada, dependendo das condições imunológicas do hospedeiro (8,9). Assim, mudanças na contagem ou na função leucocitária em sangue periférico ou no tecido podem indicar a presença de doenças infecciosas e / ou inflamatórias (10, 11,12).

1.3 ASSOCIAÇÃO ENTRE DIABETES E PERIODONTITE

A associação da periodontite com DM tem sido investigada e estudos mostram que existe uma correlação definida entre ambos (13,14). Pode-se observar que indivíduos com diabetes e controle glicêmico inadequado têm maior probabilidade de desenvolver doença periodontal grave. Em uma via de mão dupla, a periodontite também pode interferir no controle glicêmico desses indivíduos, pois tendem a ter níveis mais elevados de hemoglobina glicada (A1c) (13). Também parece existir uma relação entre a gravidade da periodontite e a dislipidemia, o estresse oxidativo (15) e as complicações cardio-renais do diabetes (16).

A periodontite avançada, quando se apresenta como comorbidade em pacientes com diabetes e doença renal crônica, aumenta o risco de mortalidade geral em 12% e em 8% o risco de morte por complicações cardiovasculares nesses mesmos pacientes, após dez anos de nefropatia (17).

O diabetes é um fator que aumenta em três vezes o risco à doença periodontal (18). A dificuldade do controle glicêmico em pacientes com diabetes aumenta a probabilidade de desenvolvimento de periodontite grave devido à redução dos mecanismos de defesa no hospedeiro e aumento da suscetibilidade a infecções (13).

Evidências indicam que a periodontite também aumenta o risco de desenvolver diabetes e suas complicações, mas os mecanismos imunopatológicos, pelos quais isto ocorre, ainda não são totalmente compreendidos (13).

1.4 CORPÚSCULOS LIPÍDICOS

A maioria das células eucarióticas contêm quantidades variadas de inclusões lipídicas citosólicas chamadas corpúsculos lipídicos ou gotículas lipídicas que regulam a hidrólise e armazenamento de lipídios neutros (19). A formação destas inclusões lipídicas durante processos infecciosos é um fenômeno bem regulado que pode ter implicação na patogênese e um importante papel na modulação da resposta imune.

Sua estrutura compreende uma monocamada externa de fosfolipídios e um núcleo central rico em lipídios neutros como triglicerídeos e ésteres de esteróis (20). São as principais organelas celulares produtoras de eicosanóides e a chave no processo de sinalização intracelular e no processo inflamatório (21). Os eicosanóides (prostaglandinas, leucotrienos e outros) são produzidos a partir da transformação do ácido araquidônico em resposta às citocinas inflamatórias, como o TNF-α, a IL-1β e a linfotoxina. Eles medeiam processos celulares, como proliferação, apoptose, metabolismo e migração celular (21).

Além dos eicosanóides, outro grupo de moléculas inflamatórias foi encontrado no interior dos corpos lipídicos: citocinas como TNF-α, RANTES, IL-16, dentre outras (22).

Apesar de células de indivíduos saudáveis apresentarem frequentemente corpúsculos lipídicos, o aumento dessas gotículas já foi descrito na sepse bacteriana, artrite, infecções micobacterianas, neoplasias, aterosclerose, síndrome do desconforto respiratório agudo (23), infecções parasitárias como a leishmaniose (24), toxoplasmose humana (25), doença de Chagas (26,27) e esquistossomose murina (28). Na infecção por *Plasmodium berghei* ANKA, o metabolismo lipídico alterado no cérebro foi associado ao desenvolvimento de malária cerebral em camundongos (29).

Esses corpúsculos são caracterizados como dinâmica e funcionalmente ativos e estudos têm mostrado que o acúmulo intracelular de lipídios nessas organelas está associado a doenças de alta relevância em saúde pública, como a hepatite C (30). A proteína da cápside do vírus da hepatite C foi encontrada associada a gotículas lipídicas, sugerindo o papel dessas organelas na produção de novas partículas virais (31). Da mesma forma, as células infectadas pelo vírus da dengue mostraram um aumento no número dessas organelas durante a infecção e o acúmulo da proteína do capsídeo maduro do vírus na superfície dos corpúsculos lipídicos (32).

1.5 FAGOCITOSE E PRODUÇÃO DE RADICAIS DE OXIGÊNIO PELOS FAGÓCITOS

Fagocitose é um mecanismo desenvolvido pelas células fagocíticas do hospedeiro contra patógenos bucais - considerada um mecanismo imune inato. Monócitos e neutrófilos são as células responsáveis, por meio da fagocitose, em eliminar micro-organismos invasores e estimular outras respostas imunológicas. A fagocitose, realizada por neutrófilos e monócitos, constitui um dos principais mecanismos de defesa contra os patógenos envolvidos na etiologia da periodontite e é iniciada pela interação de receptores da superfície celular com ligantes encontrados nos microrganismos, como lipopolissacarídeos, ou opsoninas derivadas do hospedeiro, como o complemento ou anticorpos IgG (33,34).

A resposta de morte de células fagocíticas à invasão de periodontopatógenos também inclui espécies reativas de oxigênio (ROS) (35), que contribuem para a destruição do tecido periodontal local, quando liberadas em maiores quantidades (36). Durante a periodontite, os metabólitos finais das espécies reativas de oxigênio podem ser translocados para os órgãos via circulação sangüínea, causando alterações maléficas (37,38), como nos tecidos renais (39).

1.6 TRATAMENTO PERIODONTAL NÃO CIRÚGICO

O controle da doença periodontal em indivíduos com diabetes é de extrema relevância e pode levar a um melhor controle metabólico e, consequentemente, à melhoria da qualidade de vida destes indivíduos.

A terapia periodontal mecânica envolve a remoção de agentes bacterianos nas superfícies radiculares, como aqueles presentes no cálculo supra e subgengival. Trata-se da terapia inicialmente proposta para todos os estágios da periodontite e visa a resolução da inflamação dos tecidos periodontais e, consequentemente, controle da doença periodontal (40).

Existem evidências consideráveis de que o tratamento periodontal não cirúrgico reduz o estresse oxidativo, nível de proteína C-reativa e citocinas pró-

inflamatórias (isto é, fator de necrose tumoral-alfa, interleucina-1 β e interleucina-6) (21, 41, 42, 43).

Para monitorar o sucesso do tratamento da doença periodontal e a resolução da inflamação, além do exame radiográfico, o exame clínico bucal deve incluir alguns índices periodontais essenciais que são analisados com o auxílio de uma sonda periodontal, antes e após a terapia.

Os parâmetros geralmente analisados são profundidade de sondagem (distância entre a margem gengival e a porção mais coronal do epitélio juncional, ou fundo de sulco/bolsa), nível de inserção clínica (distância entre a junção cemento-esmalte e a porção mais coronal do epitélio juncional), índice de placa visível e índice de sangramento gengival à sondagem (44).

De fato, se a periodontite realmente tiver efeitos mensuráveis na saúde geral, a terapia periodontal pode, por sua capacidade de resolução do processo inflamatório, alterar a gravidade dos desfechos.

O presente trabalho abordará uma revisão de literatura a respeito da relação do diabetes do tipo 2 associado à periodontite e os possíveis benefícios no controle das doenças oriundos do tratamento periodontal não cirúrgico.

Este trabalho também teve como objetivo identificar possíveis alterações na ocorrência de corpúsculos lipídicos em monócitos, fagocitose e produção de radical peróxido por fagócitos, além de parâmetros bioquímicos (hemograma, lipidograma, glicose, A1c e PCR) do sangue periférico de pacientes com periodontite grave associada ou não ao diabetes mellitus do tipo 2. Essas alterações podem ser importantes na demonstração de alguns mecanismos biológicos em que a periodontite poderia interferir no diabetes.

Considerando a natureza infecciosa e inflamatória das doenças periodontais e o fato de que a quantificação dos corpúsculos lipídicos ainda não ter sido avaliada na periodontite humana, isso pode sinalizar novos desfechos da relação da periodontite com repercussões sistêmicas.

A resposta imune contra periodontopatógenos poderia resultar em uma mudança na função leucocitária comparada àquela observada em indivíduos periodontalmente saudáveis ou naqueles com Diabetes Mellitus do tipo 2. Um maior número de estudos anteriores (45, 46, 47, 48,49,50, 51, 52, 53) avaliou a fagocitose por neutrófilos, enquanto a função fagocitária dos monócitos foi abordada por poucos até a atualidade (54) e, por esta razão, também foi investigada no presente estudo.

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OBJETIVOS

2. OBJETIVO GERAL

Avaliar os parâmetros hematológicos, a produção de corpúsculos lipídicos e a função dos fagócitos do sangue periférico em indivíduos com periodontite associada ou não ao diabetes mellitus do tipo 2.

2.1 OBJETIVOS ESPECÍFICOS

Na avaliação de indivíduos com periodontite associada ou não ao diabetes mellitus do tipo 2, os seguintes objetivos específicos foram estabelecidos no estudo:

- Descrever os parâmetros epidemiológicos e clínicos;
- Determinar a fagocitose, a produção de corpúsculos lipídicos e do radical superóxido;
- Determinar os parâmetros hematológicos (Hemograma completo, lipidograma completo, glicemia em jejum, níveis de hemoglobina glicada e proteína C – reativa).

REVISÃO DE LITERATURA

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Review Article

Importance of Mechanical Periodontal Therapy in Patients with Diabetes Type 2 and Periodontitis

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Periodontitis is an infectious and inflammatory disease of high prevalence worldwide and constitutes a significant oral health problem. It can lead to tooth loss. In addition, the local inflammatory process can cause the release of inflammatory mediators in the bloodstream and, consequently, contribute to the emergence of systemic effects as cardiovascular and diabetic complications. The purpose of this mini review is to alert health professionals about the risk that periodontitis represents for the onset or exacerbation of complications in individuals with type 2 diabetes mellitus and to emphasize that the mechanical treatment of periodontal disease and reestablishment of oral health are essential for the metabolic control of these patients. The periodontal therapy may help to reduce the risk of systemic complications in diabetes patients. Proper dental management should be suggested by health professionals, mainly from physicians to their patients, in order to improve the health conditions in these individuals.

3 INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease in which the body does not produce insulin or cannot use it properly. There is an estimative that there are about 422 million people with DM worldwide (1).

The two main forms of the disease are type 1 (DM1) and type 2 (DM2) diabetes. Besides, other forms are also described in the literature, such as gestational diabetes, as well as other specific types of DM such as those associated with genetic disorders, among other factors. However, DM1 and DM2 affect most of the population, where 90% of the disease's cases are concentrated in type 2 (2).

In type 1 DM, beta-pancreatic cells are mistakenly attacked by the human's immune system. So, insufficient or no insulin is released. Consequently, glucose stays in the blood instead of being used as energy by the body (2).

Because most of the studies about the relation of DM and periodontitis are related to DM2 and this is the most prevalent type of diabetes, this review will approach the aspects only involved in the treatment of periodontitis in Diabetes mellitus type 2 patients.

DM2, a global public health problem, consists of a heterogeneous group of metabolic disorders that presents chronic hyperglycemia as a result of defects in the action or the insulin secretion. DM2 results from a combination of insulin resistance and inadequate compensatory response to insulin secretion, leading to a relative deficiency in the release of this hormone (3). Insulin is the only hormone responsible for the reduction of blood glucose and is produced and released by the *β*-cells of the pancreatic islets of Langerhans (4). The major complications of diabetes are microangiopathy, nephropathy, neuropathy, macrovascular disease, and delayed wound healing. Periodontitis is considered the sixth complication of DM (2).

Periodontitis is a primarily infectious and inflammatory disease caused by anaerobic bacteria (*Porphyromonas gingivalis, Treponema denticola, Prevotella intermedia, Prevotella nigrescens, Eikenella corrodens, Aggregatibacter actinomycetemcomitans*, among others) in association or not with other periodontopathogens, in dental biofilm. It affects teeth's protection and support tissues as gingiva and alveolar bone and can lead to dental mutilation (1,5).

Periodontitis has been recently very strongly associated with the role of an overreactive immune system in the critical point of the periodontal disease

development (6,7). Periodontopathogens stimulate, among other inflammatory mediators, the production of cytokines as interleukin 1 beta (IL1- *β*), interferon gamma (IFN-*γ*), tumor necrosis factor-alpha (TNF-*α*), and chemokines (CXCL-1, CXCL-8, CCL-5) by gingival epithelium, adhesion molecules, increased permeability of the gingival capillaries, and chemotaxis of neutrophils from the junctional epithelium to the gingival sulcus. This initial response, with the production of specific cytokines and chemokines, promotes the migration of an inflammatory infiltrate composed of perivascular T cells and monocytes to the connective tissue (6, 7). If the cellular immune response fails to control the infection, recruitment of B cells that differentiate into plasma cells occurs. Plasma cells produce immunoglobulins (antibodies) that may confer protection to periodontal tissues, control the infectious process, or induce deleterious effects, leading to the destruction of connective tissue and promoting reabsorption of the alveolar bone. The effectiveness of this response varies between individuals and demonstrates importance in determining disease susceptibility (6).

Nowadays is well established the relationship between the progression of periodontitis and several factors, such as the presence of the periodontal pathogens, high levels of proinflammatory cytokines (IFN- *γ* and TNF-*α*), matrix metalloproteinases (MMP) and prostaglandin E2 (PGE2), and low levels of inflammation's inhibitory cytokines (IL-10), transforming growth factor beta (TGF-*β*), and tissue inhibitors of MMP (TIMP) (6, 8). The susceptibility and extent of tissue destruction appear to be determined by the complex cytokine balance produced by the presence of numerous associations between periodontal microorganisms. When the host's response is exacerbated, it can lead to tissue damage, causing loss of periodontal support (6, 7).

Systemic diseases, such as diabetes, can also interfere with the periodontal condition, turning the prognosis of the associated diseases unfavorable (9). Intravascular dissemination of microorganisms and their products throughout the body may occur because of the inflammation in the periodontal tissues. The total surface area of this periodontal inflammatory field is estimated to be the size of the palm of the hand. Immediate medical intervention should be done if one lesion of this size was on the skin. However, periodontitis is frequently ignored by health professionals, even though it may be associated with a range of systemic diseases and conditions (10).

The association of periodontitis with DM has been investigated and studies have shown that there is a definite correlation between both (11). It can be observed that individuals with diabetes and inadequate glycemic control are more likely to develop

severe periodontal disease and that periodontitis may interfere in the glycemic control of these individuals (12,13).

3.1 MECHANISMS OF ACTION

Patients with uncontrolled diabetes can present with micro or macrovascular complications. The prevalence of both complications occurs according to the type and duration of DM. Microvascular defects affect most intricately vascularized organs, as the retina (retinopathy), kidney (nephropathy), and peripheral nerves (neuropathy). The macrovascular defects affect large blood vessels, and consequently, some noble organs as the heart (cardiovascular disease), brain (cerebrovascular disease), and the peripheral arteries (peripheral vascular disease) (14). Vascular disorders are usually progressive. The main problem in uncontrolled diabetes is the activation of the immune system that leads to micro- and macroangiopathies and other immune reactions contributing to all major organs failure. For example, nephropathy, begins insidiously, but over time, may contribute significantly to morbidity and mortality, resulting in severe damage to the organs.

Cardiovascular diseases (CVDs) also account for increased morbidity and mortality in DM2 patients (15). CVD usually occurs about two decades earlier among DM2 patients than in those without the disease (16, 17). About 70% of individuals with DM2 die of CVD (18). The combination of the duration of diabetes (>15 years) and prior CVD was associated with a 30-fold increased risk of fatal CVD (19).

Several factors can explain the mechanisms of periodontal destruction due to DM. Initially, the hyperglycemia state can directly favor the growth of periodontal pathogens, hinder or prejudice cellular functions and, consequently, host defenses. One pathogenic consequence of hyperglycemia in diabetes is an insufficiency in detoxification of reactive carbonyl compounds. Reactive carbonyl increases due to oxidative and nonoxidative reactions where carbohydrates and lipids lead proteins to chemical modification and then, at a late stage, to oxidative stress and tissue damage. This chronic and accelerated chemical modification of proteins is associated with the AGEs (advanced glycation end products) hypothesis, which proposes that by increasing concentration of glucose in diabetes alters the structure and function of

tissue proteins, contributing and precipitating the development of diabetic complications (20).

The immunological mechanisms mediating the effect of periodontitis on the control of diabetes have moderated level of support in the current literature. Most studies demonstrate that circulating proinflammatory mediators as TNF-*α*, CRP, and mediators of oxidative stress are elevated in patients with both diseases and these subjects tend to demonstrate higher dyslipidemia, reduced beta cell function, and elevated oxidative stress (that may act synergistically in worsening cardiovascular complications in diabetes) than patients with diabetes alone. Probably these mediators affect the control of DM, but there is no sufficient information from animal studies to support this possibility (21).

There is also a substantial increase in mediators as proinflammatory cytokines and secretion of collagen degrading enzymes. Diabetes, through the formation of AGEs, can indirectly alter the union of the extracellular matrix, as well as cellular activities, amplifying inflammatory reactions, and decreasing cellular viability, which leads to deterioration of the healing process and potential change in periodontal tissues (22).

On the other hand, the mechanisms by which periodontitis promotes metabolic dysfunction are not yet fully understood. It is believed that in response to endotoxins such as lipopolysaccharide (LPS) present in periodontal microorganisms, there is an augment in the production of proinflammatory cytokines, chemokines, reactive oxygen species (ROS), and C-reactive protein (CRP) that can alter lipid metabolism and insulin resistance, leading to hyperlipidemia and hyperglycemia (22). Additionally, TNF-*α* has been identified as a potent insulin receptor blocker (23).

In severe untreated periodontitis, the ulcerated epithelium of the periodontal pockets has an estimated surface area of 8 to 20 cm^2 (24). This inflamed and ulcerated subgingival epithelial area of periodontal pockets constitutes a vast portal of entry for periodontopathogenic bacteria, their products, endotoxins such as LPS, and stimulated inflammatory mediators to reach the systemic circulation (25, 26).

Periodontal microorganisms as well as their antigens, when systemically dispersed, can cause significant systemic inflammation and contribute to DM complications. Leukocytes, endothelial cells, and hepatocytes respond to virulence factors with the secretion of proinflammatory mediators such as cytokines, chemokines, ROS, and CRP. If excessive, ROS release by phagocytes can reach

circulation and cause systemic oxidative stress. CRP is a protein mainly produced by the liver as result of increased levels of TNF-*α* and IL-6 in the inflammatory process (27). Cardiovascular disease has CRP as an independent predictor of its occurrence (28).

Data from a systematic review (29) concluded that human studies, animal experiments, and ex vivo cell culture studies provide evidence for elevated levels of interleukin-6 and interleukin-1*β* in periodontal tissues and crevicular fluid in patients with DM and periodontitis compared to systemically healthy patients.

Animal models with type 2 diabetes mellitus suggest that TNF-*α* plays an essential role in prolonging periodontal inflammation (29) and in the development of insulin resistance (23). This mediator reduces the expression of glucose transporter type 4 (GLUT4) which is an insulinregulated glucose transporter. TNF-*α* also induces serine phosphorylation of insulin receptor substrate-1 (IRS-1) that acts as an inhibitor of insulin receptor and down streams the signaling of phosphatidylinositol-3 kinase activation (23).

The increased release of proinflammatory cytokines (IL-1*β*, IL-6, and TNF-*α*), an altered RANKL/osteoprotegerin ratio, interactions between advanced glycation end products and their receptors, increased production of reactive oxygen species, and increased interaction between endothelial cells and leukocytes play a crucial role in the two-way relationship between diabetes and periodontitis. These complex changes, resulting from the presence of diabetes, modify the local inflammatory reaction in the periodontium, leading to a proinflammatory state in the gingival tissue and microcirculation (29). With continued exposure, soluble antigens react with specific circulating antibodies to form immune complexes that amplify inflammation at the sites of deposition.

Likewise, proinflammatory mediators, produced locally in the inflamed gingival tissues, can reach the systemic circulation. Proinflammatory cytokines in the circulation induce leukocytosis and acute phase proteins (e.g., CRP). In this context, the increase in the number of white blood cells is associated with an augmented risk of coronary heart disease, cardiovascular disease (CVD), atherosclerosis, thrombosis, and myocardial ischemia. This increase may be caused by the inflammatory nature of chronic infections such as periodontitis (30, 31).

Periodontitis may cause bacteremia and enhance atherosclerotic plaque formation because some microorganisms related to periodontal diseases were

detected in atherosclerotic plaques (32, 33, 34). However, other oral pathogens as *Streptococcus mutans* also have been found in atheromatous plaque samples (35). Thus, it seems that the disruption of epithelial integrity from periodontal pockets may also provide a point of entry for nonperiodontal pathogens, as those usually found in caries-affected teeth.

Periodontal bacteria, as *P. gingivalis,* or their products can also interact with platelets (direct or *via* the vascular endothelium) and promote prothrombotic effects (36).

Proinflammatory cytokines, which have been reported to be associated with periodontitis, are also involved in atherothrombogenesis (37, 38). Furthermore, periodontitis patients present many similar risk factors to those with CVD including age, lower socioeconomic status, and smoking (39). This suggests that periodontitis and CVD may share common etiological pathways and that the association between both is plausible.

Periodontitis is a risk factor for atherosclerosis through endothelial activation. Bacterial products (LPS, outer membrane vesicles, or fimbriae), cytokines, and chemokines resulted from the infectious and inflammatory periodontal process fall into the bloodstream and may stimulate a superregulation of endothelial cell surface receptors in addition to the expression of adhesion on vascular endothelium. This promotes chemotaxis for circulating monocytes. These cells adhere to the activated endothelium. Due to molecular mimicry, immunoglobulins against specific bacterial proteins act as autoantibodies and induce apoptosis in the endothelium. The monocytes then migrate into the subendothelial space and differentiate into macrophages. There, they pick up oxidized low-density lipoprotein (LDL) and become foam cells. Apoptosis of LDL-loaded macrophages results in the accumulation of lipids in the subendothelial space, contributing to the formation of atheromatous plaques. In addition, invading periodontal pathogens induce the proliferation of smooth muscle cells in the formation of the intima and neointima. The extracellular matrix development and the extravasation of T lymphocytes result in the formation of a fibrous envelope covering the atheroma. The fibrous cap and its prothrombotic components are exposed after endothelial cell apoptosis. The enzymatic degradation of the extracellular matrix results in plaque rupture with consequent exposure of its prothrombotic components and formation of thrombi, leading to vessel occlusion. Clinically, this manifest as acute

myocardial infarction, in the case of an occluded coronary artery, or a stroke in the case of an occluded cerebral vessel (40).

On the other hand, complications of DM2 because of periodontitis can be prevented or diminished if periodontal disease is treated. Studies have demonstrated that mechanical periodontal therapy can promote the reduction of inflammation's markers in the bloodstream (CRP, IL-6, among others) (41, 42, 43).

3.2 IMPORTANCE OF THE DIAGNOSIS OF PERIODONTITIS AND PERIODONTAL DEBRIDEMENT

Periodontal diseases have been associated with a reduced glycemic control in diabetes. Periodontitis increases the risk for the diabetes incidence in nondiabetic patients (21) as well as increases insulin resistance in patients with DM and disease complications, including mortality (44, 45).

The implications of periodontitis in the oral environment and maintenance of affected teeth, by themselves, would justify the relevance of seeking the complete understanding of its etiopathogenesis and, from this, implement active forms of individualized therapy. However, in addition to the implications of the disease in oral health, its meaning reaches systemic proportions, whose mechanisms are still not precise.

The effect of periodontitis on the control of DM type 2 has been studied, and there is indirect evidence to support biological mechanisms mediating this effect as reduced pancreatic islets *β*-cell function, elevated oxidative stress, and dyslipidemia. People with DM and periodontitis usually have elevated circulating proinflammatory mediators, like TNF-*α*, IL-6, CRP, and reactive oxygen species (ROS) that can interfere with diabetes metabolic control (44) and may act synergistically in worsening cardiovascular complications in diabetes (45).

Mechanical periodontal therapy involves the removal of bacterial agents from periodontium, supra, and subgingival calculus, by scaling and root planning with periodontal curettes or ultrasonic devices. It is the conventional treatment for periodontitis for resolution of inflammation from periodontal tissues and consequently, control of periodontal disease (46).

There is considerable evidence that nonsurgical periodontal treatment reduces oxidative stress, C-reactive protein level, and proinflammatory cytokines (i.e., tumor necrosis factor-alpha, interleukin-1*β*, and interleukin-6) (21, 42, 47, 48).

To monitor the success of the treatment of periodontal disease and the resolution of inflammation before and after therapy, besides radiographic examination, the oral clinical examination must include some essential periodontal indexes which are analyzed with the aid of a periodontal probe.

The parameters usually analyzed are probing depth (PD), clinical attachment level (CAL), visible plaque index (PI), and gingival bleeding on probing (BOP) index (49).

In fact, if periodontitis truly has measurable effects on general health, treatment of this infection may alter the severity of the outcomes, with the resolution of inflammation.

The importance of periodontal treatment is not only to promote the reduction of local clinical inflammation, but it has also been associated with a subsequent decrease in serum levels of IL-6, TNF-*α*, CRP, and ROS (50–55). This evidence supports the mechanistic link between periodontitis and diabetes through inflammatory mediators. It is important to emphasize that diabetes can interfere with the homeostatic interaction between microorganisms and host at periodontal sites, where host immune response to diabetes can trigger a destructive inflammatory pathway against previously well-tolerated microorganisms. Experimental models demonstrate that the development of periodontitis in diabetic rats involves a high expression of proinflammatory cytokines (TNF-*α*, IL-1*β*, IL-6) and destructive tissue factors as advanced glycation end products (AGEs) without significant changes in commensal oral microbiota (56).

Patients with DM2 usually have glycated hemoglobin HbA1C elevation in serum, so current studies have shown that periodontal therapy can improve the control of HbA1C levels in patients with both diseases. Periodontal treatment can also successfully reduce circulating levels of TNF-*α*, CRP in patients with DM associated with periodontitis (57, 58); however, research about the impact of successful long-term periodontal treatment does not exist and should be done.

The magnitude of reported HbA1C reductions ranges from 0.27% to 0.48% at 3-4 months following periodontal therapy (58), which means the same quantity of short-term HbA1C reduction obtained to that often achieved by adding a second medication to a pharmacological regimen (59). If such decreases can be sustained over the longer

term after periodontal therapy, it may contribute to reduced morbidity and mortality associated with DM.

It is challenging to estimate the social cost of the morbidities related to patients living with diabetes. Many individuals are unable to continue their work activities because of chronic complications of the disease or remain with some limitations in their professional performance, causing significant losses regarding productivity. Thus, the control of periodontal disease in patients with DM through mechanical debridement (scaling and root planning) is crucial and may lead to better metabolic control and, consequently, to the improvement of the quality of life of these people.

It is of extreme relevance that health-care professionals, as physicians, to be aware of periodontitis and its implications for glycemic control and complications in individuals with diabetes. Diagnosis of periodontitis should be an integral part of a diabetes care visit. A periodontal examination should be done as part of their ongoing management of DM by a dentist.

Even without diagnosed periodontitis initially, an annual periodontal review is recommended. All patients with DM should be provided with oral health education as part of their overall educational program (21).

In the other hand, dentists should pay attention in identifying both prediabetes and undiagnosed diabetes mellitus because periodontitis could increase the risk of many diabetes complications as retinopathy, nephropathy, neuropathic foot ulceration, cardiovascular diseases and mortality (60).

In conclusion, periodontitis and diabetes establish a two-way pathway, and each one, if untreated, could promote or exacerbate complications of each other. Periodontal screening must be part of the overall clinical examination of patients with diabetes and, if diagnosed, periodontal disease must be treated appropriately to avoid or exacerbate diabetes complications besides improving glycemic control in these individuals.

3.3 ADDITIONAL POINTS

Clinical Significance. To alert health professionals about the relation of diabetes mellitus and periodontitis and encourage them to conduct a multidisciplinary treatment/ assistance.

3.4 CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

3.5 ACKNOWLEDGMENTS

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ESTUDO OBSERVACIONAL DE CORTE TRANSVERSAL

QUANTIFICATION OF LIPID BODIES AND PHAGOCYTIC ACTIVITY OF THE INNATE IMMUNE SYSTEM CELLS FROM PERIODONTITI'S PATIENTS WITH OR WITHOUT DIABETES TYPE 2.

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Running Title: Lipid bodies, phagocytosis and superoxide anion in the association of periodontitis and diabetes.

Clinical Relevance

Scientific rationale for the study: There are several studies focused on the association between periodontitis and systemic complications in diabetes. However, biological mechanisms of the immune responses involved in this relation remain still unclear.

Principal findings: We found that severe periodontitis induced an increment in the frequency of monocytes with lipid bodies in their cytoplasm compared to healthy individuals. Patients with the association of periodontitis and diabetes showed an increased number of lipid bodies within monocytes compared to diabetes alone. It was also observed impairment in phagocytic activities from neutrophils and monocytes in patients with periodontal disease compared to healthy individuals. C-reactive protein (CRP) levels were augmented in blood from patients with diabetes type 2 with periodontitis compared to those with diabetes alone.

Practical implications: This study showed that periodontitis may lead to a systemic susceptibility or fragility of the immune system, increasing the risk of acquired diseases

in healthy individuals and contribute to the development of systemic complications in individuals with diabetes type 2, such as cardiopathic complications.

Abstract

Aim: This study aimed to investigate, for the first time, whether periodontitis can alter the occurrence of lipid bodies from monocytes of systemically healthy or type 2 diabetes patients. Phagocytic activity and the production of superoxide anion by neutrophils and monocytes were also assessed. Differential leucocytes count, lipid, fast glucose, A1c and CRP levels analyses were performed to investigate if the periodontal disease was contributing to biochemical modifications in the systemic circulation of these patients.

Materials and Methods: 58 participants, 23 males and 35 females, with diabetes type 2 or systemically healthy were divided into four groups: Control (n=16), periodontitis (n=14), diabetes (n=11) and diabetes with periodontitis (n=17) were evaluated. The following clinical periodontal parameters were assessed: probing depth, clinical attachment level, visible plaque index and gingival bleeding on probing index. Lipid bodies were evaluated by cytochemical tagging method with red oil O (Oil-Red-O). Immediately after obtaining the peripheral blood samples, the tests of phagocytic activity by adherence in a slide of light microscopy and Nitro blue tetrazolium (NBT) were performed. Total leukocyte, neutrophils, monocytes, eosinophils and basophils counts were evaluated in the hemogram. Total lipidogram, fasting glucose, glycated hemoglobin (A1c) and CRP levels were also evaluated by biochemical analysis of blood samples.

Results: When periodontitis' group was compared with healthy individuals, there was an increase the frequency of monocytes with corpuscles in their cytoplasm in samples containing opsonized yeasts ($p = 0.012$) or not ($p = 0.003$), Kruskal-Wallis. Additionally, corpuscular index was increased in patients with diabetes and periodontitis ($p < 0.001$ non-sensitized; $p = 0.022$ sensitized; Kruskal-Wallis) compared to diabetes alone. Regarding phagocytosis of the control group, a significant reduction in the phagocytic activity of neutrophils in non-opsonized samples in periodontitis ($p = 0.008$, Kruskal-Wallis) was observed and, in the opsonized samples, this reduction occurred in patients with diabetes and periodontitis ($p = 0.029$, Kruskal-Wallis) when a 20:1 phagocyte yeast stimulus was used. In the sensitized samples, monocytes showed a reduction in the phagocytic function of the individuals with periodontitis in the

proportions 5:1 and 20:1 (yeast: monocyte), in relation to the controls (p = 0.018 and 000.7, respectively; Kruskal-Wallis). There was also a significant reduction in monocyte phagocytic activity in individuals with diabetes and periodontal disease compared to healthy subjects (p = 0.0007, sensitized; Kruskal-Wallis, 20:1). There was no significant difference in the production of superoxide between the evaluated groups. Patients with both diseases also presented higher CRP levels (p < 0.001, Kruskal-Wallis) compared to patients with diabetes.

Conclusions: Our results suggest that periodontitis may contribute to a systemic susceptibility or fragility of the innate immune responses and a major risk of acquired diseases in healthy individuals. Besides, periodontitis may favor the emergence of systemic complications in diabetes type 2 patients.

Keywords: leukocytes; lipid bodies; phagocytosis; superoxide anion; periodontitis; diabetes mellitus.

4 INTRODUCTION

Diabetes mellitus (DM) type two [2] is a metabolic disorder that results in defects of the insulin secretion or its' action, leading to chronic hyperglycemia (1).

Periodontitis is an infectious and inflammatory disease that affects teeth's protection and support tissues (1,2). The disease constitutes a great risk for dental mutilation, masticatory dysfunction and is considered the sixth complication of DM (3).

Periodontopathogens stimulate cells from the innate immune system as neutrophils and monocytes, which can lead to an exacerbated local or systemic inflammatory response, depending on the host health conditions (4, 5, 6). These microorganisms and their products from periodontal pockets can disseminate intravascularly throughout the human body (1).

The association of periodontitis with DM has been investigated and studies have shown that there is a definite correlation between both (7,8). Individuals with diabetes and inadequate glycemic control are more likely to develop severe periodontal disease. In a two-way via, periodontitis may also interfere with the glycemic control of these individuals, because they tend to have higher levels of glycated hemoglobin (A1c) (7).

There also appears to exist a relationship between the severity of periodontitis and dyslipidemia, oxidative stress (9), and cardio-renal complications of diabetes (10).

An important innate immune response to oral pathogens is phagocytosis, a mechanism developed by the host's phagocytic cells - including monocytes and neutrophils, to eliminate invading microorganisms and stimulate other immune responses. Phagocytosis is initiated by the interaction of cell surface receptors with ligands found on the microorganisms, such as lipopolysaccharides, or host-derived opsonins, such as complement or IgG antibodies (11,12).

The killing response of phagocytic cells to periodontal pathogens invasion also includes reactive oxygen species (ROS) (13) as superoxide anion that contribute to local periodontal tissue destruction, when released in larger amounts (14). During periodontitis, the end-metabolites from ROS can be translocated to the organs via blood circulation causing changes (15,16) such as in renal tissues (17).

Most eukaryotic cells contain varying amounts of cytosolic lipid inclusions called lipid bodies or lipid droplets which regulate the hydrolysis and storage of neutral lipids (18). Their structure comprises an external monolayer of phospholipids and a central nucleus rich in neutral lipids as triglycerides and sterol esters (19). The formation of these lipid inclusions during infectious processes is a well-regulated phenomenon that may have implication in the pathogenesis and an important role in the modulation of the immune response.

These corpuscles are characterized as dynamically and functionally active and studies have shown that intracellular accumulation of lipids in these organelles is associated with diseases of high public health relevance as hepatitis (20) and dengue (21).

This work aimed to identify possible alterations in lipid body occurrence in monocytes, phagocytosis and superoxide production by phagocytes and biochemical parameters (hemogram, lipidogram, glucose, A1c and CRP levels) of the peripheral blood from patients with periodontitis associated or not to type 2 diabetes mellitus. These alterations can be important in showing some biological mechanisms in which periodontal diseases would interfere with diabetes.

Considering the infectious and inflammatory nature of periodontal diseases and the fact that lipid bodies quantification has never been evaluated during human periodontitis, this is the first study to investigate this association.

4.1 MATERIAL AND METHODS

4.1.1 Groups of study

This study was approved by the Human Research Ethics Committee, University of Brasília (UnB) (CAAE 46609515.7.0000.0030), in accordance with Brazilian law, which complies with the Declaration of Helsinki, as revised in 2013. All individuals in the four groups were individually informed about the proposed study and agreed to participate by signing a written informed consent form.

Subjects were recruited between December 2015 and April 2017 at the Clinic of Periodontology, University Hospital of Brasilia (HUB), Distrito Federal, Brazil. The 58 participants, 23 males (39.7%) and 35 (60.3%) females, with diabetes type 2 or systemically healthy, non-smokers underwent periodontal examination by a single examiner (PFN).

The periodontitis (P) and diabetes mellitus with periodontitis (DMP) groups consisted of patients ≥ 30 years old with clinical and radiographic diagnosis of periodontitis at ≥ 2 non-adjacent teeth [PD ≥ 4mm, ≥ stage III; grades A (P), B or C (DMP), according to % A1c], according to the new classification of periodontitis (2) and based on pathophysiology of the disease and, a minimum of 12 teeth. The diabetes group (DM) was composed by individuals with controlled (A1c < 7%) or uncontrolled (A1c ≥ 7%) DM2, previously diagnosed and confirmed in this study, without periodontal diseases and, a minimum of 12 teeth. For the control group (C) buccally and systemically healthy individuals were chosen with a minimum of 20 teeth. For both DM and C groups, the authors selected individuals without evidence of periodontal diseases (with no interproximal or buccal CAL ≥ 3mm, PD ≤ 3 mm and BOP < 10%) and no radiographic evidence of bone loss.

The following conditions were considered exclusion criteria: periodontal treatment in the last 12 months, antimicrobial therapy for systemic conditions or topical oral use in the last 12 months, use of medications as anti-inflammatory, corticoid and immunosuppressive therapy, pregnant or lactating women, autoimmune, infectious, allergic, renal and gastrointestinal diseases, cancer, morbid obesity [body mass index (BMI) > 40kg/m^2] or malnutrition (BMI < 18,5 kg/m^2) (22).

All volunteers were initially interviewed to obtain clinical and demographic data, including age, sex, and tobacco use. Individuals who had never smoked or had stopped smoking more than five years before the interview were considered non-smokers. In the clinical examination, probing depth in six sites in each tooth (buccal, mesio or distobuccal, lingual/palatine, mesio or distopalatine/lingual) was recorded. They were analyzed with the aid of a periodontal probe (Michigan O probe with Williams markings), excluding third molars. The parameters analyzed were probing depth (PD), clinical attachment loss (CAL), visible plaque index (PI) (23) and gingival bleeding on probing (BOP) index (24).

The participants who had periodontitis underwent mechanical periodontal therapy. Those without periodontal diseases underwent dental prophylaxis. All participants received recommendations about oral hygiene and on the interrelation of periodontal diseases with diabetes. All studied groups underwent peripheral blood collection at two different moments.

4.1.2 Blood sampling for cell count and biochemical analysis

Blood samples were collected and analyzed at Laboratory Sabin, Brasilia – Distrito Federal, Brazil. The followed parameters were evaluated: complete blood count, complete lipidogram, fasting glycemia, glycated hemoglobin (A1c), and C-reactive protein. (CRP).

These samples were collected at the same week of collection of those from the immunoassays, as described below.

4.1.3 Obtaining peripheral blood mononuclear cells

Separation of peripheric blood mononuclear cells (PBMC) by Percoll gradient centrifugation was performed. For this, 10 milliliters (mL) of venous blood was collected in a heparinized vacutainer tube. After centrifugation, the supernatant serum was collected, aliquoted and frozen at -80 °C. The precipitate composed of red blood cells,

leukocytes and platelets was resuspended with phosphate buffered saline (PBS) at approximately 4°C, pH 7.2, in the same volume of removed serum. Then the resuspended content was gently placed on the percoll (density 1077, 3 milliliters of percoll per 5 mL of blood) followed by centrifugation at 750 g (3000 rpm) for 15 minutes at 4°C. The mononuclear cell layer was then transferred to a new tube. For removal of percoll residues, the content was resuspended in 10 mL of PBS at approximately 4°C, pH 7.2, and centrifuged at 400 g (2000 rpm) for 10 minutes at 4°C. The supernatant was then discarded, resuspended in 10 mL of refrigerated FTS and centrifuged at 200 g (1000 rpm) for 10 minutes at 4°C for removal of platelets. The supernatant was discarded, and the pellet resuspended in 2 mL of incomplete RPMI medium, pH 7.2 and maintained in a box with ice until finalization of the cell count.

For the counting of obtained mononuclear cells after the homogenization of the solution with Pasteur pipette, 20µL of the solution were removed and diluted with 80µL of 0.05% nigrosin in PBS (dilution = 5). The cell counts were made in Neubauer's chamber and the number of live cells, the number of dead cells and the total number of cells were obtained.

To evaluate the percent viability of the recovered cells, the number of recovered viable cells suspended in 2 mL RPMI was calculated. The number of total cells consisted of the product of the mean number of live cells by the volume of the suspension in mL [2], dilution [5] and depth of the Neubauer chamber [10]. Finally, the value obtained was further multiplied by 1000 (to convert mm to mL). Then, the percentage of recovery of the cells was obtained in relation to the total number of leukocytes in the blood. The cells were separated from methanol-fixed percoll, stained with 10% Giemsa and the purity of the preparation determined.

4.1.4 Quantification of lipid corpuscles

The quantification of lipid bodies was performed by the red oil cytochemical labeling technique (Oil red – O), which is a dye with great solubility for lipophilic substances, and which presents in red color in monocyte cytoplasm.

Cells were resuspended in RPMI without fetal bovine serum (FBS) (10^6 PBMC per 500 µL) and the solution distributed in 24 excavations culture plates with circular

sterile glass laminules previously inserted into each well. The plate was incubated for 2 hours at a humid chamber with 5% CO_2 at 37°C for adhesion of the cells to the laminules and subsequently washed (twice) with sterile FBS at 37°C. After washing, yeast (*Saccharomyces cerevisiae*) in the proportion of five yeasts to 1 monocyte (5:1) was added in complete RPMI medium and incubated for 30 minutes. A new wash with FBS at 37 ° C was performed.

The cells were then fixed with 4% paraformaldehyde for 15 minutes and then washed twice with PBS, pH 7.2, and once with 60% isopropyl alcohol. PBMCs were then stained for 15 minutes with a red oil solution three times filtered on a 0.22 µm filter and prepared at the time of use from a stock solution of 0.5% (2 parts stock solution to 3 parts of distilled water). Excess dye was removed, and the excavations were washed once with one mL of 60% isopropyl alcohol and then twice with one mL of distilled water. The cell nuclei were stained with Mayer's hematoxylin for five minutes, rinsed again with distilled water and the laminules were mounted on glass slides containing a thin layer of gelatinous medium (10 g of gelatin, 60 mL of distilled water, 70 mL of glycerol and 0.25 g of phenol).

By this method, lipid droplets appear as circular structures that redden in the cytoplasm of macrophages. The percentage of monocytes presenting lipid corpuscles in the cytoplasm (MØ%) was determined, and the mean number of corpuscles inside the monocytes (MCL) was counted. Then, the corpuscular index (CI) was established by the multiplication of MCL and the MØ%. For each patient, 200 sensitized, non-sensitized and basal monocytes were blinded evaluated by a single examinator (MR). The slides with basal samples had no stimulus and no yeast. The slides with sensitized samples had yeast and opsonin from the sample donor itself while the non-sensitized samples slides had yeast but did not have opsonin.

4.1.5 Evaluation of phagocytosis

A technique previously described (25) was adapted to assess phagocytosis of dead *Saccharomyces cerevisiae* via pattern-recognition receptors or facilitated by opsonins.

Whole blood (40 µL/area) was placed in clean glass slides containing eight marked areas of 7 mm diameter each and incubated in a wet chamber for 45 minutes at 37°C. The slides were then rinsed with 0.15M PBS, pH 7.2 at 37°C, to remove non-adherent cells. After washing, monocytes remained adhered to the slide approximately in the same proportion as they were in the whole blood. Adherent cells (12,534 ± 5,050 cells/area; 5.63 ± 0.85% phagocytes) were incubated with a suspension of $2.5x10^5$ *S. cerevisiae* in 20 µL Hanks-tris (Sigma, USA), pH 7.2, with 10% heat-inactivated fetal calf serum (FCS) (Gibco) for 30 min in a wet chamber at 37°C. Slides were then rinsed with 0.15M PBS at 37°C to eliminate non-phagocytosed *S. cerevisiae* and the final washing was done with 30% FCS in Hanks-tris. The slides were then fixed with absolute methanol and stained with 10% Giemsa solution. The number of *S. cerevisiae* phagocytosed by 200 neutrophils or monocytes in single preparations was assessed by optical microscopy. Microscopic fields distributed throughout the slide were randomly selected and all phagocytes in each particular field were blinded examined by a single examinator (ACGC). The phagocytic index was calculated as the average number of phagocytosed *S. cerevisiae* per phagocytosing neutrophils or monocytes, multiplied by the percentage of these cells engaged in phagocytosis (25).

Yeasts to be phagocytosed by phagocytes were used with or without previous incubation with fresh serum from the donor for 30 min at 37°C. In the former case, yeast cells were considered sensitized, because complement molecules present in serum opsonized them and phagocytosis occurred through phagocytes CR1 and CR3 receptors that bind to C1 and C3 components of complement adhered on the surface of the yeasts (25). The yeast cells that were not pre-incubated with fresh serum from the donor, but were incubated with inactivated fetal bovine serum, were considered as non-opsonized, and their phagocytosis occurred via the pattern-recognition receptors (PRR) of neutrophils or monocytes (26).

4.1.6 Evaluation of superoxide production

Nitro blue tetrazolium (NBT) test was adapted from a technique previously described (27). This technique evaluates the microbicidal mechanism of phagocytes by their ability to generate toxic oxygen radicals capable of reducing the compound

NBT to an insoluble form, named formazan, which can be identified under optical microscopy by a blue color in the cytoplasm of the cell (28).

The quantity of NBT reduced is directly proportional to the amount of oxygen radicals produced by phagocytes, and these molecules are among the principal microbicidal agents produced by phagocytic cells (29).

Briefly, the phagocytes adhered on the slide, as previously described, were incubated with 0.05% NBT solution in Hanks-tris (Sigma, USA) for 20 minutes at 37°C in a humidified chamber.

The slides were then washed, fixed with methanol and stained with a solution of 1.4% safranin and 28.6% glycerol in distilled water. NBT reduction was also stimulated by sensitized *S. cerevisiae.* The percentage of phagocytes with reduced NBT in the cytoplasm was blinded assessed by optical microscopy (ACGC).

4.1.7 Statistical analysis

The sample calculation (n = 31) was based on the annual mean number of patients seen in the clinic of Periodontics at HUB (n = 60), considering a sample error of 5%, a 95% confidence level and a prevalence of 19.4 % of Periodontitis, in the adult population, according to the literature (30).

Statistical analysis was performed using the Prism® software (GraphPad, USA). Variables in the samples were previously verified for normality by the Kolmogorov-Smirnov test.

To verify the differences among three or more groups, the analysis of variance (ANOVA), followed by the Turkey test or Kruskal-Wallis test followed by the Dunn's method, was performed for variables with Gaussian or non-Gaussian distribution and similar or different variances, respectively. The level of significance was set at $p < 0.05$.

4.2 RESULTS

4.2.1 Clinical and demographic data

A convenience sampling was obtained and all individuals that volunteered to participate were chosen to be part of the study sample. Fifty-eight [58] participants, 23 males and 35 females, were enrolled at the study.

Four groups were assessed as follows: 1) Diabetes type 2 with periodontitis group (DM-P) consisted of 17 patients, 7 females (F) and 10 males (M), ages 53±7 [mean± standard deviation (SD)]; 2) Diabetes type 2 group (DM), without periodontal diseases, consisted of 11 patients (9F; 2M), age 51±10; 3) Periodontitis group (P) consisted of 14 patients (8F; 6M), age 45±9; 4) Control group (C) consisted of 16 healthy volunteers (11F; 5M), age 42±9.

The age of diabetes patients (mean 53 or 51 years, with or without periodontitis, respectively) was about eight or nine years older when compared to systemically healthy individuals (mean 45 or 42, with or without periodontitis respectively). Body mass index (BMI) of the patients with diabetes (mean 30 or 31 kg/m^2, with or without periodontitis; p = 0.0137, ANOVA) was higher when compared to the BMI of the control group (mean 26 kg/m^2) or periodontitis alone (mean 28 kg/m^2). The control group had a significantly higher number of teeth (mean 27; p = 0.0108; Kruskal-Wallis followed by Dunn's comparison) in relation to patients with diabetes (mean 22 or 21, with or without periodontitis) or periodontitis (mean 23), but without significance.

Concerning clinical parameters, patients with periodontitis (with or without diabetes) had a significant increase in probing depth (PD), clinical attachment loss (CAL), plaque index (PI), and bleeding on probing (BOP) when compared to groups without periodontal disease ($p < 0.0001$; Kruskal-Wallis) (Table 1).

Table 1 - Clinical periodontal data from the participants of the study.

	C	P	DM	DM-P	*p-Value*
PI (%)	≤15	92/79 ± 27.4	≤15	84/77 ± 23.3	< 0.0001
BOP (%)	≤10	44/48 ± 26.1	≤10	35/38 ± 25.8	< 0.0001
CAL (mm)	0	4.9/5.4 ± 1.5	≤2	4.4/4.6 ± 1.1	< 0.0001
CAL^{+} (mm)	0	5.8/6.3 ± 1.9	≤2	5.1/5.5 ± 1.6	< 0.0001
PD (mm)	≤3	5.1/5.5 ± 1.1	≤3	5/5.1 ± 0.8	< 0.0001
PD^{+} (mm)	≤3	6/6.1 ± 1.7	≤3	5.1/5.5 ± 1.2	< 0.0001
Extension (%)	0	49/51 ± 29.8	0	36/42 ± 26.3	< 0.0001

Kruskal-Wallis test. $^{+}$ Deeper pockets or major CAL per tooth. Values are expressed as median/mean ± standard deviation; *p*-value significant when < 0.05. Legends: C = control group; P = periodontitis group; DM = diabetes mellitus group; DMP = diabetes mellitus with periodontitis group; PI = plaque index; BOP = bleeding on probing; CAL = clinical attachment loss; PD = probing depth.

4.2.2 Evaluation of laboratorial parameters

All values obtained for the hematological examinations were within the normality or reference values, except for the glycemic indices for both diabetes groups or triglycerides in the DM-P group (Table 2).

Although triglyceride values in subjects with diabetes were within normal ranges, this was not observed in subjects with diabetes associated with periodontitis, whose concentrations were above-established standards (Table 2).

Table 2 - Biochemical profile of individuals according to the study's groups.

Groups		**C**	**P**	**DM**	**DMP**	**RV**
		(Median; Mean ± SD)				
Lipids	Total cholesterol (mg/mL)	173 188±34	189 192±35	179 178±28	187 195±39	<190
	HDL (mg/mL)	52 50±10	51.0 53±10	46.0 47.8±10.8	47 47±8	> 40
	LDL (mg/mL)	103 113±31	110 113±25	95 95.5±24.7	106 107±42	< 130
	Triglicerydes (mg/mL)	108 124±55	121 131±75	129.0 186±150	210* 214±102	< 150
Blood glucose	A1c (%)	5 5±0	5 5±0	7*# 8±2	7*# 7±1	4 to 6
	Glucose (%)	87.0 88±9	94.0 97±12	137.0* 139±50	133*# 138±43	70 to 99
Hemogram	Hematocrit (%)	41 42±4	42 69±102	39 41±4	44 66±94	36.0 a 54.0 ♂ 33.0 a 47.8 ♀
	Total leukocytes (mm^3)	6100 6359±1510	6370 6746±1942	5680 5866±1503	7000 7918±2738	3600 to 11000

Kruskal-Wallis test. *****Values differ from control group ($p < 0.05$); # Values differ from periodontitis group ($p < 0.05$). Legends: C = control group; P = periodontitis group; DM = diabetes mellitus group; DMP = diabetes mellitus with periodontitis group; HDL = high density lipoprotein; LDL = low density lipoprotein; A1c = glycated hemoglobin. RV = Reference values for normality with fasting from 8 to 12 hours.

4.2.3 C reactive protein (CRP) levels

Among patients with diabetes, those with periodontitis as co-morbidity had higher and significant CRP levels (5/4.4±2.7; mean/median ± SD) compared to the control group (03/0.5±0.4) or diabetes without periodontitis group (0.4/0.7±0.9) (Figure 1).

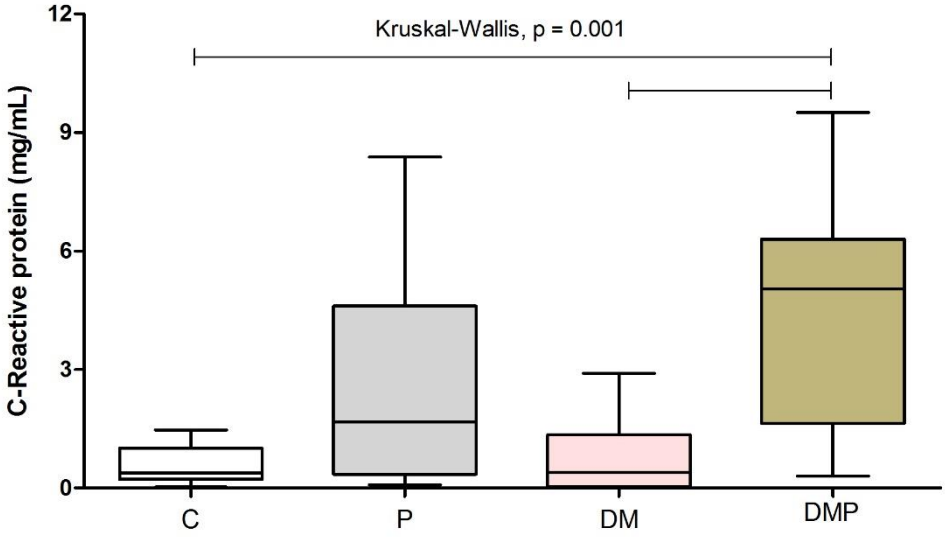

Figure 1. CRP levels among the different study's groups. Kruskal-Wallis test. Legends: C = control group; P = periodontitis group; DM = diabetes mellitus group; DMP = diabetes mellitus with periodontitis group. p significative < 0.05. Results are expressed as median with max and minimum quartiles.

4.2.4 Lipid bodies

The corpuscular index (CI) showed a significant increase in the DMP group (non-sensitized: median/mean±SD 13.5/15±10.3) related to the diabetes group (non-sensitized: 0.3/2.6±3.7). The CI from the periodontitis's patients also showed a significant increase (non-sensitized: 12/13.5±5.7 or sensitized: 26/27.5±16.5) when compared to the diabetes group (non-sensitized: 0.3/2.6±3.7 or sensitized: 8/6.5±4.5, respectively).

The percentage of monocytes presenting corpuscles in the cytoplasm (MØ%) showed a significant increase between the periodontitis group (non-sensitized: 8.5/8.5.5±4 or sensitized: 15/14.7±9.3) and the control group (non-sensitized: 1.5/2.3±2.5 or sensitized: 2.5/5.8±5.3). When sensitized periodontitis' group was compared to the DM group, there was a significative increase in MØ% (15/14.7±9.3 and 3.5/6.1±6.2, respectively). There was no difference in the mean number of corpuscles per monocyte (MBL) among the different groups studied. Figure 2 shows the quantification of lipid bodies per groups according to MØ%, MBL and CI. Detailed resuts are in described in Chart 1C.

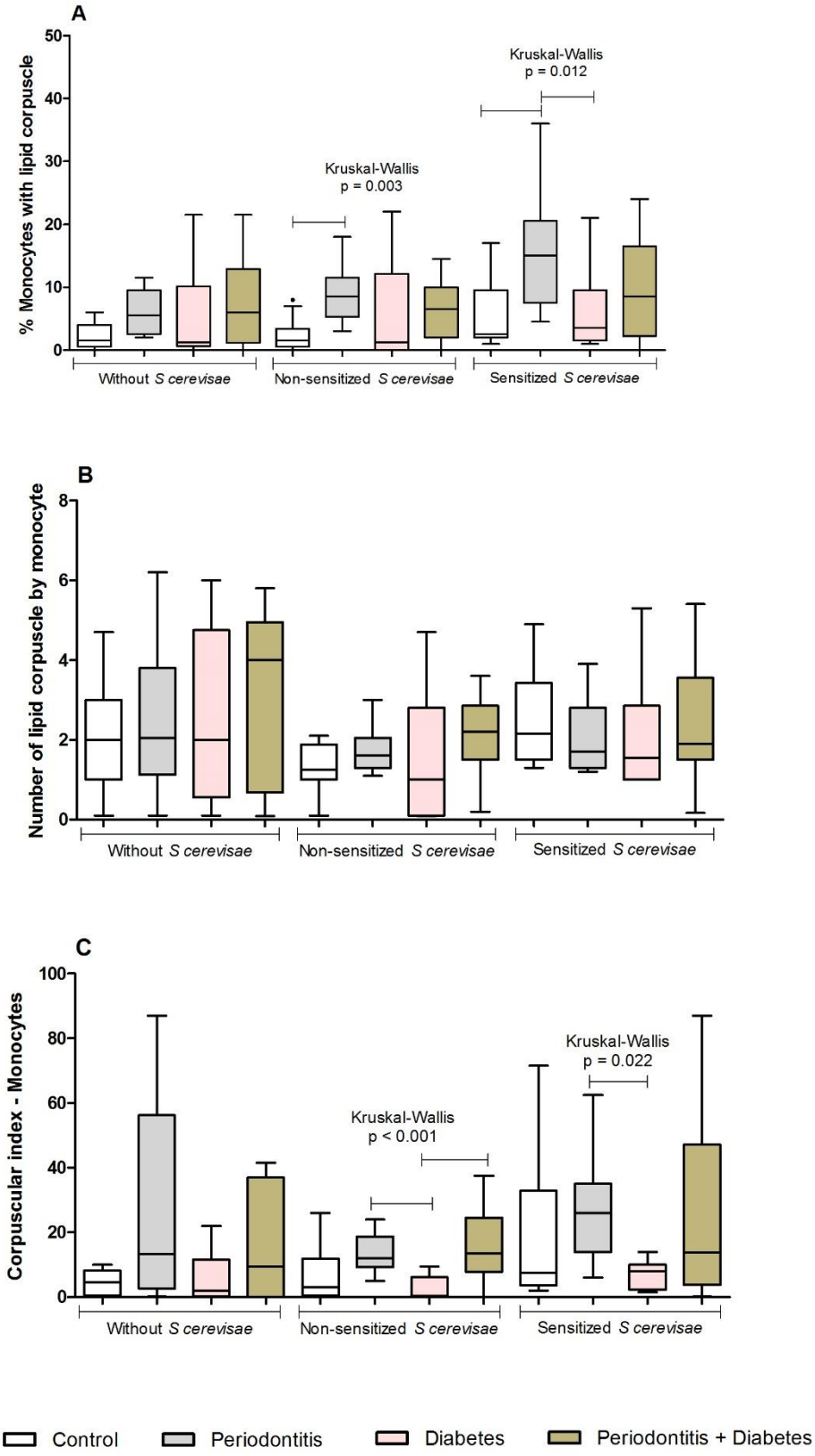

Figure 2. Production of lipid bodies by peripheral blood monocytes from individuals of the four studied groups evaluated by corpuscular index (MØ% x MBL). Values are shown as medians, quartiles, maximum and minimum values.

4.2.5 Phagocytic activity by neutrophils

There was no difference ($p<0.05$) in the phagocytic index among the groups, when sensitized or non-sensitized yeasts were used in the proportion 5:1 levedures per neutrophil, in spite of the reduction ($p=0.007$, ANOVA) in the number of sensitized phagocytized yeasts per neutrophils in periodontitis (median/mean±SD 1.6/1.6±0.5) and DMP groups (1.7/1.8±0.6) compared to controls (2.3/2.4±0.7).

When occurred the increase of the stimulus by levedure in the proportion 20:1, it was observed a reduction ($p=0.008$, Kruskal-Wallis) in the phagocytic index in periodontitis patients (3.5/3.5±2.4) compared to controls (5/16.6±16.9) and DM patients (24.3/26.7±22.1) in the nonsensitized samples, mainly due to the reduction ($p=0.04$, Kruskal-Wallis) in the frequency of neutrophils involved with phagocytosis in periodontitis (2.3/7±15.8) compared to DM (11.8/12.5±9.8) patients.

Healthy individuals also had a significantly higher ($p=0.029$, Kruskal-Wallis) phagocytic index (225.5/223.5±56.1) when compared to patients with diabetes associated to periodontitis (85.3/126.8±101.1) in the opsonized samples, in the proportion of 20 levedures per neutrophil. There was a higher number ($p=0.008$, ANOVA) of phagocytized *S. cerevisiae* per neutrophil in controls (3/2.9±0.7) compared to patients with DMP (1.8/2±0.7) or periodontitis alone (1.7/1.9±0.7). Results are summarized in Figure 3 and Chart 2C.

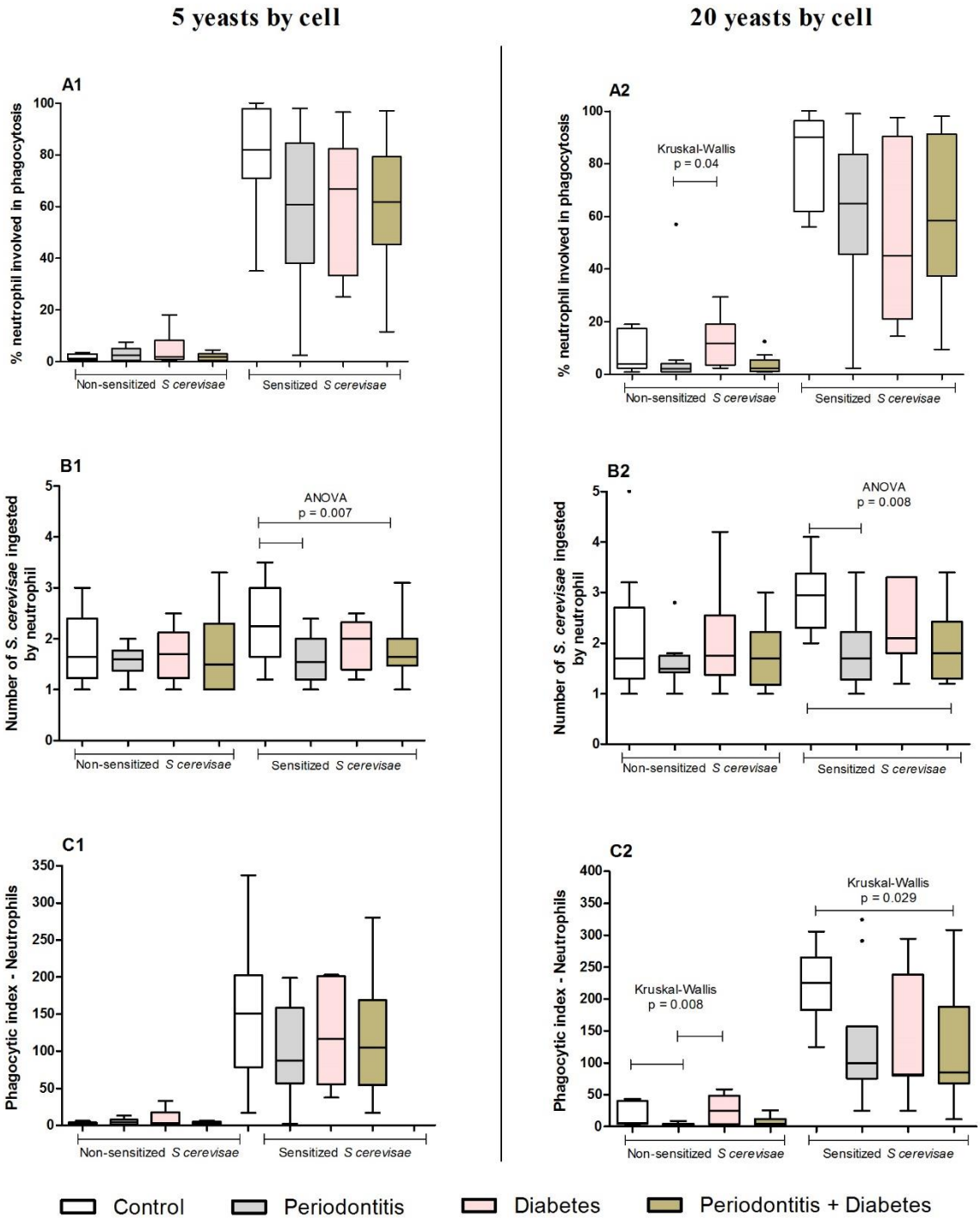

Figure 3. Phagocytic capacity of neutrophils obtained from the peripheral blood of individuals in the control (C) periodontitis (P), diabetes (DM) and periodontitis + diabetes (DMP) groups evaluated by the phagocytic index (C1 and C2), which is the product of percentage of cells involved in phagocytosis (A1 and A2) by the mean of yeasts (sensitized or not with the individual's own serum) ingested per cell (B1 and B2). The results showed lower phagocytic capacity of the periodontitis group than of the control and DM groups when 20 non-sensitized yeasts/cell (C2) was used. Also, the phagocytic index decreased in

the DMP group (with yeast sensitization) in comparison with their respective controls (C2). The medians, quartiles, maximum and minimum values are shown.

4.2.6 Phagocytic activity of monocytes

There was no difference ($p<0.05$) in the phagocytic index among the groups, when non-sensitized yeasts were used in the proportion 5:1 levedures per monocyte. However, when sensitized yeasts were used, there was a reduction ($p=0.018$, Kruskal-Wallis) in PhI from periodontitis patients (median/mean±SD 61.3/82.4±59.2) compared to controls (123/132.9±27.5). It was also observed a reduction ($p=0.001$, Kruskal-Wallis) in the percentage of cells engaged in phagocytosis in periodontitis (48.5/51.5±17.9) and DMP (52.3/51.8±21.7) groups compared to controls (75.8/73.5±8.1) in sensitized samples.

In the 20:1 proportion of levedures per monocyte, there was no difference ($p<0.05$) in the phagocytic index among the groups, when non-sensitized yeasts were used. When sensitized levedure was used, a reduction ($p=0.0007$, Kruskal-Wallis) in PhI occurred in both periodontitis and DMP groups compared to controls.

In the 20:1 proportion, there was a reduction ($p=0.0001$, Kruskal-Wallis) in both the percentage of cells engaged in phagocytosis in DMP (56.8/52.4±23.9)/ periodontitis patients (52.5/53.6±15.4) and in the mean number of yeasts ingested by monocytes from periodontitis group (1.7/1.7±0.4; $p=0.02$, ANOVA) than in controls (87.5/84.5±10.4, % monocytes; 2.3/2.4±0.7, number of phagocytized yeasts).

All results concerning phagocytosis by monocytes are summarized in Figure 4 and Chart 3C.

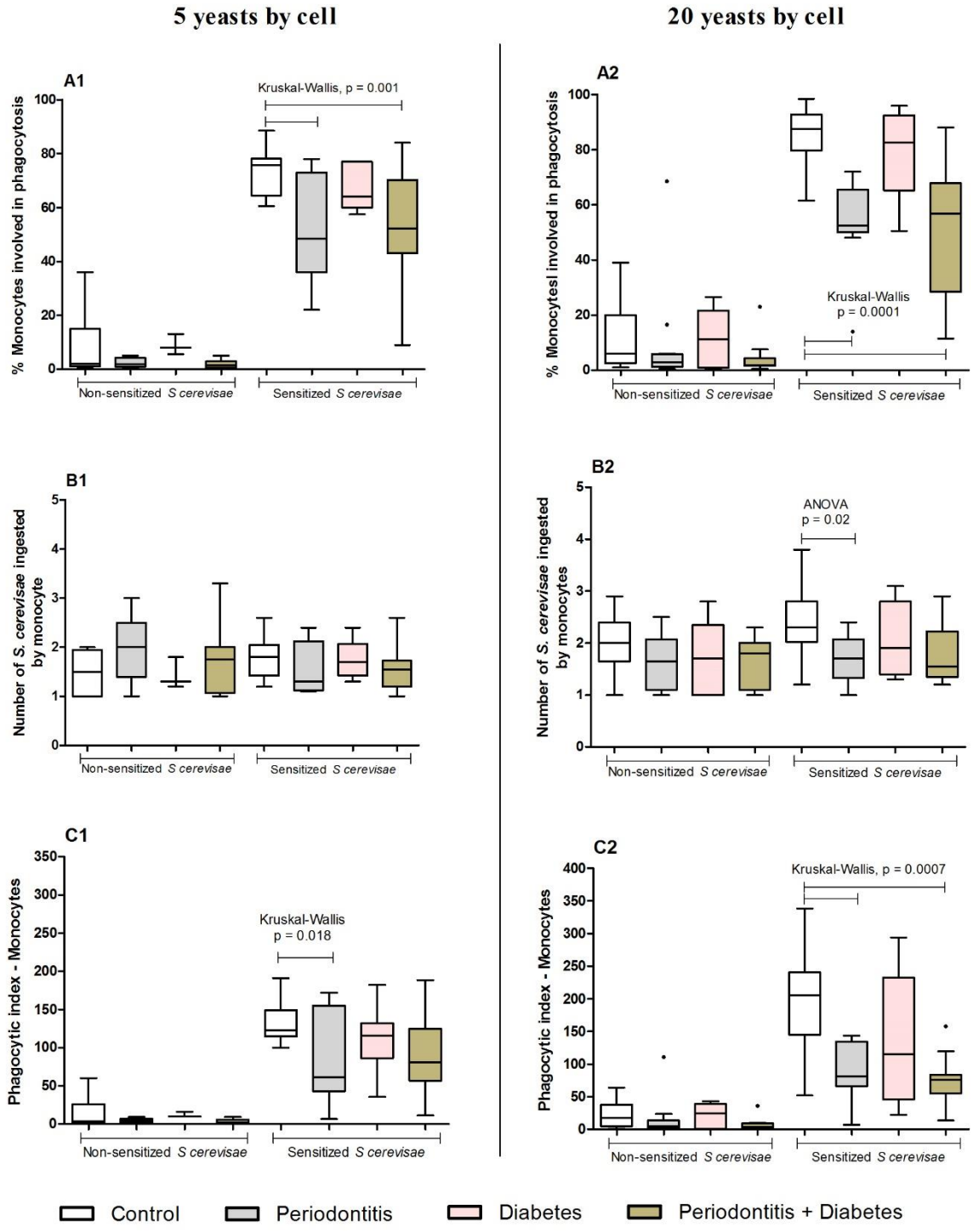

Figure 4. Phagocytic capacity of monocytes obtained from the peripheral blood of individuals in the control (C) periodontitis (P), diabetes (DM) and periodontitis + diabetes (DMP) groups evaluated by the phagocytic index (C1 and C2), which is the product of percentage of cells involved in phagocytosis (A1 and A2) by the mean of yeasts (sensitized or not with the individual's own serum) ingested per cell (B1 and B2). The results showed lower phagocytic capacity of the periodontitis' group than of the control group when both 5 or 20 sensitized yeasts/cell (C1 or C2, respectivelly) were used. Also, the phagocytic index decreased in the DMP group (with yeast sensitization) in comparison with their respective controls (C2). The medians, quartiles, maximum and minimum values are shown.

4.2.7 Superoxide radical

Neither periodontal disease nor diabetes has influenced the capacity of basal or stimulated production of superoxide when compared to the control group or between diabetes groups, as shown in figure 5.

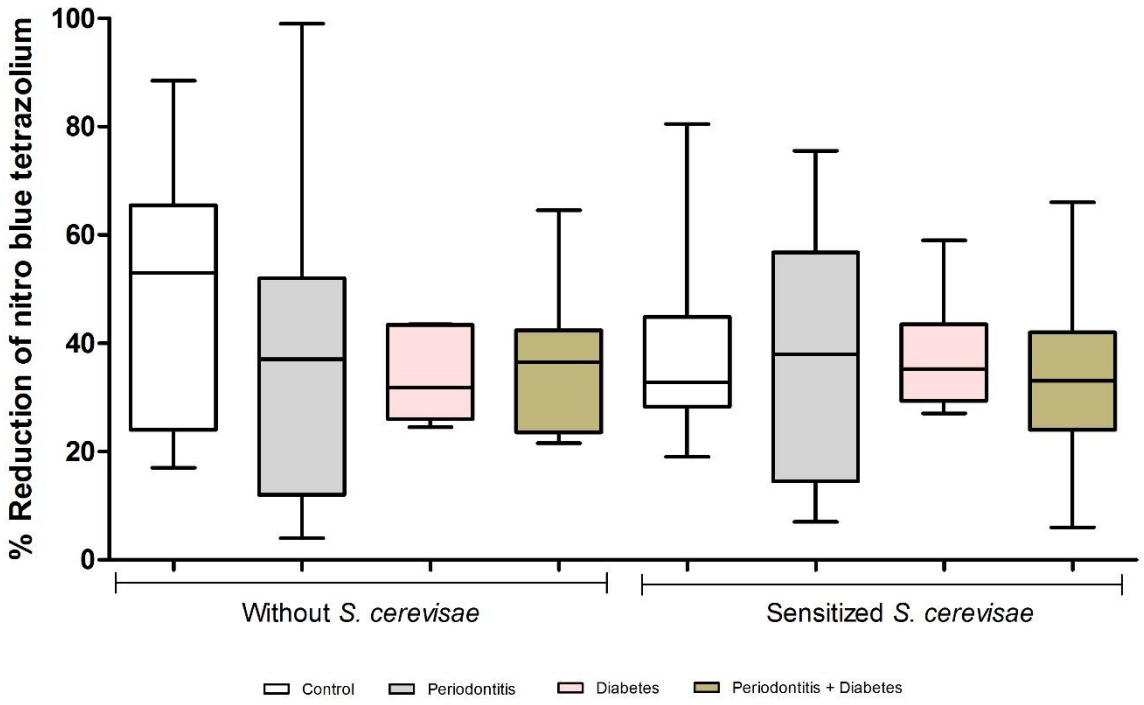

Figure 5. Percentage of reduction of NBT (nitro blue tetrazolium) test by peripheral blood leukocytes from individuals in the control (C) periodontitis (P), diabetes (DM) and periodontitis + diabetes (DMP) groups. The results showed that both basal (Kruskal-Wallis test) and *S. cerevisiae* stimulated (ANOVA) production did not differ between groups. The medians, quartiles and extreme values are shown.

4.3 DISCUSSION

This study evaluated, for the first time, the production of lipid bodies by peripheral monocytes during periodontitis associated or not to diabetes type 2. It also evaluated the influence of periodontitis on the monocyte and neutrophil functions of healthy or DM2 adult individuals, as phagocytosis and production of superoxide

anions, in order to contribute to a better understanding of the action of this oral disease on the main effector cells of the innate immune system.

A significant increase in the frequency of monocytes with corpuscles in their cytoplasm was observed in patients with periodontitis compared to controls after phagocytosis of non-opsonized and opsonized *S. cerevisiae*. However, it was not observed difference regarding the average number of corpuscles present in each cell, neither to the corpuscular index (CI) between these populations.

There was also a significant increase in the CI of the DMP patients compared to the DM group, in the non-sensitized samples. Probably, diabetes or periodontitis alone, per si, were not capable of modifying the total quantity of lipid bodies. However, when the diseases were associated, there was a significant increase in CI in this group (DMP), which reinforces the idea that periodontitis may, through different mechanisms not fully elucidated, contribute to metabolic uncontrol in people with these associated diseases.

Lipid bodies are organelles commonly found in healthy human cells dispersed in the cytoplasm, but some pathological conditions may be associated with its increased number such as neoplasia, atherosclerosis, bacterial sepsis, acute respiratory distress syndrome, arthritis and mycobacterial infections (31). They are the main cellular eicosanoid-producing organelles and the key in the process of intracellular signaling and the inflammatory process (32). Eicosanoids (prostaglandins, leukotrienes and others) are produced from the transformation of arachidonic acid in response to inflammatory cytokines, such as TNF-α, IL-1β and lymphotoxin. They mediate cellular processes such as proliferation, apoptosis, metabolism and cell migration (32).

Besides the eicosanoids, another group of inflammatory molecules was found within lipid bodies: cytokines as TNF-α, RANTES, IL-16, among others (33).

Considering the inflammatory and infectious nature of periodontal diseases, our findings agree with other studies (31,34) which report a direct association between parasite's or infectious diseases as Chagas's disease, malaria, hepatitis C and tuberculosis, and the increased number of lipid bodies. In this study, this increase was attributed to higher frequency of cells presenting this organelle, but without changes in the total CI in periodontitis alone.

LBs exist in various types of inflammatory cells besides in noninflammatory ones, but some studies have shown that LBs in leukocytes, especially macrophages,

increase during inflammation (35, 36), suggesting LBs may function as great sites to produce prostaglandins and other lipid mediators (33).

Concerning the metabolic diseases as diabetes, it is increasingly evident that they can be influenced by changes in the regulation of metabolism and physiology of LBs. Some LBs-associated proteins as perilipin 1 (PLIN 1) and other four proteins: ADRP (also referred to as Plin2), TIP47 (Plin3), S3–12 (Plin4), and OXPAT (Plin5), constitutes the perilipin/ADRP/TIP47 (PAT) family. All these proteins are critical regulators of lipid metabolism (37) and alteration of them in cells as adipocytes can lead to insulin resistance.

Reduced expression of PLIN1, among other proteins in adipocytes, results in the constitutive release of fatty acids (FA). FA can reach the bloodstream activating inflammatory pathways and promote ectopic lipid deposition in peripheral tissues with consequent insulin resistance (37). Insulin resistance has been associated with increased rates of adipocyte lipolysis (38, 39, 40, 41) and increased expression of proinflammatory cytokines as TNF-α, in obesity.

Regarding the clinical characteristics of the studied population, it was observed that healthy individuals had more teeth than patients with diabetes (with or without periodontitis) or periodontitis. This is likely to reflect that general health care is, in addition to other factors, associated with oral health care, since both DM2 and periodontitis are often associated with risk factors such as health-threatening habits and lifestyle (7, 30).

Systemically healthy subjects, including those with periodontitis, had a body mass index (BMI) max of 28 kg /m^2, and the groups with diabetes had a BMI higher than 30 kg /m^2. This indicates that most of the study's participants were already overweight or in degree one of obesity according to the World Health Organization (22,42), respectively. It is worth mentioning that BMI between 17 and 25 kg /m^2 provides a better survival rate and that the relative risk of mortality increases in individuals outside this range.

Differently from our results regarding to patients with periodontitis, we did not find differences in the hemogram and the leukogram parameters between them and controls, but other studies showed increased levels of total leukocytes counts (43, 44, 45) and lower levels of erythrocytes and hemoglobin in severe chronic periodontitis patients compared to controls.

Differently from participants with DM who had triglyceride levels similar to controls, those with DMP presented higher levels of triglycerides when compared to healthy individuals, even exceeding reference values. This may be an indication that periodontal disease is capable of predisposing patients with DM to a higher risk of complications such as the development of cardiovascular diseases since the literature positively associate these high levels of triglycerides with higher cardiac risk (46, 47).

The role of circulating levels of CRP, TNF-α and IL-6 in patients with periodontitis and diabetes have been shown conflicting results in human studies (48). While many studies showed elevated serum levels of these inflammatory mediators in patients with both diseases (49,50,51,52,53,54,55,56) others failed to show an association (57, 58, 59, 60). The elevated levels of proinflammatory cytokines and excessive release of ROS can interfere with diabetes metabolic control (61) and worse cardiovascular complications in DM (62).

Besides the rise of pro-inflammatory mediators as cytokines due to periodontal infection, the systemic oxidative stress has been suggested as an activator of systemic pro-inflammatory pathways which could influence diabetes control in DM patients (48, 49, 50). Unfortunately, there is no scientific model, closer to human model such as animal studies, to support this possibility.

It is not fully understood the mechanisms by which periodontitis promotes metabolic dysfunction. Probably in response to endotoxins such as lipopolysaccharides found in periodontal microorganisms, there is an increase in the production of proinflammatory cytokines that can alter lipid metabolism and insulin resistance (1).

Periodontitis has been associated with a more reduced glycemic control in diabetes (1), but we did not find differences in the results presented in this study between subjects with DMP and DM alone concerning to glycemic levels (blood glucose or % A1c).

A cohort longitudinal study in Germany (63), over 15 years in patients with severe periodontitis, showed that A1c levels just after five years from their baseline levels, were significantly higher than in healthy individuals. Additionally, after a 10-year follow-up period study (64), the authors reported greater increase in the prevalence of periodontal disease among patients who developed glucose intolerance than those who had not. This poorer control in glycemic indices increases the risk for the diabetes

incidence in non-diabetic patients, insulin resistance in patients with DM and disease complications, including mortality (1).

Regarding innate immune defense, leukocytes are the major mechanisms of the body against microbial invasion (65). At the beginning of a bacterial infection, neutrophils are the predominant cells involved in the host defense and they also play an important role in the course of pathogenesis and inflammation of the infectious diseases (66). Leukocytes respond to virulence factors with the secretion of pro-inflammatory mediators such as cytokines, chemokines, reactive oxygen species (ROS) and C-reactive protein (CRP) (67). CRP is mainly produced by the liver, and cardiovascular disease has CRP as an independent predictor of its occurrence (68).

The present study found no significant differences in leukocytes counts between periodontitis patients and controls, however other studies showed different results (69,70). Patients with periodontitis may present a significantly higher number of leukocytes compared to healthy people and this may indicate an increased risk of myocardial infection (69). A study conducted by Pejčić et al. (2011) (70) concluded that an increase in total leukocyte count in patients with periodontitis, especially in its severe form, may be an indicator of possible exposure to a systemic disease, and may represent an important warning for physicians to refer their patients to a dentist. Phagocytes, such as neutrophils and monocytes, stand out as leukocytes that act on the initial defense response against periodontal pathogens.

Acute phase proteins and leukocytosis are induced by pro-inflammatory cytokines or bacterial components released into the circulation. The raised number of white blood cells has been associated with the risk of coronary heart disease, cardiovascular disease, atherosclerosis, thrombosis and myocardial ischemia. In this context, the inflammatory nature of chronic infections such as periodontitis could be associated with leukocytosis and major CRP levels into the bloodstream (71,72).

Previous studies (73) have shown that serum inflammatory biomarkers, as leukocytes and CRP, are elevated in periodontitis patients (74,75,76) and this can predict the incidence of DM2 (77,78). In line with these findings, our study demonstrated that peripheral blood CRP levels increased when diabetes was associated with periodontitis compared to DM alone. The risk of coronary heart disease is estimated to be 1.5–2-fold higher in people with periodontitis than in those without the disease (79,80).

However, the current knowledge about the association of both diseases shows that CRP serum levels decrease with the administration of mechanical periodontal treatment in periodontal disease (81), supporting the view that severe periodontitis induces systemic micro-inflammation.

In the present study, there was no difference in phagocytosis among different groups when non-opsonized *S. cerevisea* was used as antingen in the proportion of 5:1 yest per cell, for both neutrophils and monocytes. But, when we chalange cells with increased levedure stimulus (20:1 yest per cell), it was observed an impairement in phagocytic function of neutrophils from periodontitis patients in non-sensitized samples compared to controls.

However, interestingly when opsonized yests (with own serum from cell's donors) there was a signifcant reduction in phagocytosis of monocytes from periodontitis patients compared to controls in both 5:1 and 20:1 proportions. This reduction in phagocytic functions of monocytes was mainly due to the reduction in the percent of cells engaged with phagocytosis in both yeasts proportions and also due to the minor number of phagocytized yeasts per cells (only in 20:1 proportion).

Perhaps, periodontitis, by mechanisms not yet elucidated, promotes opsonic defects in patients and causes this less effective phagocytosis by neutrophils and especially, monocytes.

Compared to healthy individuals, DMP patients also showed reduction in phagocytosis from neutrophils and monocytes, when opsonized yests (20:1) were used. Neutrophils had reduced phagocytic capacity becase of a reduction in the capacity to englobe levedures, and monocytes because of the reduction in the frequency of monocytes envolved in this function.

These data may reinforce the fact that periodontitis, *per se*, is capable of impairing innate defense mechanisms of individuals, which could contribute to the development of complications from diabetes mellitus.

There are few studies on phagocytic activity by neutrophils or monocytes from the peripheral circulation. These reports have controversial results, with reduction (82,83,84,85,86) or even increase (87,88) of this function in individuals with periodontitis. However, our finds agree with those found by Carneiro et al. (2012) (82), who showed a deficiency in this same function in individuals with severe periodontitis, indicating that maybe periodontal disease may modify immune responses, as phagocytic activities, even in systemic healthy individuals.

One important phagocyte-derived oxidant has been identified as critical controlling periodontitis infection: superoxide anion (O_2^-) (89). Neutrophils and monocytes are examples of cells that produce superoxide anion against bacterial agents (90,91). We did not find any alteration in the production of superoxide among all groups. However, some studies that have evaluated reactive oxygen species, including superoxide, in the systemic circulation of individuals with periodontitis are not consensual in the literature, showing equal (92) or even enhanced (89,93,94,95) production. The production of ROS by phagocytic cells is enhanced at periodontal pockets (96). Excessive local ROS can affect the oxidative status of tissues, activating and sustaining phagocytes to kill phagocytized pathogens. However, this may be harmful not only to the microorganisms but also to the host cells (97).

ROS is also one of the factors that have a possible impact in insulin resistance, based upon two aspects. One is the association of obesity and diabetes with markers of oxidative stress (98,99) and the other is that there is evidence that direct treatment with agents that induce ROS accumulation can induce insulin resistance (100, 101).

All findings achieved here lead to observe that periodontitis, per se, is probably capable of modifying the innate immune response mediated by neutrophils or monocytes in individuals with type 2 diabetes or systemically healthy, a fact that may contribute to a greater systemic susceptibility to diseases, or even worsen the health status of patients with DM2.

A possible limitation of this study is the extrapolation of an *in vitro* laboratory finding for a clinical condition that involves a multiplicity of interplaying functional factors. To this respect, it should be pointed that there is no alternative way to that presently employed in order to demonstrate the effects of different factors acting directly on phagocytic cells in humans, *in vivo.*

Another limitation of this study is the sample size. Although a minimum number of participants (n = 31) was calculated for each group and, even after an extensive screening of candidates, we were unable to reach this number. It was due to the rigorous inclusion and exclusion criteria of the study.

Most individuals interviewed had a variety of comorbidities or complications associated with diabetes, which could compromise the association between periodontitis with changes in individual immune responses and create *bias* in the study. In addition, many candidates, especially those with diabetes, had total or partial edentulism (with fewer teeth than the minimum required for each group). Another

complicating factor is that there was a low demand for dental care by the population affected by periodontitis, perhaps since the disease hardly causes painful associated symptomatology. Often patients only seek specialized dental care when their teeth already have marked mobility with indication for multiple exodontia.

Probably new studies would obtain more significant results related to the changes in the immune response of these individuals if the above-related difficulties were overcome.

4.4 CONCLUSIONS

The increase in the frequency of monocytes with lipid bodies in their cytoplasm in periodontitis patients may be related to the fact that these organelles participate in some way in the etiopathogenesis of periodontal disease: periodontitis. This finding, associated with the reduction of phagocytosis in these patients, contributes to reinforcing the theory that periodontitis may predispose healthy individuals to a series of systemic complications such as diabetes mellitus.

Since patients with DM had few changes in the immunological parameters in the present study, the data suggest a greater impact of the disease when associated with periodontitis as showed by higher levels of C-reactive protein (CRP) and increased number of lipid bodies from blood of patients with DMP compared to DM alone or reduced phagocytic activity of neutrophils and monocytes from diabetes with periodontitis patients compared to health individuals.

So, it is reasonable to conclude that periodontitis may cause important immunological changes in cells from human peripheral blood whose impact on the systemic condition of patients with or without diabetes mellitus needs to be better studied.

4.5 ACKNOWLEDGMENTS

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4.6 CONFLICT OF INTEREST AND SOURCE OF FUNDING STATEMENT

The authors declare that there is no conflict of interest in this study.

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5 PERSPECTIVAS

A produção de corpúsculos lipídicos durante a ocorrência de algumas doenças infecciosas ou inflamatórias tem sido amplamente investigada. Nosso estudo mostrou, pela primeira vez, a presença dos mesmos em monócitos de indivíduos com processos ativos de periodontite associada ou não ao diabetes. Tais resultados reforçam que o aumento no número destas organelas no citoplasma de determinados tipos celulares pode estar associado a processos inflamatórios ocasionados por algumas doenças.

Somado a isso, os resultados referentes à função fagocitária de neutrófilos e monócitos deste estudo vêm ao encontro de outros previamente publicados, aumentando as evidências sobre a relação da periodontite com a maior fragilidade ou susceptibilidade do sistema imune frente a novos desafios e, predispondo o indivíduo a maiores riscos de compromentimento de sua saúde geral.

A relevância dos resultados do nosso estudo deverá, ainda, ser complementada por pesquisas adicionais que afiram os efeitos da infecção periodontal nestas células, de forma local – i.e., nos tecidos periodontais e, sistêmica, utilizando para isso micro-organismos de importância na etiologia da periodontite como estímulo à fagogocitose e à produção de corpúsculos lipídicos.

Os mecanismos pelos quais a deficiência da função fagocítica e aumento do número de corpúsculos lipídicos podem levar ao comprometimento da saúde sistêmica, também precisam de maior elucidação. A influência dos mediadores inflamatórios e de polimorfismos genéticos envolvidos nestas relações deverá ser mais profundamente explorada.

O maior conhecimento da relação entre os agentes microbianos e a resposta imunológica poderá resultar na implementação de formas de tratamento que tenham como alvo não os micro-organismos e sim, a redução da resposta inflamatória para evitar a destruição dos tecidos de suporte dos dentes em relação aos efeitos locais da periodontite. Esta proposta considera a modulação da resposta do hospedeiro por mecanismos farmacológicos ou biológicos, produzindo um mínimo ou nenhum efeito adverso ou colateral ao indivíduo. Todavia, ressalta-se que a importância da desorganização do biofilme é um princípio claramente estabelecido, cujo sucesso requer, imprescindivelmente, sua associação à terapia mecânica periodontal.

Em nível sistêmico, este conhecimento pode ser grande valia para a prevenção de potenciais riscos à saúde geral dos indivíduos com periodontite associada ou não a outras doenças sistêmicas como o diabetes, aumentando tanto a qualidade quanto a expectitiva de vida destas pessoas.

ANEXO A

Comprovante de envio ao Comitê de Ética

SECRETARIA DE ESTADO DE SAÚDE DO DISTRITO FEDERAL - FEPECS/ SES/ DF

PARECER CONSUBSTANCIADO DO CEP

Elaborado pela Instituição Coparticipante

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Efeito da terapia periodontal não cirúrgica no perfil microbiológico, imunocelular, de imunoglobulinas e na dosagem de citocinas pró e anti-inflamatórias provenientes de sangue periférico e saliva de indivíduos com diabetes tipo 2

Pesquisador: MARIA DO CARMO MACHADO GUIMARÃES

Área Temática: Genética Humana:
(Trata-se de pesquisa envolvendo Genética Humana que não necessita de análise ética por parte da CONEP;);

Versão: 1

CAAE: 46609515.7.3001.5553

Instituição Proponente: Faculdade de Ciências da Saúde da Universidade de Brasília

Patrocinador Principal: FUNDACAO UNIVERSIDADE DE BRASILIA

DADOS DO PARECER

Número do Parecer: 1.576.279

APÊNDICE A

Termo de consentimento livre e esclarecido

UNIVERSIDADE DE BRASÍLIA
PÓS GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (TCLE)

Convidamos o (a) senhor (a) para participar do projeto de pesquisa "**Efeito da terapia periodontal não cirúrgica no perfil microbiológico, imunocelular, de imunoglobulinas e na dosagem de citocinas pró e anti-inflamatórias provenientes de sangue periférico e saliva de indivíduos com Diabetes tipo 2**", sob a responsabilidade da pesquisadora Priscilla Farias Naiff. O projeto avaliará as suas condições de saúde bucal por meio de um exame clínico (exame de sua boca) além de coletar um pouquinho de seu sangue, saliva, líquido e biofilme subgengivais (de baixo da gengiva), para fazer algumas medidas.

O objetivo desta pesquisa é o de medir a quantidade de bactérias, anticorpos, células de defesa do corpo e citocinas (substâncias produzidas pelas células do seu corpo) presentes no sangue, saliva, biofilme dental e no fluido gengival de pessoas com ou sem diabetes do tipo 2 e com ou sem periodontite crônica (inflamação da gengiva e do osso ao redor dos dentes). Além disso, este estudo irá verificar a frequência de determinadas variantes do DNA (material genético que informa como nosso corpo é formado).

O (a) senhor (a) receberá todos os esclarecimentos necessários antes e no decorrer da pesquisa e lhe asseguramos que seu nome não aparecerá, sendo mantido o mais rigoroso sigilo pela omissão total de quaisquer informações que permitam identificá-lo (a)

A sua participação consistirá em responder uma ficha de identificação e autorizar, por duas vezes (uma antes e outra após o tratamento periodontal), a coleta de 40 mL (aproximadamente quatro seringas) de sangue. O procedimento é o mesmo utilizado para realização de diversos outros tipos de exame de sangue. Será utilizado material novo, estéril e descartável. A coleta do sangue será feita em uma veia de seu braço, podendo causar uma leve dor na hora e um pequeno hematoma (mancha roxa) que desaparecerá em 3 a 4 dias após a coleta. Também serão coletados um pouco de sua saliva, biofilme (placa dental) e líquido do sulco gengival por duas vezes (uma antes e outra após o tratamento periodontal). A coleta de saliva levará dez minutos e o (a) senhor (a) deverá cuspir a saliva em um tubo plástico durante este tempo. Para a coleta de placa e liquido subgengival, será inserida uma fina tira de papel específica e apropriada para esse fim entre seu dente e gengiva, por no máximo, um minuto. As coletas de saliva, biofilme e fluido gengival não gerarão nenhum desconforto para o (a) senhor (a).

A coleta das amostras será pela manhã, em jejum, e antes de qualquer procedimento de higiene bucal (escovação, uso do fio dental ou bochecho). Após a coleta, o (a) senhor (a) receberá orientações

sobre higiene bucal e será realizada profilaxia bucal (limpeza) e tratamento periodontal, se necessário, com um tempo estimado de 6 meses a 1 ano para sua realização.

Se for necessário o tratamento periodontal, por meio de raspagem, imediatamente após a realização do mesmo, há possibilidade de apresentar hiperestesia dentinária (sensibilidade ou dor transitória nos dentes, normalmente associada ao frio), por um período de até 4 semanas, mas esperamos que isso não aconteça.

Se você autorizar esta coleta, as amostras serão utilizadas para este estudo e o que não for usado será guardado para realização de outras pesquisas, de acordo com o estabelecido pela Resolução n◦ 441, de 12 de maio de 2011 do Conselho Nacional de Saúde.

Os riscos decorrentes de sua participação na pesquisa, como já descritos anteriormente são, a sensibilidade após o tratamento periodontal e dor leve no procedimento de coleta de sangue. O (A) senhor (a) foi escolhido (a) porque tem a (s) doença (s) ou porque não tem doença nenhuma. Se você aceitar participar, estará contribuindo para confirmarmos se o tratamento periodontal auxilia na melhoria da qualidade de vida dos pacientes com diabetes, por meio da alteração de mediadores inflamatórios.

Os resultados dos seus exames poderão ser entregues pelo (a) pesquisador (a) responsável mediante a sua solicitação, a qualquer momento, desde que as amostras já tenham sido processadas e analisadas. Esta solicitação poderá ser feita agora durante a assinatura deste TCLE, por e-mail ou telefone (presentes neste TCLE), e o (a) pesquisador (a) agendará uma reunião para a entrega do resultado.

Os resultados da pesquisa serão analisados e publicados, mas sua identidade não será divulgada, sendo guardada em segredo para sempre. Os resultados deste trabalho poderão ser apresentados em encontros ou revistas científicas, entretanto, ele mostrará apenas os resultados obtidos como um todo, sem revelar seu nome ou qualquer informação que esteja relacionada com sua privacidade.

Toda nova pesquisa a ser feita com o material guardado foi submetida para aprovação de um Comitê de Ética em Pesquisa (CEP) e, quando for o caso, da Comissão Nacional de Ética em Pesquisa.

O (a) senhor (a) pode se recusar a responder (ou participar de qualquer procedimento) qualquer questão que lhe traga constrangimento, podendo desistir de participar da pesquisa em qualquer momento sem nenhum prejuízo para o (a) senhor (a). Sua participação é voluntária, isto é, não há pagamento por sua colaboração. Se depois de autorizar a coleta, o (a) senhor (a) não quiser que seu sangue, saliva, fluido ou biofilme subgengival sejam usados, tem o direito e a liberdade de retirar seu consentimento em qualquer fase da pesquisa, seja antes ou depois da coleta das amostras, independente do motivo e sem prejuízo do atendimento que irá receber, bastando para isso entrar em contato com um (a) dos (as) pesquisadores (as) responsáveis.

Todas as despesas que você tiver relacionadas diretamente ao projeto de pesquisa (tais como, passagem para o local da pesquisa, alimentação no local da pesquisa ou exames para realização da pesquisa) serão cobertas pelo pesquisador responsável.

Caso haja algum dano direto ou indireto decorrente de sua participação na pesquisa, você poderá ser indenizado, obedecendo-se as disposições legais vigentes no Brasil.

Se o (a) senhor(a) tiver qualquer dúvida em relação à pesquisa, por favor telefone para: Drª Maria do Carmo Machado Guimarães ou Priscilla Farias Naiff, no Departamento de Odontologia, da UnB no telefone (61) 3107-1802 no horário de 8 às 17h e/ou pelos e-mails: mmgcarmo@gmail.com ou pri_naiff@yahoo.com. O Departamento de Odontologia está localizado na Faculdade de Ciências da Saúde, Campus Universitário Darcy Ribeiro, Universidade de Brasília, Asa Norte.

Este projeto foi Aprovado pelo Comitê de Ética em Pesquisa da Faculdade de Ciências da Saúde (CEP/FS) da Universidade de Brasília. O CEP é composto por profissionais de diferentes áreas cuja função é defender os interesses dos participantes da pesquisa em sua integridade e dignidade e contribuir no desenvolvimento da pesquisa dentro de padrões éticos. As dúvidas com relação à assinatura do TCLE ou os direitos do participante da pesquisa podem ser esclarecidos pelo telefone (61) 3107-1947 ou do e-mail cepfs@unb.br ou cepfsunb@gmail.com, horário de atendimento de 10:00hs às 12:00hs e de 13:30hs às 15:30hs, de segunda a sexta-feira. O CEP/FS se localiza na Faculdade de Ciências da Saúde, Campus Universitário Darcy Ribeiro, Universidade de Brasília, Asa Norte.

Este documento foi elaborado em duas vias, uma ficará com o pesquisador responsável e a outra com o Senhor(a).

CONSENTIMENTO PÓS–INFORMAÇÃO

Eu,_____, RG _____, CPF _____ fui informado (a) sobre o que o (a) pesquisador(a) quer fazer e porque precisa da minha colaboração e entendi a explicação. Por isso, eu concordo em participar do projeto, sabendo que não vou ganhar nada e que posso retirar minha autorização quando quiser. Estou recebendo uma cópia deste documento, assinada, que vou guardar.

_____, _____ de _____ de _____

_____

Assinatura do participante

_____

PRISCILLA FARIAS NAIFF

APÊNDICE B

Ficha de anamnese e exame clínico periodontal

QUESTIONÁRIO DE SAÚDE

Nome: _____

Data de nascimento: _____ Sexo ( ) feminino ( ) masculino

Naturalidade (Cidade/ estado): _____

Idade: _____ Altura: _____ Peso: _____

Escolaridade: ( ) ensino fundamental ( ) ensino médio ( ) nível superior

Endereço: _____

Telefone: _____

Sabe se tem alguma doença na boca? Qual?

_____

_____

_____

_____

Quantos dentes tem na boca? _____

Sente dor nos dentes ou gengivas? ( ) SIM ( ) NÃO

Suas gengivas sangram? ( ) SIM ( ) NÃO

Tem dentes moles? ( ) SIM ( ) NÃO

Já tomou anestesia na boca para tratamento dentário? ( ) SIM ( ) NÃO

Teve alguma reação a esta anestesia? ( ) SIM ( ) NÃO

Já arrancou algum dente antes por ter ficado mole? ( ) SIM ( ) NÃO

Pratica exercício físico? ( ) SIM ( ) NÃO

Fuma? ( ) SIM ( ) NÃO

Já fumou ( ) SIM ( ) NÃO Quando parou de fumar? _____

Consome bebida alcoólica frequentemente? ( ) SIM ( ) NÃO

Tem alergia a algum remédio? ( ) SIM ( ) NÃO Qual? _____

Se for mulher, está grávida? ( ) SIM ( ) NÃO

Se for mulher, está amamentando? ( ) SIM ( ) NÃO

Já fez alguma cirurgia? ( ) SIM ( ) NÃO Qual? _____

Você está fazendo algum tratamento médico? ( ) SIM ( ) NÃO. Para quê?

_____

_____

_____

Toma algum medicamento? ( ) SIM ( ) NÃO Qual (is) e para quê?

_____

_____

_____

Quando foi sua última visita ao dentista? Que tratamento fez lá?

_____

Assinale um **X** nas doenças que você tem ou já teve:

( ) diabetes tipo 1( ) tipo 2 ( ) Não sabe ( )

( ) pressão alta ou baixa

( ) anemia, hemorragia ou leucemia

( ) alergia. A quê?_____

( ) problemas no fígado? Qual? _____

( ) doença no coração. Qual? _____

( ) problema nos rins. Qual? _____

( ) hepatite – (a) (b) (c)

( ) câncer. Onde? _____

( ) doenças articulares

( ) lúpus

( ) aids

FICHA CLÍNICA PERIODONTAL

Nome:_____ **Idade:**_____ **Data:**____/____/____

Dente	Profundidade de bolsa (NIC- valor entre parênteses)						Lesão de furca	Mobilidade	Gengivite	Periodontite			Prognóstico			Índice gengival	Índice de placa
	MV	V	DV	ML	L	DL				Leve	Modera	Grave	Bom	Duvidoso	Ruim		
18																	
17																	
16																	
15																	
14																	
13																	
12																	
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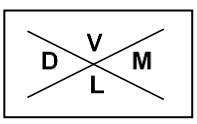
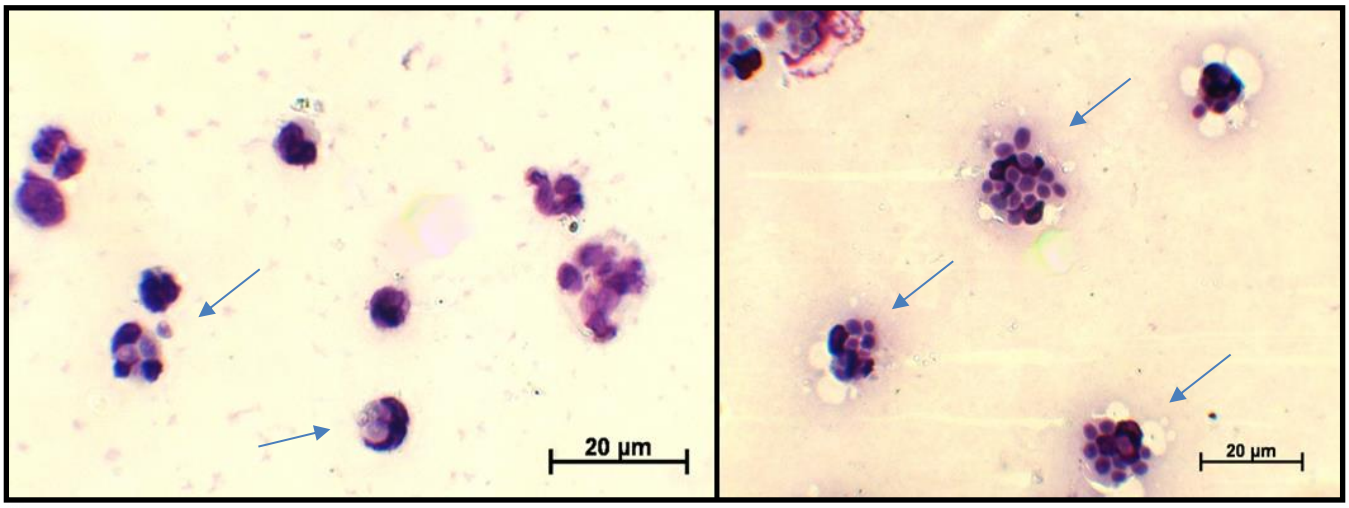


APÊNDICE C

Figuras dos experimentos realizados



A **B**

Figura 1. Fagocitose de *Saccharomyces cerevisiae* **A)** incubadas com soro bovino inativado **B)** incubadas com soro do indivíduo (opsonizadas).

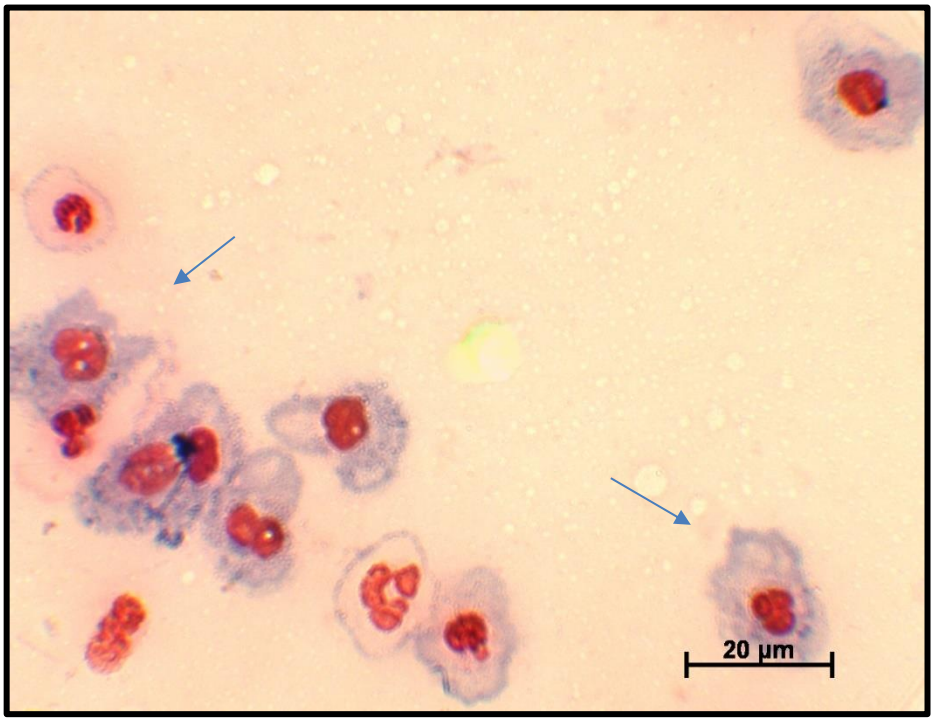


Figura 2. Produção indireta de ânion superóxido. O processo de redução converte o NBT de um composto solúvel em um material insolúvel de coloração azul, visível no citoplasma do fagócito.

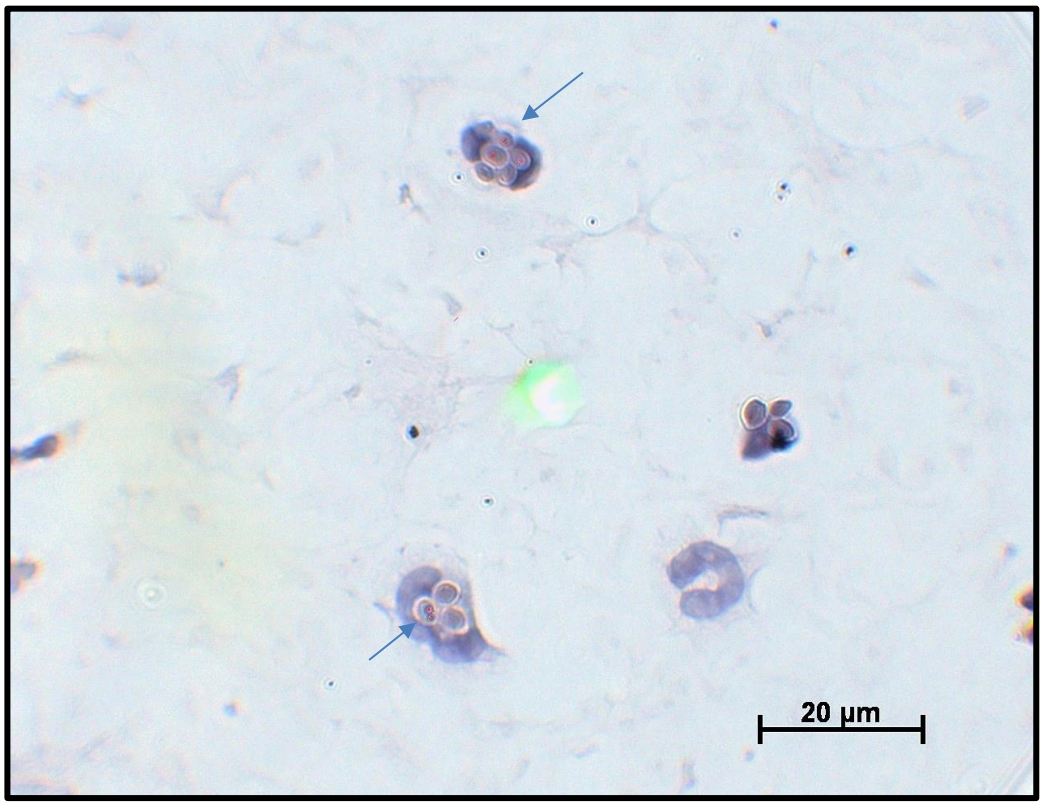


Figura 3. Presença de corpúsculos lipídicos em monócitos.

APÊNDICE D

Tabela e quadros complementares

Tabela 1C - Perfil epidemiológico dos indivíduos

GRUPO CONTROLE					GRUPO PERIODONTITE				
Indivíduo	***Sexo***	***Idade***	***IMC (kg/m²)***	***Nº de dentes***	***Indivíduo***	***Sexo***	***Idade***	***IMC (kg/m²)***	***Nº de dentes***
WSD	M	32	22	25	ATS	M	54	27.31	20
GOC	M	33	27	30	FLS	M	30	22.79	22
RAF	M	48	26	26	GPS	M	37	25.24	26
LAL	M	46	27	30	PSP	M	42	22.20	28
JFNS	M	31	27	28	FAM	M	48	26.20	13
CRD	F	64	23	25	NLS	M	44	24.00	29
PFN	F	35	24	27	ZMF	F	58	23.83	27
MMCR	F	46	25	24	AMMS	F	53	34.15	28
MRSA	F	41	20	28	RSV	F	32	23.74	31
NRG	F	47	31	23	SPS	F	45	33.32	22
AFS	F	39	26	28	CAO	F	50	37.32	11
MNL	F	38	24	26	EVS	F	40	37.11	22
IG	F	30	26	28	NSRS	F	37	22.50	26
TML	F	53	27	28	MER	F	58	-	15
MAAS	F	46	31	27	-	-	-	-	-
NGM	F	44	25	23	-	-	-	-	-
Média±DP	**-**	**42±9**	**26±3**	**27±2**	**Média±DP**	**-**	**45±9**	**28±6**	**23±6**

GRUPO DIABETES					GRUPO DIABETES + PERIODONTITE				
Indivíduo	***Sexo***	***Idade***	***IMC (kg/m²)***	***Nº de dentes***	**Indivíduo**	***Sexo***	***Idade***	***IMC (kg/m²)***	***Nº de dentes***
DVA	M	54	29.6	21	MBC	M	52	34.0	21
CGSL	M	38	25.4	26	ARD	M	44	32.8	22
MSM	F	53	39.8	28	JML	M	50	34.7	11
JAL	F	53	27.2	12	PPS	M	56	27.6	26
GCAA	F	41	35.3	20	ESA	M	60	27.0	14
KMBS	F	40	38.3	24	EAP	M	61	28.0	16
JGP	F	45	30.7	18	MRO	M	41	34.6	26
SPG	F	45	28.1	17	RML	M	57	27.7	21
CCF	F	70	32.4	29	VCP	M	48	32.0	17
LRPS	F	55	29.6	21	HMS	M	59	29.4	23
VCSA	F	62	23.1	19	TAS	F	60	41.7	19
-	-	-	-	-	MVB	F	48	25.7	21
-	-	-	-	-	DAC	F	49	30.1	28
-	-	-	-	-	HESB	F	53	31.5	29
-	-	-	-	-	SSN	F	46	23.8	25
-	-	-	-	-	NEC	F	64	24.7	26
-	-	-	-	-	BSM	F	50	26.4	23
Média±DP	**-**	**51±10**	**31±5**	**21±5**	**Média±DP**	**-**	**53±7**	**30±5**	**22±5**

Quadro 1C - Corpúsculos lipídicos

		% monócitos com CL										
%	C basal	P basal	DM basal	DMP basal	C-NS	P-NS	DM-NS	DMP-NS	C-S	P-S	DM-S	DMP-S
Mediana	1.5	5.5	1.2	6.0	1.5	8.5	1.2	6.5	2.5	15.0	3.5	7.5
Média	2.4	6.2	5.5	7.5	2.3	8.5	5.6	6.4	5.8	14.7	6.1	8.7
Desvio padrão	2.1	3.5	7.5	7.7	2.5	4.0	8.5	4.5	5.3	9.3	6.2	7.7
		Média de CL/célula										
%	C basal	P basal	DM basal	DMP basal	C-NS	P-NS	DM-NS	DMP-NS	C-S	P-S	DM-S	DMP-S
Mediana	2.0	2.0	2.0	4.0	1.2	1.6	1.0	2.2	2.1	1.7	1.5	1.9
Média	2.0	2.4	2.4	2.9	1.2	1.7	1.6	2.1	2.4	2.0	2.1	2.5
Desvio padrão	1.3	1.9	2.1	2.2	0.6	0.5	1.6	0.9	1.1	0.8	1.3	1.5
		Índice corpuscular										
%	C basal	P basal	DM basal	DMP basal	C-NS	P-NS	DM-NS	DMP-NS	C-S	P-S	DM-S	DMP-S
Mediana	4.5	13.2	2.0	9.5	3.0	12.0	0.3	13.5	7.5	26.0	8.0	13.7
Média	4.5	28.2	5.6	15.1	6.3	13.5	2.6	15.0	18.0	27.5	6.5	27.6
Desvio padrão	4.0	32.5	8.1	17.0	7.8	5.7	3.7	10.3	21.0	16.5	4.5	30.3

Quadro 2C - Fagocitose por neutrófilos

5 leveduras/célula	% neutrófilos envolvidos na fagocitose							
	C-NS	P-NS	DM-NS	DMP-NS	C-S	P-S	DM-S	DMP-S
Mediana	1,3	2,5	2,0	2,0	82,0	60,8	66,8	61,8
Média	1,7	2,8	4,8	1,9	80,9	58,5	61,8	60,0
Desvio padrão	1,2	2,5	6,7	1,3	18,2	29,3	26,8	24,7
	Média de leveduras/célula							
	C-NS	P-NS	DM-NS	DMP-NS	C-S	P-S	DM-S	DMP-S
Mediana	1,7	1,6	1,7	1,5	2,3	1,6	2,0	1,7
Média	1,8	1,6	1,7	1,7	2,4	1,6	1,9	1,8
Desvio padrão	0,7	0,3	0,5	0,8	0,7	0,5	0,5	0,6
	Índice fagocitário							
	C-NS	P-NS	D-NS	D +P-NS	C-S	P-S	D-S	D +P-S
Mediana	2,8	4,0	3,0	2,5	151,0	87,5	117,0	105,3
Média	2,8	4,4	8,9	3,0	151,5	102,5	123,9	115,1
Desvio padrão	1,9	4,2	12,6	2,1	86,8	66,9	72,2	74,7
20 leveduras/célula	% neutrófilos envolvidos na fagocitose							
	C-NS	P-NS	DM-NS	DMP-NS	C-S	P-S	DM-S	DMP-S
Mediana	4,0	2,3	11,8	2,5	90,0	65,0	45,0	58,5
Média	8,0	7,0	12,5	3,9	81,6	61,0	53,6	58,8
Desvio padrão	7,2	15,8	9,8	3,4	17,0	28,4	32,8	30,8
	Média de leveduras/célula							
	C-NS	P-NS	D-NS	D +P-NS	C-S	P-S	D-S	D +P-S
Mediana	1,7	1,5	1,8	1,7	3,0	1,7	2,1	1,8
Média	2,2	1,6	2,0	1,8	2,9	1,9	2,6	2,0
Desvio padrão	1,2	0,5	1,1	0,6	0,7	0,7	1,5	0,7
	Índice fagocitário							
	C-NS	P-NS	D-NS	D +P-NS	C-S	P-S	D-S	D +P-S
Mediana	5,0	3,5	24,3	4,5	225,5	99,5	81,5	85,3
Média	16,6	3,5	26,7	7,7	223,5	137,7	133,4	126,8
Desvio padrão	16,9	2,4	22,1	7,9	56,1	92,8	97,5	101,1

Quadro 3C - Fagocitose por monócitos

5 leveduras/célula	% monócitos envolvidos na fagocitose							
	C-NS	P-NS	DM-NS	DMP-NS	C-S	P-S	DM-S	DMP-S
Mediana	2,0	1,8	8,0	1,5	75,8	48,5	64,0	52,3
Média	8,4	2,3	8,8	1,9	73,5	51,5	66,6	51,8
Desvio padrão	13,1	1,7	3,8	1,5	8,1	17,9	7,8	21,7
	Média de leveduras/célula							
	C-NS	P-NS	DM-NS	DMP-NS	C-S	P-S	DM-S	DMP-S
Mediana	1,50	2,00	1,30	1,75	1,80	1,30	1,70	1,55
Média	1,48	1,94	1,43	1,79	1,78	1,57	1,76	1,56
Desvio padrão	0,42	0,63	0,32	0,75	0,38	0,52	0,38	0,43
	Índice fagocitário							
	C-NS	P-NS	DM-NS	DMP-NS	C-S	P-S	DM-S	DMP-S
Mediana	3,5	4,0	10,0	2,3	123,0	61,3	115,5	81,0
Média	13,7	4,4	12,0	3,5	132,9	82,4	110,4	85,5
Desvio padrão	20,5	3,0	3,5	2,8	27,5	59,2	42,5	49,3
20 leveduras/célula	% monócitos envolvidos na fagocitose							
	C-NS	P-NS	DM-NS	DMP-NS	C-S	P-S	DM-S	DMP-S
Mediana	6,0	2,8	11,3	1,8	87,5	52,5	82,5	56,8
Média	11,4	9,4	11,8	4,2	84,5	53,6	79,6	52,4
Desvio padrão	12,3	19,1	11,0	6,2	10,4	15,4	17,4	23,9
	Média de leveduras/célula							
	C-NS	P-NS	DM-NS	DMP-NS	C-S	P-S	DM-S	DMP-S
Mediana	2,0	1,7	1,7	1,8	2,3	1,7	1,9	1,6
Média	2,0	1,7	1,7	1,7	2,4	1,7	2,1	1,8
Desvio padrão	0,5	0,5	0,7	0,5	0,7	0,4	0,7	0,6
	Índice fagocitário							
	C-NS	P-NS	DM-NS	DMP-NS	C-S	P-S	DM-S	DMP-S
Mediana	17,5	4,8	24,8	3,8	205,5	81,3	115,0	75,8
Média	22,0	15,8	22,0	6,9	200,0	87,1	142,2	75,6
Desvio padrão	22,1	30,7	19,2	9,7	79,6	43,6	99,7	37,1

Quadro 4C - Percentual de redução do NBT

	C/basal	P/basal	DM/basal	DMP/basal	C/est	P/est	DM/est	DMP/est
Mediana	53.0	37.0	31.7	36.5	31.7	38.0	35.2	33.0
Média	47.1	37.9	34.1	36.2	32.7	37.6	37.3	33.6
Desvio padrão	23.8	27.3	8.1	12.3	14.1	22.6	9.9	18.8