UNIVERSIDADE DE BRASÍLIA – UnB
FACULDADE DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

Valéria Martins de Araújo Carneiro

ANÁLISE SISTÊMICA DA FUNÇÃO FAGOCITÁRIA E RADICAIS DE OXIGÊNIO, ANTES E APÓS TERAPIA PERIODONTAL, EM PACIENTES COM PERIODONTITE

Orientadora: Profª. Dra. Ana Cristina Barreto Bezerra

Brasília
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Tese apresentada como requisito parcial para a obtenção do Título de Doutor em Cências da Saúde pelo Programa de Pós-Graduação em Ciências da Saúde da Universidade de Brasíia.

Orientadora:
Profª. Dra. Ana Cristina Barreto Bezerra

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DEDICATÓRIA

A Deus, Pai e Criador, por ter dado tanto a mim.

A meu marido, Luís Arnaldo, e, minha filha, Luiza, pelo apoio e compreensão pelas horas preciosas em que fiquei distante para conclusão deste objetivo.

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“Pois quem é que te faz sobressair? E que tens tu que não tenhas recebido? E, se o recebeste, por que te vanglorias, como se o não tiveras recebido?”

1 Coríntios 4:7

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LISTA DE ABREVIATURAS E SIGLAS

A.a.	*Aggregatibacter actinomycetemcomitans*
BOP	Bleeding on Probing
BMI	Body Mass Index
CAL	Clinical Attachment Level
CD11	Cluster of Differentiation 11
CD11a	Cluster of Differentiation 11a
CD11b	Cluster of Differentiation 11b
CD18	Cluster of Differentiation 18
CD38	Cluster of Differentiation 38
CRP	C Reactive Protein
DBP	Diastolic Blood Pressure
Fc	Fragment crystallizable
FCS	Fetal Calf Serum
Fcy RIIIb- NA2	Receptor gamma RIIIb- neutrophil antigen 2
FMLP	N-formyl-L-methionyl-L-leucyl-L-phenylalanine
GP 110	Glicoproteína 110
HDL	High-density lipoprotein cholesterol
FN	Interferon
IL-1	Interleucina 1
IL-1α	Interleucina 1alfa
IL-1β	Interleucina 1 beta
IL-6	Interleucina 6
IL-8	Interleucina 8
IL-10	Interleucina 10
LDL	Low Density Lipoprotein
LPS	Lipopolissacárides
MCP-1	Monocyte chemotactic protein-1

NADPH	Nicotinamida adenina dinucleotídeo fosfatase
NBT	Nitroblue Tetrazolium Test
PBS	Phosphate-Buffered Saline
PCR	Proteína-C-Reativa
PD	Probing Depths
P.g.	*Porphyromonas gingivalis*
PGE_2	Prostanglandina E_2
Ph	Percentage of cells involved in phagocytosis
PhI	Phagocytic Index
PI	Plaque Index
P.i.	*Prevotella intermedia*
PMA	Porbol Miristato Acetato
PMNs	Polimorfonucleares
RvE1	Resolvina E1
SBP	Systolic Blood Pressure
T.f.	*Tannerella forsythia*
TGF-1β	Transforming growth factor 1 beta
TLR4	Toll-like receptor 4
TNF	Fator de Necrose Tumoral
TNF- α	Fator de Necrose Tumoral Alfa
TNF- β	Fator de Necrose Tumoral beta
WHO	World Health Organization

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INTRODUÇÃO

INTRODUÇÃO

As doenças periodontais são doenças complexas, cuja expressão fenotípica envolve interações entre o biofilme bacteriano e a resposta imunoinflamatória do hospedeiro e têm, por isto, um caráter inflamatório de natureza infecciosa[1] . Quando subsequentes alterações na homeostasia dos tecidos periodontais acarretam perda gradual dos tecidos de suporte, incluindo reabsorção óssea, observa-se um quadro de periodontite, diferentemente da gengivite, que se limita aos tecidos periodontais de proteção.

O último modelo conceitual acerca da patogênese da doença periodontal foi proposto por Page & Kornman[2] (1997), no qual a microbiota específica periodontopatogênica é necessária para o início e perpetuação da doença. As formas mais severas de periodontite estão associadas às espécies *Aggregatibacter actinomycetemcomitans* (*A.a.*), *Porphyromonas gingivalis* (*P.g.) e Tannerella forsythia* (*T.f.)*[3,4]. A prevalência destas formas está em torno de 10% e seu curso culmina em perda dentária, quando terapia periodontal adequada não é implementada.

De acordo com o modelo de Page & Korman[2] (1997), o grau e severidade da manifestação clínica dependem da forma como os componentes da susceptibilidade do hospedeiro, especialmente resposta imunoinflamatória, são modulados pelos fatores ambientais e de risco adquiridos, além dos genéticos.

Entre os componentes da resposta imunoinflamatória do hospedeiro à microbiota das periodontites, têm sido descritos eventos inflamatórios locais caracterizados por intenso recrutamento de polimorfonucleares (PMNs) para os tecidos e bolsa periodontal[2]. Cabe ressaltar que o leucócito polimorfonuclear e monócito/macrófago são células da primeira linha de defesa, que apresentam função protetora por meio da sua habilidade em fagocitar e matar microrganismos e constituem o principal sistema de células fagocitárias na defesa do hospedeiro contra agentes infecciosos. O contato fagócito-microrganismo é acompanhado por sinais intracelulares que desencadeiam processos celulares tão diversos como o rearranjo do citoesqueleto, modificações no tráfico de membrana, ativação de

mecanismos de morte microbiana, produção de citocinas pró e anti-inflamatórias e quimiocinas, ativação da apoptose e produção de moléculas necessárias para apresentação eficiente de antígeno ao sistema imune adaptativo[5]. A função protetora destas células é também observada na manutenção da integridade do periodonto. A magnitude do papel dos PMNs na proteção bucal está evidenciada quando há ausência ou falha de sua função adequada, como em algumas síndromes que apresentam desordens quantitativas e qualitativas em neutrófilos. Nestas, além de variados graus de suscetibilidade às infecções em geral, há característica manifestação bucal de doença periodontal agressiva severa, com consequente perda prematura dos dentes em ambas as dentições, decídua e permanente[6].

Por outro lado, não obstante, ao crucial papel dos PMNs contra os constantes desafios bacterianos presentes na cavidade bucal, especificamente no sulco gengival, ao exercerem a função protetora, estas células liberam enzimas lisossomais e radicais de oxigênio que, paradoxalmente, podem causar destruição dos tecidos periodontais. Os radicais de oxigênio são considerados de natureza antibacteriana porém, têm a capacidade de se fixarem aos componentes celulares e teciduais e, consequentemente, modulam várias atividades celulares que causam lesão tecidual[6,7].

Reativos intermediários do oxigênio são moléculas oxidantes potentes utilizadas pelos fagócitos para realizar a morte microbiana. Uma vez estimulado o neutrófilo, há grande aumento do consumo de oxigênio da célula, conhecido como surto respiratório ou oxidativo. O complexo enzimático responsável por este aumento é a nicotinamida adenina dinucleotídeo fosfatase (NADPH) oxidase, encontrado na membrana de neutrófilos e fagócitos mononucleares. Uma grande variedade de partículas solúveis e moléculas pode ativar a NADPH oxidase. Este complexo é responsável pela produção de espécies oxigênio reativo, incluindo ânion superóxido e radicais livres de oxigênio. Os ânions superóxidos formados são potencialmente tóxicos, no entanto, muitos destes são convertidos em peróxido de hidrogênio por uma enzima citoplasmática conhecida como superóxido dismutase. O neutrófilo pode liberar a enzima mieloperoxidase, estocada nos grânulos primários (azurófilos), que converte peróxido de hidrogênio em ácido hipoclorito, sendo o mais potente sob o ponto de vista antimicrobiano. O ácido hipoclorito é considerado o principal radical de oxigênio, responsável pela toxicidade mediada pelo neutrófilo[8].

Várias condições patológicas são consequências de agressões causadas por substâncias oxidantes derivadas de neutrófilos, como artrite reumatóide, infarto do miocárdio, diabetes, derrame e doença inflamatória pulmonar[9].

Estes outros elementos da resposta dos fagócitos frente ao acúmulo microbiano no sulco gengival explicam, em grande parte, o desenvolvimento da periodontite. No entanto, o entendimento atual da imunopatogenia das doenças periodontais implica que diferenças individuais na resposta imunológica frente à presença da placa bacteriana estão relacionadas a fatores genéticos[10]. Em alguns indivíduos, observa-se certo equilíbrio entre a resposta dos fagócitos e o acúmulo bacteriano. Neste caso, a manifestação clínica da infecção periodontal e sua progressão se dão de forma leve ou moderada e não acarretam grande morbidade ou mortalidade dentária. Em outros indivíduos, um acúmulo mínimo do biofilme suscita intensa resposta dos fagócitos com desvastadora destruição dos tecidos periodontais, que muito comumente leva à perda dentária. Estes apresentam as chamadas formas severas de periodontite, hoje, consideradas doenças multifatoriais que se desenvolvem como resultado de interações complexas entre genes específicos do hospedeiro e o meio. A exposição ambiental aos patógenos potenciais, dotados de fatores de virulência específicos é a condição primária para o estabelecimento da doença[11].

A literatura comporta evidência da expressão do gene de monócitos do sangue periférico na periodontite severa. As diferenças na expressão deste gene e sua associação com o estado inflamatório foram avaliadas por Papapanou et al.[12] (2007) em pacientes com periodontite severa, nos quais se detectou, antes da terapia periodontal, onze espécies bacterianas periodontopatogênicas pelo método *checkerboard DNA-DNA hybridization*. Ao investigarem a correlação entre vários mediadores inflamatórios e o estado inflamatório, observaram que em um terço dos pacientes avaliados, o tratamento resultou em mudanças nas funções biológicas dos monócitos relacionadas à imunidade inata, apoptose e sinais de transdução celular. Estas mudanças foram também consistentes com a melhora nos níveis dos mediadores inflamatórios. No entanto, deve-se considerar que o tempo de avaliação dos parâmetros pós-terapia neste estudo foi de um mês apenas e isto pode ter influenciado no número de pacientes que apresentou mudanças nas funções dos monócitos.

As implicações da doença periodontal severa no ambiente bucal e no prognóstico dos dentes, por si só, justificariam a relevância de se buscar o completo entendimento da sua etiopatogenia e, a partir deste, implementar formas eficazes de terapêutica individualizada. No entanto, além de sua importância na saúde bucal, seu significado alcança proporções sistêmicas, cujos mecanismos ainda não estão totalmente esclarecidos.

Em um sítio com periodontite severa não tratada, o epitélio ulcerado das bolsas periodontais tem área de superfície estimada entre 8 a 20 cm^2, conforme descrito por Hujoel et al.[13] (2001). Esta área epitelial subgengival inflamada e ulcerada das bolsas periodontais constitui uma larga porta de entrada para que bactérias periodontopatogênicas, seus produtos, endotoxinas como os lipopolissacárides (LPS) e mediadores inflamatórios estimulados alcancem a circulação sistêmica[14,15]. Bacteriemia e endotoxemia podem ser induzidas por procedimentos odontológicos e atividades cotidianas, como a mastigação, e são capazes de induzir um estado inflamatório, caracterizado pela presença de níveis aumentados de marcadores inflamatórios na corrente circulatória e detectados no soro plasmático[16,17]. Assim, mediadores e citocinas proinflamatórias produzidos localmente no periodonto, como IL-1, IL-6, TNF e PGE_2, são lançados na corrente circulatória, e consequentemente, podem causar efeitos em tecidos diversos e distantes, entre estes, células sanguíneas (leucócitos e eritrócitos), endoteliais e hepatócitos. Da mesma forma, podem provocar infecções ou processos inflamatórios crônicos que, por meio da ativação do sistema imune inato, induzem a resposta de fase aguda, marcada pela produção e presença sérica das proteínas de fase aguda[15]. Estudos têm relatado níveis aumentados de mediadores inflamatórios em indivíduos com periodontite[18,19], de modo que, particularmente aqueles colonizados por bactérias periodontopatogênicas Gram-negativas, como *P.g., T.f.* e *Prevotella intermedia (P.i.)* apresentam níveis significativamente aumentados de PCR, IL-6 e fibrinogênio, em comparação com indivíduos sem periodontite[20]. Em culturas de sangue total de indivíduos saudáveis, *P.g.*, principal periodontopatógeno, induziu uma população mista de leucócitos a produzir níveis elevados de citocinas IL-1, TNF, IL-6, IFN, quimiocinas IL-8 e MCP-1, indicando que, ao ativar células imune circulatórias, a infecção periodontal pode servir como estímulo e/ou perpetuação do estado sistêmico inflamatório crônico[21], como por exemplo àquele associado à patogênese do diabetes tipo 2. Dessa forma, a infecção periodontal

pode constituir um fator de risco para um controle metabólico inadequado nestes pacientes[22], bem como para alterações cardiovasculares[23]. O valor do tratamento periodontal, por sua vez, está não só em promover a redução da inflamação clínica local, como também tem sido associado à subsequente diminuição sérica de IL-6 e PCR[18,19,24]. Seus efeitos sobre o controle do diabetes e o impacto sobre o risco à doença cardiovascular e parto prematuro, por outro lado, ainda não são conclusivos[25,26].

A terapia periodontal mecânica apresenta eficácia, claramente estabelecida, sobre a resolução da inflamação e cura dos tecidos periodontais. Entretanto, não foram ainda determinados os efeitos desta terapia sobre a função fagocitária relativa à fagocitose por monócito. Quanto à fagocitose por neutrófilo, poucos estudos implementaram terapia periodontal objetivando analisar os seus efeitos sobre esta célula[27,28]. Johnstone et al.[28] (2007) sugerem que a permanência de uma resposta hiperativa após terapia pode ser devido ao fenótipo hiperativo do neutrófilo ou à resolução incompleta da infecção. O estudo de Kimura et al.[27] (1992) não detalha os efeitos do tratamento relativos à resolução clínica da infecção e atribuem à função fagocitária alterada uma resposta intrinsicamente hipoativa.

A respeito dos efeitos da terapia periodontal sobre a produção de radical de oxigênio, os estudos de Matthews et al.[29](2007); Fredriksson et al.[30] (2003) encontraram produção elevada mesmo após terapia periodontal. Os estudos mencionados estabelecem um período pré-definido de três meses pós-tratamento para reavaliar a função fagocitária. Porém, este período de tempo nem sempre corresponde ao ideal para cada paciente, pois em alguns destes, a resolução das bolsas pode se dar mais tardiamente. Para se analisar os efeitos da terapia sobre a função celular, a resolução do processo inflamatório deve ser primeiramente alcançada. A determinação do período de reavaliação da função fagocitária deverá ser feita de forma individualizada, de acordo com o processo de resolução da inflamação e concomitante redução ou eliminação das bolsas (processo de cura).

Considerando os aspectos acima relatados sobre a inter-relação doença periodontal e resposta imune inata, este estudo objetivou analisar sistemicamente a fagocitose por neutrófilo e monócito, como também a produção de radical de oxigênio. O segundo objetivo foi avaliar os efeitos da terapia periodontal sobre a fagocitose e produção de radical de oxigênio. Para assegurar uma avaliação adequada dos efeitos da terapia, estabeleceu-se de forma individualizada, o tempo

pós-tratamento no qual foram coletadas novas amostras de sangue para comparação da função fagocitária. Este tempo foi estabelecido de acordo com a resolução clínica da inflamação em cada paciente.

OBJETIVOS

OBJETIVO GERAL

- Avaliar os efeitos da infecção periodontal na fagocitose por neutrófilo, monócito e na produção de radical de oxigênio.

OBJETIVOS ESPECÍFICOS

- Avaliar sistemicamente, antes e após terapia periodontal mecânica, as seguintes funções da imunidade inata em pacientes com periodontite severa (agressiva localizada, agressiva generalizada, crônica generalizada):
    - Capacidade fagocitária de neutrófilos;
    - Capacidade fagocitária de monócitos;
    - Produção de radicais de oxigênio em pacientes com periodontite.

ARTIGO CIENTÍFICO 1

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Decreased phagocytic function in neutrophils and monocytes from peripheral blood in periodontal disease

Valéria Martins Araújo CARNEIRO[a], Ana Cristina Barreto BEZERRA[b], Maria do Carmo Machado GUIMARÃES[a], Maria Imaculada MUNIZ-JUNQUEIRA[c].

a- Periodontics Division, University of Brasilia, DF, Brazil.

b- Pediatric Dentistry Division, University of Brasilia, DF, Brazil.

c- Laboratory of Cellular Immunology, Faculty of Medicine, University of Brasilia, DF, Brazil.

Corresponding address: Valéria Martins Araújo Carneiro, Universidade de Brasília-UnB-Departamento de Odontologia, Campus Universitário Darcy Ribeiro, Brasilia-70910-900, DF-Brasil- Phone:+55 61 3307 2514, Fax number: +55 61 3107 1974 e-mail: valeriamartins@unb.br.

Corresponding address: Ana Cristina Barreto Bezerra, Universidade de Brasília-UnB-Departamento de Odontologia, Campus Universitário Darcy Ribeiro, Brasilia-70910-900, DF-Brasil- Phone:+55 61 3307 2514, Fax number: +55 61 3107 1974 e-mail: acrisbarreto@gmail.com.

Corresponding address: Maria do Carmo Machado Guimarães, Universidade de Brasília-UnB-Departamento de Odontologia, Campus Universitário Darcy Ribeiro, Brasilia-70910-900, DF-Brasil- Phone:+55 61 3307 2514, Fax number: +55 61 3107 1974 e-mail: mmgcarmo@gmail.com.

Corresponding address: Maria Imaculada Muniz-Junqueira, Universidade de Brasília-UnB- Faculdade de Medicina, Patologia, Laboratório de Imunologia Celular, Campus Universitário Darcy Ribeiro, Brasilia-70910-900, DF-Brasil- Phone:+55 61 3307 2273, Fax number: +55 61 3273-3907 e-mail: mimjunqueira@unb.br.

Decreased phagocytic function in neutrophils and monocytes from peripheral blood in periodontal disease

ABSTRACT

Phagocytosis by neutrophils and monocytes constitutes the main defense mechanism against bacterial challenges in periodontitis. Phagocytosis by neutrophils has already been evaluated, whereas phagocytic function of monocytes has hardly been addressed so far. Objectives**:** The aim of this study was to assess phagocytosis by neutrophils and monocytes in periodontitis. Material and Methods: The sample included 30 subjects with severe periodontitis and 27 control subjects without periodontal disease. The phagocytic index (PhI) was calculated as the mean number of adhered/ingested *Saccharomyces cerevisiae* per phagocytozing monocyte or neutrophil multiplied by the percentage of phagocytes involved in phagocytosis. Results**:** A significant reduction in phagocyte functions was observed in individuals with periodontitis. The median of PhI of neutrophils using non-sensitized *S. cerevisiae* was 3 for the control group, and 1.5 for the periodontitis group, *P*=0.01, Mann Whitney test. The median of PhI of monocytes with non-sensitized *S. cerevisiae* was 26.13 for the control group, and 13.23 for the periodontitis group, *P*=0.03, Mann Whitney test. The median of PhI of monocytes assessed with sensitized *S. cerevisiae* was 97.92 for the control group and 60.1 for the periodontitis group, *P*=0.005, *t*-test. Conclusion: The data demonstrated a reduction in the function of phagocytes, suggesting a decrease in immune defenses in periodontitis.

Key Words: Periodontitis, Neutrophils, Monocytes, Phagocytosis.

INTRODUCTION

It has been considered that periodontal infection or periodontitis results from the imbalance between the direct and indirect effects of pathogenic bacteria and the host immune response[7]. Data support the microbial etiology of periodontal disease and the role played by specific pathogenic species[5,25]. The polymorphonuclear

neutrophil constitutes the first line of defense against these pathogens in the subgingival area. In the presence of plaque, an inflammatory infiltrate is frequently in the gingival tissues, showing mononuclear phagocytes, lymphocytes, and polymorphonuclear leukocytes[16,22]. Additionally, inflammatory response changes the microenvironment of the biofilm and selects for specific organisms[28]. The interaction between LPS and these predominant cell types in the inflammatory infiltrate stimulates the production and elevation of prostanoids, mainly prostaglandin E_2 in the gingival fluid of sites that present attachment loss[30]. In addition to the local effects, the bacterial virulence factors and inflammatory mediators arising from this parasite-host interaction may create and sustain a chronic systemic inflammatory process in the bloodstream[17]. In the most aggressive forms of periodontal disease, the role played by the elevated serum levels of TNF- α -alpha and IL-1 has been considered partly responsible for altering the neutrophil function[1]. In addition, new perspectives have indicated that periodontitis occurs as a hyperactive immune/inflammatory response to the specific bacteria of plaque in predisposed individuals, involving the excessive generation of oxygen radicals and the release of proteases[14]. However, despite this new paradigm that PMN is not "hypofunctional" or "deficient", but "hyperfunctional"[14], phagocytosis in this context is not yet elucidated.

Data on phagocytosis by neutrophils from peripheral blood in individuals with periodontitis are controversial. There are reports both of reduction[2,4,12,29] and increase of phagocytosis by neutrophils[13,21]. Studies addressing the phagocytosis by monocytes in these patients are still scarce. Therefore, it still has to be determined whether phagocytosis by monocytes in individuals with periodontitis is different compared to individuals without periodontal disease. For this reason, it is important to investigate the immune function represented by phagocytosis in periodontitis to gain insight into the defense against pathogens involved in disease conditions and the pathophysiological mechanism involved, including those of infectious nature, which are present during the course of the disease. Therefore, this study evaluated the phagocytic function of monocytes and neutrophils in periodontal disease, in comparison with control individuals without periodontal disease.

MATERIAL AND METHODS

Subjects and study groups

This study was approved by the Institutional Review Board of the Health Sciences Faculty – UnB – University of Brasilia (045/2008). The subjects were evaluated and selected for inclusion in the present study at the periodontal clinic of the University Hospital of Brasilia, Brazil. The sample included 30 subjects with periodontal disease and 27 subjects with healthy periodontium, all otherwise healthy and non-smokers. The periodontitis group consisted of 20 women and 10 men (age range 21-45, median 34.5) with at least 18 present teeth. The severe periodontitis was diagnosed according to the following inclusion criteria[23]: radiographic evidence of bone loss extending to $\geq$ 30% of the root length in multiple teeth, age $\geq$ 18 years, presence of $\geq$ 2 teeth/quadrant with a pocket depth of $\geq$ 6mm and concomitant attachment loss of > 3mm. The control group consisted of 18 women and 9 men (age range 21-44, median 34) with clinical probing depths (PD) ≤ 3mm and clinical attachment level (CAL) ≤ 3 mm, ≤ 10% sites with bleeding on probing and no radiographic evidence of bone loss. The following exclusion criteria were considered: previous mechanical periodontal therapy and antimicrobial therapy for systemic or topical oral use in the last 12 months, pregnant or lactating women, diabetes, morbid obesity, autoimmune, infectious, allergic, and gastrointestinal diseases, malnutrition, renal alterations, cancer or any other clinical situation that might alter the function of the immune system, use of medications that could alter the level of inflammatory mediators, and smokers.

Clinical examination

The initial periodontal evaluation of each patient included periapical radiographic documentation by the parallelism technique. The clinical examinations were performed by an experienced examiner and included visible plaque accumulation (PI) without the use of any disclosing agent, bleeding on probing (BOP), probing depth (PD) and clinical attachment level (CAL). The measurements were assessed at four sites around each tooth, namely buccal, lingual and proximal sites (the greatest depth was recorded for each proximal surface) using a manual probe (Michigan O probe with Williams markings), excluding third molars. Analyses of degrees of mobility and furcation involvement were recorded.

Phagocytosis Test

Phagocytosis of *Saccharomyces cerevisiae* was adapted from a technique previously described[20]. Briefly, samples of 40 µL per marked area of heparinized whole peripheral blood obtained by venipuncture from each subject were placed on clean glass slides containing 8 marked areas with 7-mm diameter each, in duplicate preparations, and incubated in a wet chamber for 45 min at 37°C. The slides were then rinsed with 0.15M phosphate-buffered saline (PBS) pH 7.2 at 37°C to remove non-adherent cells. After washing, neutrophils and monocytes remained adhered onto the slide approximately in the same proportion as they were in the whole blood. Adherent cells (12,534 ± 5,050 cells/marked area; 5.63 ± 0.85% monocytes and 93.5 ± 1.08% neutrophils) were incubated with a suspension of $2.5x10^5$ *S. cerevisiae* in 20 µL Hanks-tris (Sigma, St Louis, MO, USA) pH 7.2, with 10% heat-inactivated fetal calf serum (FCS) (Gibco) for 30 min in a wet chamber at 37°C. To evaluate the influence of complement molecules on phagocytosis in periodontitis the *S. cerevisiae* were incubated at 37° for 30 min with 10% fresh serum from the donor in Hanks-Tris solution. Slides were then rinsed with 0.15M PBS at 37°C to eliminate nonphagocytosed *S. cerevisiae* and the final washing was done with 30% FCS in Hanks-tris. The slides were fixed with absolute methanol and stained with 10% Giemsa solution. The number of *S. cerevisiae* phagocytozed by 200 monocytes or by 200 neutrophils in individual preparations was assessed by light microscopy. Microscopic fields distributed throughout the slide were randomly selected and all monocytes or neutrophils in each particular field were examined. The phagocytic index was calculated as the mean number of phagocytozed *S. cerevisiae* per phagocytosing monocytes or neutrophils, multiplied by the percentage of these cells engaged in phagocytosis[19].

Baking yeast (*Saccharomyces cerevisiae*) was prepared according to a technique previously described[20].

Statistical Analysis

Statistical analysis was performed using the Prism® software (Graphpad, USA, 2005). Beforehand the variables in the samples were previously verified for normality, using the Skewness and Kurtosis and Kolmogorov-Smirnov tests. The t-test was used for comparison between two variables with normal distribution, and the

Mann Whitney test was used for those that did not present normal distribution. The differences between variables were considered statistically significant when the bi-caudal probability of their occurrence due to chance (error type I) was lower than 5% ($p<0.05$). As several data showed non-normal distribution, for homogeneity, all data were graphically expressed as median, quartiles and extremes.

RESULTS

Clinical and Demographic Characteristics

The clinical and demographic characteristics of the two groups are summarized in Table 1. Distribution of age and gender was similar between control and periodontitis groups. Subjects with periodontitis had significantly higher body mass index than control ($P= 0.001$). Statistically significant differences were also observed for all periodontal parameters. Subjects with periodontitis showed severe destructive periodontal disease when observed by the percentage of sites with PD ≤ 4mm to ≥ 7mm and CAL ≤ 4mm to ≥ 7mm. The hematological characteristics of the groups are listed in Table 2. No statistically significant difference was observed between groups, except for C reactive protein (CRP). The mean of serum levels of CRP was 0.21±0.25 for the control group and 0.51±0.62 for the periodontitis group, $P=0.01$.

Table 1 – Demographic Characteristics and Full-mouth Clinical Parameters.

Characteristics / Parameters	Control (n=27)	Periodontitis (n=30)	(p) Test
Age (years; mean ± SD)	33.2 ± 6.4	33.5 ± 6.8	0.8441**
Gender (males/females: n)	9/18	10/20	1.0000***
Numbers of teeth (mean ± SD)	28 ± 1.3	27.5 ± 4.8	0.3545*
BMI	20.3 ± 1.28	26.53 ± 5.49	0.0001*
SBP(mmHg)	120.9 ± 4.8	122.4 ± 14.8	0.9188*
DBP (mmHg)	80.5 ± 2.5	80.9 ± 11.6	0.0744*
PI (%; mean ± SD)	4.8 ± 2.1	61.37 ± 33.59	0.0001*
BOP (%; mean ± SD)	2.7 ± 1.2	41.83 ± 30.04	0.0001*
PD (mm; mean ± SD)			
≤3mm	100%	70.2 ± 15.4	
4 mm	0.0%	3.8 ± 3.97	
5-6 mm	0.0%	16.1 ± 9.33	
≥ 7 mm	0.0%	9.8 ± 8.8	
CAL (mm; mean ± SD)			
≤3mm	100%	64.2 ± 18.7	
≤ 4 mm	0.0%	4.9 ± 4.7	
5-6 mm	0.0%	17.9 ± 9.2	
≥ 7 mm	0.0%	13.0 ± 11.4	

** Mann Whitney test*

*** t-test*

**** Chi-Square test, BMI (body mass index), SBP (Systolic Blood Pressure), DBP (Diastolic Blood Pressure)*

PI (plaque index), BOP (bleeding on probing).PD (probing depth), CAL (clinical attachment level).

Table 2 – Biochemical and Hematological Characteristics.

Characteristics / Parameters	Control (n=27)	Periodontitis (n=30)	(p) Test
Triglycerides (mg/dl)	86.1 ± 35.4	98.9 ± 46.2	0.2675*
Total Cholesterol (mg/dl)	172 ± 31.4	173.9 ± 3.3	0.8222**
HDL-cholesterol (mg/dl)	48.2 ± 12	42.5 ± 12.3	0.0747*
LDL-cholesterol (mg/dl)	106 ± 24.4	111.5 ± 27.3	0.4348**
Glucose (mg/dl)	85.3 ± 6.7	90.6 ± 14.1	0.1528*
Eosinophils	146.1 ± 103.3	222.9 ± 175.8	0.0646*
Basophils	14.7 ± 31.8	8.1 ± 21.3	0.627*
Lymphocytes	2239 ± 530.7	2.137 ± 499.5	0.4607**
Monocytes	422.6 ± 140.9	388.5 ± 160.1	0.2369*
Total Leukocytes	6093 ± 1216	6.308 ± 1.473	0.5111*
C-Reactive Protein	0.21 ± 0.25	0.51 ± 0.62	0.0105*

** Mann Whitney test*

*** t-test*

RESULTS OF THE PHAGOCYTOSIS TEST

Phagocytic Index of Neutrophils and Monocytes for non-opsonized S. cerevisiae

The median of the phagocytic index of neutrophils from individuals with periodontitis (1.5) was significantly lower than that of normal controls (3.0), (p=0.01, Mann Whitney test) (Fig. 1A). This occurred because there was a reduction in the percentage of neutrophils involved in phagocytosis in the periodontitis group (0.50%) when compared to the normal control group (2.25%) (p=0.006, Mann Whitney test) (Fig. 1C), whereas no statistically significant difference was observed between groups for the mean number of yeasts adhered to/ingested by neutrophils (p=0.50, Mann Whitney test) (Fig 1B).

The same situation was verified when phagocytosis by monocytes was tested. The median of the phagocytic index of monocytes from individuals with periodontitis (13.23) was significantly lower than that of the normal controls (26.13) (p=0.02, Mann Whitney test) (Fig. 1D), because a statistically significant reduction in the percentage

of monocytes involved in phagocytosis was observed in the periodontitis group (8.50%) when compared to the control group (17.25%) (p= 0.01, Mann Whitney test) (Fig. 1F). Conversely, no difference was observed between groups for the mean number of yeasts adhered to/ingested by monocytes (p=0.71, t test) (Fig 1E).

Phagocytic Index of Neutrophils and Monocytes for opsonized S. cerevisiae

A different situation occurred when phagocytosis was evaluated using sensitized *S. cerevisae*. No statistical difference was observed in the phagocytic index of neutrophils between individuals with periodontitis (117.9) and normal control individuals (157.0) (p=0.14, t-test) (Fig. 2A). Periodontitis had no influence on the mean number of yeasts adhered/ingested by neutrophils (p=0.15, Mann Whitney test) as illustrated in Fig. 2B. There was no statistical difference in the involvement of neutrophils in phagocytosis between the two study groups (p=0.16, Mann Whitney test) (Fig. 2C). The median proportion of neutrophils involved in phagocytosis in the periodontitis group was 65.50%, compared to 71.50% in the control group.

However, a different result was verified when phagocytosis was tested by monocytes, where the median of PhI of monocytes from individuals with periodontitis (60.10) was significantly lower than that from the normal controls (97.92) (p=0.005, t-test) (Fig. 2D). This decrease was caused by a lower percentage of monocytes involved in phagocytosis, namely 36.25% for the periodontitis group and 51.75% for the control group (p= 0.0016, t-test) (Fig. 2F). The medians of the mean number of yeasts adhered to/ingested by monocytes were statistically similar between both groups, as illustrated in Fig. 2E.

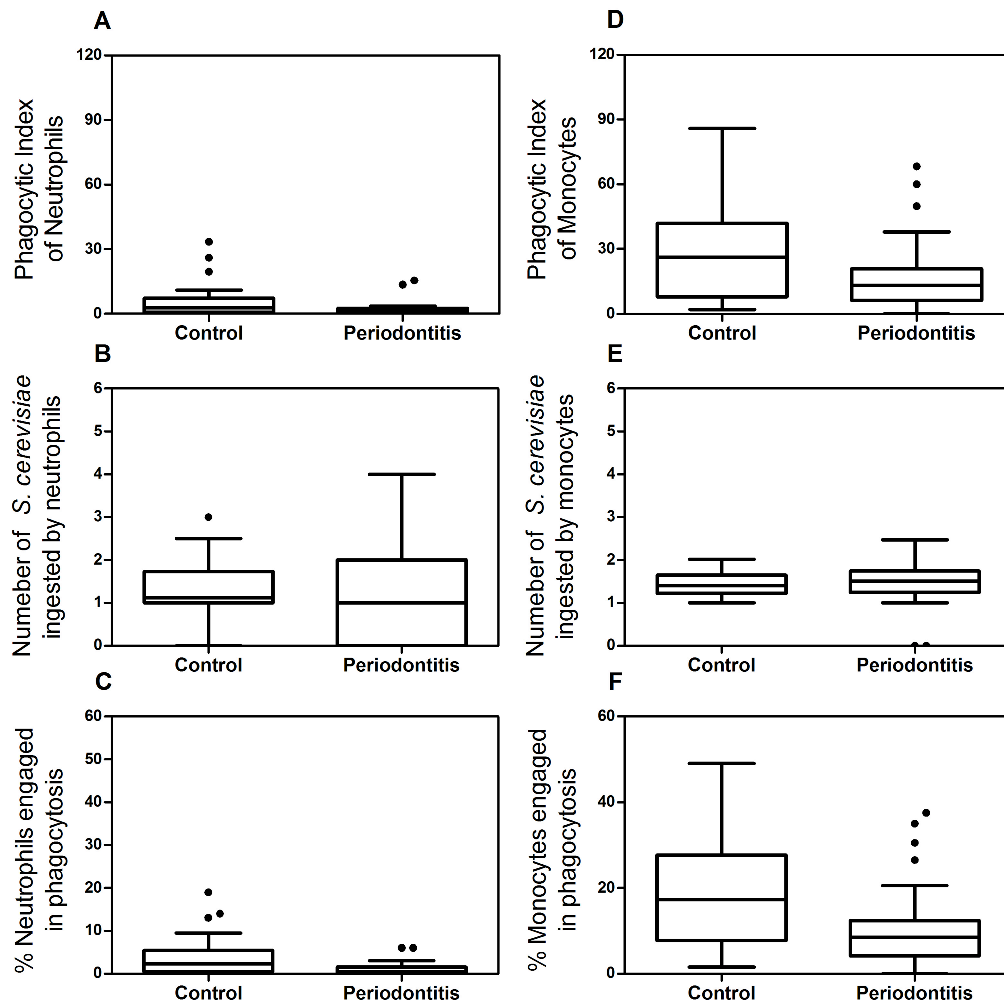

Figure 1 *– In vitro evaluation of the phagocytic capacity of neutrophils (left) or monocytes (right) in individuals with periodontal disease (P) and normal control individuals, using 2.5 x* 10^5 *non sensitized yeast per well. In A: Reduction of the phagocytic index (p = 0.01 Mann Whitney test). In B: Mean number of yeasts adhered to/ingested by neutrophils (p = 0.50, Mann Whitney test). In C: Reduction of the percent of neutrophils involved in phagocytosis (p = 0.006, Mann Whitney test). In D: Reduction of the phagocytic index of monocytes (p = 0.0,2 Mann Whitney test). In E: Mean number of yeasts adhered to/ingested by monocytes (p = 0.71 t-test). In F: Reduction in the percentage of monocytes involved in phagocytosis (p = 0.01, Mann Whitney test). Data were expressed as median, quartile and extreme. Outlier values are marked*

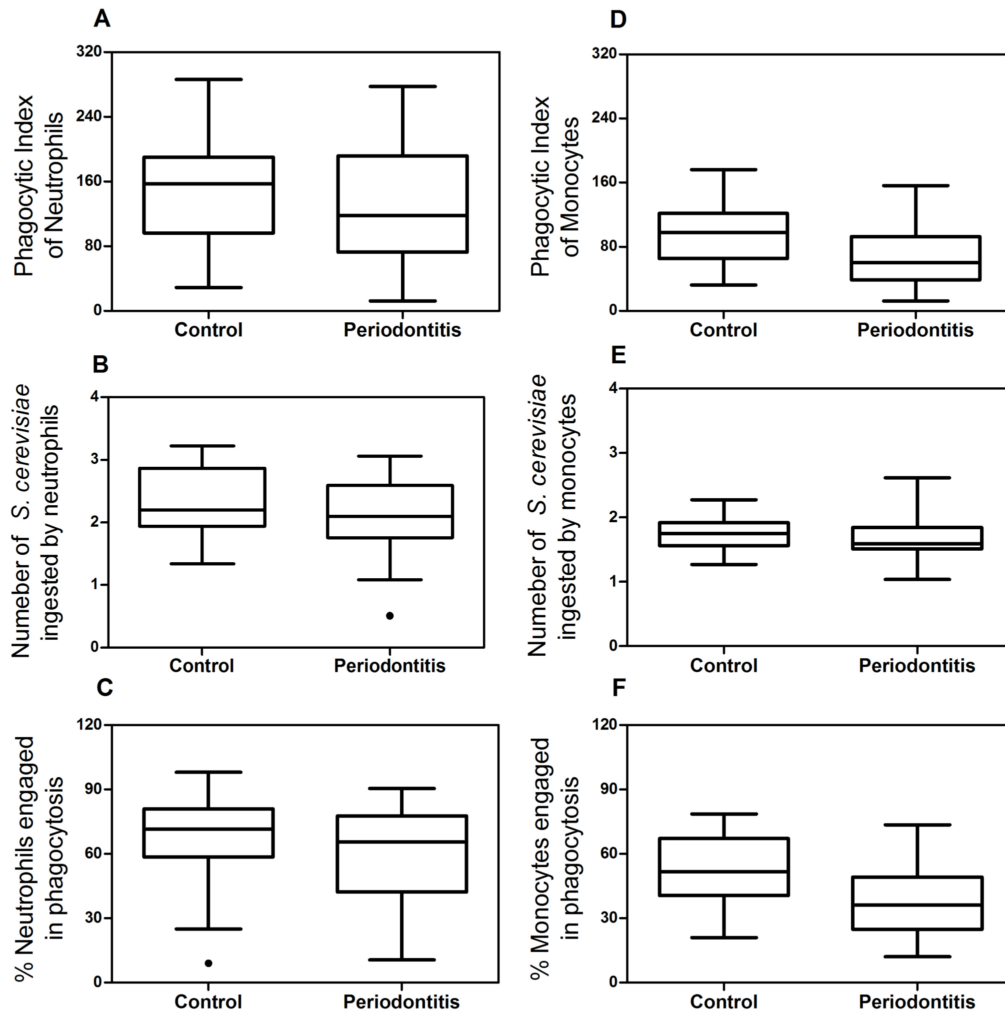

Figure 2 *– In vitro evaluation of the phagocytic capacity of neutrophils (left) or monocytes (right) in individuals with periodontal disease (P) and normal control individuals , using 2.5 x 10^5 sensitized yeast per well. In A: Phagocytic Index (p = 0.14,t- test). In B: Mean number of yeasts adhered to/ingested by neutrophils (p = 0.15 Mann Whitney test). In C: Percentage of neutrophils involved in phagocytosis (p = 0.16, Mann Whitney test). In D: Reduction of the Phagocytic Index of monocytes (p = 0.005, t- test). In E: Mean number of yeasts adhered to/ingested by monocytes (p = 0.19 Mann Whitney test). In F: Percentage of monocytes involved in phagocytosis (p = 0.001 t-test). Data were expressed as median, quartile and extreme. Outlier values are marked.*

DISCUSSION

This is the first description of decreased phagocytic capacity of monocytes in human periodontal diseases. In the present study phagocytosis by monocytes and neutrophils were compared between individuals with healthy periodontium and those with periodontitis. In this work, killed *Saccharomyces cerevisiae* was used because receptors involved in their uptake are involved in phagocytosis by neutrophils and monocytes of pathogenic bacteria present in periodontal disease[27]. When using live bacteria, their virulence factors may influence phagocytosis. Thus, when investigating the phagocytosis, two lines of reasoning can be defined: the direct action of bacteria in phagocytosis and the effects of host-parasite interactions in phagocytosis. As our aim was to evaluate the effects of this interaction in the host, this justifies another stimulus provided to the cell, including yeasts. The use of different stimuli is reported in the literature: N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP)[29], *Staphylococcus aureus*[5], C. albicans[2, 25], *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis*[13, 21].

The present study showed a significant reduction in the phagocytic capacity of monocytes, both using opsonized and non-opsonized *S. cerevisiae* (Fig. 1D and 2D). However, for neutrophils, the disease decreased phagocytosis only when it was assessed using non-opsonized yeasts (Fig.1A). This lower phagocytic capacity of neutrophils in periodontitis has also been reported by other researchers[2,4,12,29]. In this study, lower phagocytosis using non-opsonized *S. cerevisiae* was observed in the group of individuals with periodontitis, both by neutrophils (Fig 1A) and monocytes (Fig. 1D). These data suggest that the phagocytic function of both neutrophils and monocytes was intrinsically affected because, in the absence of serum components, the level of phagocytosis was lower in the periodontitis group. Regarding the monocytes, for both analyses, the phagocytic index was lower in individuals with periodontal disease (Fig. 1D and 2D). A reduced number of monocytes involved in phagocytosis in this group was also evident (Fig. 1F and 2F).

The mechanisms for the deficient response of phagocytes have not been explained yet, however some hypotheses can be suggested. It is known that many periodontal pathogens develop particular strategies for subverting the mechanisms of phagocytosis[27]. Among the mechanisms of immunodepression, the role of LPS has

been considered. Studies have indicated that the LPS of *P. gingivalis* appears to be antagonists of the toll-like receptor-4, thus competing with LPS of other species to couple with that receptor. This is a possible mechanism of deficiency of the innate immune system of the host, since recognition of the bacterial pathogens identified by the toll-like receptor-4 would be blocked[6]. The recruitment of neutrophils and macrophages in mice infected with *Aggregatibacter actinomycetemcomitans* and lacking the toll-like receptor-2 led to the reduction in the influx of these cells in the peritoneal cavity. Infection with *Aggregatibacter actinomycetemcomitans* in this experimental model caused a significant decrease in the cytokine and chemokine levels and reduction in the phagocytic capacity of neutrophils and monocytes, in addition to alveolar bone loss[11].

Another possibility that has been described is the direct toxicity of *Aggregatibacter actinomycetemcomitans* to neutrophils and monocytes by the production of leukotoxin[26]. Strains of *Aggregatibacter actinomycetemcomitans* have various mechanisms that control phagocytosis, such as inhibition of chemotaxis and immunosuppressive and cytotoxic factors that suppress both the unspecific and specific immune responses, as well as preventing fibroblast proliferation. They also present the additional capacity to invade epithelial and endothelial cells[15]. Baehni et al[3]. (1979) observed by electron microscopy that the cytotoxic effects on PMN by strains of *Aggregatibacter actinomycetemcomitans* were independent of phagocytosis. The authors suggested that soluble bacterial products may be released by bacteria. Carvalho et al. (2009) found high frequency of *P. gingivalis, T. forsythia* and *A. actinomycetemcomitans* in individuals with aggressive periodontitis. They observed that in these individuals the frequency and quantity of *P. gingivalis* and *T. forsythia* presented negative correlation with phagocytosis by PMN. The depression of phagocytosis observed in the present study in the periodontal disease group can be justified by the action of these pathogens usually found in more severe forms of periodontal disease. In the analysis of phagocytosis by neutrophils using yeasts sensitized with fresh serum from the individual, although the phagocytic index in individuals from the periodontitis group presented a trend toward reduction, the results did not differ statistically from those of the control group (Fig. 2A). This suggests that there was no considerable change in complement an immunoglobulin receptors on neutrophils, as well as considerable influence of other serological factors in this group.

Different from the present results, studies in individuals with more severe forms of periodontal disease demonstrate that regulatory factors in serum may modulate functions of PMNs. Depression of the chemotactic response in patients with localized aggressive periodontitis may not be an abnormality associated with the cell, but rather a consequence of the elevation of the serum concentration of cytokines produced during the host-parasite interaction. The TNF-α and IL-1 cytokines may cause a reduction in chemotactic receptors[1]. However, increased local production of cytokines in response to pathogens in the periodontium may result in bone loss and tissue damage typically observed in more severe forms of periodontitis[10,30]. Although there are no studies showing the relationship between serum levels of inflammatory mediators and phagocytosis, there may be an inverse correlation between them because depressed phagocytosis is always found in periodontal pockets[9,24].

Although with small difference, the present data demonstrated that the serum level of C-reactive protein (CRP) was increased in individuals with periodontitis (Table 2). In more severe forms of periodontal disease, the association between the LPS of periodontopathogenic bacteria and inflammatory mediators has been well established[8], which leads to the increase of CRP with consequent cardiovascular alterations[18]. Similarly, it is possible that systemic immunosuppression of phagocytic cells, as observed in this study, may reduce the defense against bacteria and fungi. It is also a possibility the fact that the absence of systemic manifestations, observed in the present study, occur due to the redundancy of immune system functions. The long-term consequences of the reduction of phagocytosis in the group with periodontitis should be further evaluated.

CONCLUSIONS

This study showed for the first time that the monocytes of peripheral blood from individuals with periodontitis present decreased phagocytosis of opsonized and non-opsonized *S. cerevisiae* in comparison with control individuals. Concerning the neutrophils, decreased phagocytosis was observed only for non-opsonized yeasts. Although the individuals did not show clinical parameters of immunodeficiency, a laboratorial decreased function of phagocytes was characterized in this work, which

may be a consequence, not the cause, of periodontitis. More studies should be conducted to investigate the immune inflammatory events implicated in phagocytosis by neutrophils and monocytes in periodontitis.

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ARTIGO CIENTÍFICO 2

Artigo encaminhado para avaliação ao periódico *Oral Health and Preventive* *Dentistry*

Effects of Periodontal Therapy on Phagocytic Activity of Peripheral Blood Neutrophils- Evidence of an Extrinsic Cellular Defect

Valéria Martins Araújo CARNEIRO[a], Ana Cristina Barreto BEZERRA[b], Maria do Carmo Machado GUIMARÃES[a], Maria Imaculada MUNIZ-JUNQUEIRA[c].

a- Periodontics Division, University of Brasilia, DF, Brazil.

b- Pediatric Dentistry Division, University of Brasilia, DF, Brazil.

c- Laboratory of Cellular Immunology, Faculty of Medicine, University of Brasilia, DF, Brazil.

Corresponding address: Valéria Martins Araújo Carneiro, Universidade de Brasília-UnB-Departamento de Odontologia, Campus Universitário Darcy Ribeiro, Brasilia-70910-900, DF-Brasil- Phone:+55 61 3307 2514, Fax number: +55 61 3107 1974 e-mail: valeriamartins@unb.br.

Corresponding address: Ana Cristina Barreto Bezerra, Universidade de Brasília-UnB-Departamento de Odontologia, Campus Universitário Darcy Ribeiro, Brasilia-70910-900, DF-Brasil- Phone:+55 61 3307 2514, Fax number: +55 61 3107 1974 e-mail: acrisbarreto@gmail.com.

Corresponding address: Maria do Carmo Machado Guimarães, Universidade de Brasília-UnB-Departamento de Odontologia, Campus Universitário Darcy Ribeiro, Brasilia-70910-900, DF-Brasil- Phone:+55 61 3307 2514, Fax number: +55 61 3107 1974 e-mail: mmgcarmo@gmail.com.

Corresponding address: Maria Imaculada Muniz-Junqueira, Universidade de Brasília-UnB- Faculdade de Medicina, Patologia, Laboratório de Imunologia Celular, Campus Universitário Darcy Ribeiro, Brasilia-70910-900, DF-Brasil- Phone:+55 61 3307 2273, Fax number: +55 61 3273-3907 e-mail: mimjunqueira@unb.br.

Effects of Periodontal Therapy on Phagocytic Activity of Peripheral Blood Neutrophils- Evidence of an Extrinsic Cellular Defect

Purpose: The aim of this study was to compare phagocytic activity of peripheral blood neutrophils from subjects with and without periodontal disease and evaluate the effects of periodontal therapy.

Materials and Methods: We compared phagocytic activity of neutrophils in peripheral blood collected from 27 control subjects with healthy periodontium and 28 periodontitis subjects, prior to and post-treatment. We quantified phagocytosis of killed *Saccharomyces cerevisiae*, pre-sensitized or non-sensitized with patient blood and calculated a phagocytic index as the mean number of yeast cells phagocytosed by the percentage of neutrophils involved in phagocytosis.

Results: Periodontal therapy significantly improved in clinical periodontal status. Prior to periodontal treatment, patients with periodontitis exhibited significantly lower neutrophil phagocytic activity than control patients with healthy periodontium. Periodontal treatment resulted in significantly increased phagocytosis of both pre-sensitized (from 65.0 pre- to 79.0 post-treatment, P = 0.02) and non-sensitized *S. cerevisiae* (from 1.5 pre- to 3.5 post-treatment, P = 0.001), to levels observed in control patients.

Conclusions: Subjects who underwent non-surgical periodontal treatment and strict supportive therapy for 6 months showed improved phagocytic activity in peripheral blood neutrophils.

Key Words: Periodontitis, Neutrophils. Phagocytosis, Periodontal Therapy.

INTRODUCTION

Periodontitis is a complex disease, in which clinical expression involves intricate interactions of the biofilm with the host immunoinflammatory response and

subsequent alterations in bone and connective tissue homeostasis (Offenbacher et al, 2007). Periodontal diseases are initiated by dental plaque and result in progressive destruction of the periodontium in susceptible subjects. While associations between periodontal disease and inflammatory or humoral responses have been reported (Kinane et al, 2001), establishing whether periodontal disease is a cause or effect of immune dysfunction remains challenging.

Neutrophils constitute over 65% of leukocytes in peripheral blood and are phagocytic cells involved in the first line of host defense. Neutrophil function may be altered in periodontal disease, but current data are insufficient (Nussbaum and Shapira, 2011).

Mechanical periodontal therapy is of fundamental importance in the resolution of inflammation and healing of periodontal tissues. One approach to study immune function in periodontal disease is to evaluate the impact of periodontal treatment on specific functions. However, studies that have evaluated the effect of periodontal therapy on neutrophil phagocytic function have not carefully controlled for the degree of resolution of the infection and showed mixed results (Kimura et al, 1992; Johnstone et al, 2007). Variation between patients in the amount of time required for complete resolution of periodontal disease complicates standardization of post-treatment evaluation.

Given the role of neutrophils in the host innate immune system, as well as in the inflammatory process, this study aimed to evaluate the effects of periodontal therapy on phagocytic activity by peripheral blood neutrophils. We recruited patients in a periodontal maintenance program and used pre-determined outcome criteria to minimize differences between patients in level of resolution of inflammation at the end of treatment.

MATERIAL AND METHODS

Subjects and study groups

This study protocol was approved by the Research Ethics Committee of the Health Science Faculty – University of Brasilia (045/2008). The subjects were evaluated and selected for inclusion in the present study at the Periodontal Clinic of the University Hospital of Brasilia, from August 2008 until August 2010. The sample included 28 subjects with periodontal disease and 27 subjects with healthy

periodontium; all were otherwise healthy; none were smokers. The periodontitis group consisted of 19 women and 9 men (ages ranging from 20-45, median 34.5), with the presence at least 18 teeth. Classification of periodontal disease was according to Armitage (1999) and Armitage and Cullinan (2010). The control group consisted of 18 women and 9 men (ages ranging from 21-44, median 34), with clinical probing depths (PD) ≤ 3mm and clinical attachment level (CAL) ≤ 3 mm, ≤ 10% sites with bleeding on probing and no radiographic evidence of bone loss. Exclusion criteria were as follows: subjects who had prior periodontal therapy, had received antimicrobial therapy for systemic conditions or had used topical oral antibiotics in the last 12 months, women who were pregnant or lactating women, had diabetes, an autoimmune disease, acute infections, severe allergies, renal and gastrointestinal diseases, cancer, were morbidly obesity [body mass index (BMI) >40 kg/m^2] or malnurished (BMI < 18,5 kg/m^2) (WHO, 2000), or who presented with any condition that investigators judged might alter the function of the immune system, including use of immunosuppressive medications such as corticoid therapy that could alter the level of inflammatory mediators.

Clinical examination

The clinical examinations, performed by a single experienced examiner, included visible plaque accumulation (PI), without the use of any disclosing agent, bleeding on probing (BOP), probing depth (PD) and clinical attachment level (CAL). The measurements were assessed at four sites around each tooth, buccal, lingual and proximal sites (for each proximal surface the greatest depth was recorded) using a manual probe (Michigan O probe with Williams markings), excluding third molars. The calibration and measurements of PD and CAL were repeated within 24 hours and demonstrated agreement over 80%. The BOP was calculated by the Kappa coefficients and the intraexaminer agreement was >0.85. The clinical examinations were assessed at baseline and at the end of the period of 6 months of the maintenance therapy.

Treatment Protocol

The subjects of the periodontitis group were treated in three stages: 1- mechanical periodontal therapy, 2- reinstrumentation of sites, 3- supportive periodontal therapy. Stage 1 was performed in ≤ 14 days. One month later, stage 2,

was performed in all patients for treatment of persistent deep pockets, bleeding on probing and calculus. In this stage, meticulous scaling and root planing was done until one of the following predetermined periodontal conditions were met: probing depth up to 4mm at three or fewer sites, probing depth up to 5 mm at two or fewer two sites, plaque index ≤15% and bleeding on probing ≤10%. At stage 3, the subjects were scheduled biweekly or monthly depending on need for plaque control. Supportive periodontal therapy was performed for 6 months.

Phagocytosis test

The assay of neutrophil phagocytic activity was adapted from a previously described technique (Muniz-Junqueira et al, 2003). Nonpathogenic *Saccharomyces cerevisiae* (Baker's yeast) cells are used because receptors involved in their uptake are involved in phagocytosis by neutrophils of pathogenic bacteria present in periodontal disease (Underhill and Ozinsky, 2002). Killed *S. cerevisiae* cells were prepared as previously described (Muniz-Junqueira et al, 2003).

Samples of heparinized whole peripheral blood were obtained by venopuncture from all subjects. On clean glass slides, 8 marked fields 7-mm in diameter were created with oil ink mixed with epoxy resin and 40 µL of whole blood was placed in each field. Slides were then incubated in a wet chamber for 45 min at 37°C and rinsed with 0.15M PBS pH 7.2 at 37°C to remove non-adherent cells. Neutrophils were the main cell that remained adhered onto the slide after washing (12.534 ± 5.050 cells/marked area; 93.5 ± 1.08% neutrophils) (Muniz-Junqueira et al, 2003). Adherent cells were incubated in duplicate preparations with 2.5×10^5 *S. cerevisiae* cells in 20 µl of Hanks-Tris solution (pH 7.2; Sigma, St. Louis, MO.) in a wet chamber at 37°C for 30 min. Prior to addition to prepared slides with adherent cells, killed *S. cerevisiae* cells were either pre-sensitized by incubating cells for 30 min at 37°C in Hanks-Tris solution containing 10% fresh serum from the each subject, or pre-incubated for 30 min at 37°C with Hanks-Tris solution containing 10% heat-inactivated fetal calf serum ("non-sensitized"). Pre-sensitized yeast cells are phagocytosed mainly through complement receptors while non-sensitized yeast cells are phagocytosed through pathogen-associated molecular pattern receptors (Muniz-Junqueira, et al, 2003). Slides were then rinsed with 0.15M PBS at 37°C to eliminate nonphagocytosed *S. cerevisiae* and the final washing was done with 30% fetal calf serum in Hanks-Tris solution. The slides were fixed with absolute methanol and

stained with 10% Giemsa solution. The number of *S. cerevisiae* phagocytosed by 200 neutrophils in each individual field was assessed by optical microscopy.

Microscopic fields for enumeration were randomly selected in all eight marked fields in each preparation and all neutrophils in the selected fields were counted by observers blinded to the study groups. The number of *S. cerevisiae* phagocytosed by 200 neutrophils in each individual preparation was assessed. The phagocytic index for each individual was calculated as the average number of phagocytosed *S. cerevisiae* per phagocytosing neutrophil, multiplied by the percentage of neutrophils engaged in phagocytosis (Muniz-Junqueira et al, 1992). For patients with periodontal disease, phagocytosis assays were conducted before therapy and at the end of maintenance program.

STATISTICAL ANALYSIS

Statistical analysis was performed using the Sigma Stat 32.Ink® software and Prism® software (Graphpad, USA, 2005). Sample size was determined for a desired power of 90% and an alpha level of significance of 0.05, which showed a minimum number of 25 individuals by group, by employing the Sigma Stat software. For comparisons between periodontitis and control subjects, we used Student's t-test for normally-distributed variables and the Mann-Whitney test for non-normally distributed values. For comparison of pre- and post-treatment observations for periodontitis subjects, we used paired t-tests or Wilcoxon tests for normally and non-normally distributed values, respectively. Normality was assessed using the Skewness and Kurtosis and Kolmogorov-Smirnov tests. Statistical significance was considered as a two-tailed $P < 0.05$.

RESULTS

Retention

Of the 28 subjects in periodontitis group, 14 were diagnosed with Generalized Chronic Periodontitis, 8 with Generalized Aggressive Periodontitis and 6 with Localized Aggressive Periodontitis. Five periodontitis subjects did not complete treatment and follow-up: 10 with Generalized Chronic Periodontitis, 7 with

Generalized Aggressive Periodontitis and 6 with Localized Aggressive Periodontitis. Twenty-three subjects concluded the 3 stages of the periodontal protocol. Among the 23 subjects who completed the periodontal treatment, 10 (43%) completed treatment in 9 months, 10 (43%) in 10 months and 3 (14%) in 12 months. None of the periodontitis patients reported experiencing adverse effects of treatment such as fever or indisposition.

Clinical and Demographical Characteristics

Information about the subjects enrolled in the study and clinical outcomes of periodontal treatment are summarized in Table 1. Distribution of age and gender was similar among subjects with or without periodontal disease. Subjects with periodontitis had significantly higher body mass index than subjects without periodontal disease (P = 0.001). Periodontal treatment resulted in significant improvement in patients' plaque indexes, prevalence of bleeding on probing and probing depth at 6 months post-supportive therapy (Table 1).

No differences were observed in blood triglycerides or cholesterol, or in peripheral blood leukocyte count, comparing subjects with periodontal disease prior to treatment and subjects without periodontal disease (Table 2). Among periodontitis subjects, levels of HDL cholesterol increased slightly following treatment (P = 0.003, Wilcoxon test).

Table 1 – Demographic characteristics and full-mouth clinical parameters at baseline and post-supportive therapy						
Characteristics / Parameters	**Control (n=27)**	**Pre-treatment (n=28)**	**Post-treatment (n=23)**	**Test (p) Pre x Post**	**Test (p) Control x Pre**	**Test (p) Control x Post**
		% or median (minimum – maximum)				
Age – years	34.0 (21.0-44.0)	34.5 (20.0-45.0)	35.5 (21.0-45.0)	0.47[4]	0.84[4]	0.37[4]
Gender (males/females: n)	9/18	9/19		-	1.0000[5]	-
Numbers of teeth	28 (26.0-32.0)	29 (18.0-32.0)	25 (12.0-32.0)	0.0009[1]	0.74[3]	0.03[3]
Body Mass Index (kg/m^2)	22.0 (18.0-27.0)	26.0 19.0-39.0)	25.5 (18.0-37.5)	0.40[1]	0.001[3]	0.0086[3]
Systolic Blood Pressure (mmHg)	120 (110.0-132.0)	120 (98.0 -165.0)	116 (97.0-140.0)	0.07[1]	0.92[3]	0.11[3]
Diastolic Blood Pressure (mmHg)	80 (76.0-86.0)	79 (65.0-115.0)	76 (62.0-102.0)	0.48[1]	0.02[3]	0.002[3]
Plaque Index, %	4.0 (2.0-10.0)	63 (10.0-100.0)	2.0 (0.0-22.0)	0.0001[1]	0.001[3]	0.07[3]
Bleeding on Probing, %	2.0 (1.0-7.0)	37.5 (2.0-100.0)	0.0 (0.0-10.0)	0.0001[1]	0.0001[3]	0.0001[3]
Probing Depth, mm						
≤3mm	100.0	77.5 (29.0-113.0)	99.9 (94.4-100.0)	0.0002[2]	-	-
4 mm	0.0	4.0 (0.0-12.0)	0.0 (0.0-2.8)	0.0002[1]	-	-
5-6 mm	0.0	16.5 (2.0-40.0)	0.0 (0.0-4.0)	0.0001[1]	-	-
≥ 7 mm	0.0	9.0 (1.0-34.0)	0.0 (0.0-2.6)	0.0001[1]	-	-
Clinical Attachment Level, mm						
≤3mm	100.0	73.0 (10.0-111.0)	NA	NA	-	-
4 mm	0.0	4.0 (0.0-15.0)	NA	NA	-	-
5-6 mm	0.0	17.0 (4.0-36.0)	NA	NA	-	-
≥ 7 mm	0.0	11.0 (1.0-42.0)	NA	NA	-	-

[1] Wilcoxon test, [2] Paired t test, [3] Mann Whitney test, [4] t test, [5] Chi square test , NA – not applicable.

Table 2 – Hematological characteristics at baseline and post-supportive therapy.

Characteristics / Parameters	Control (n=27)	Pre-treatment (n=28)	Post-treatment (n=23)	Test (p) Pre x Post	Test (p) Control x Pre	Test (p) Control x Post
		Median (minimum – maximum)				
Triglycerides (mg/dl)	77. (47.0-190.0)	96.5 (31.0-240.0)	102.5 (30.0-183.0)	0.89[2]	0.29[4]	0.12[4]
Total Cholesterol (mg/dl)	168 (124.0-242.0)	169 (112.0-231.0)	186 (128.0-280.0)	0.08[2]	0.82[3]	0.32[3]
HDL-cholesterol (mg/dl)	44 (30.0-72.0)	39 (24.0-74.0)	42.5 (29.0-112.0)	0.003[1]	0.11[3]	0.66[4]
LDL-cholesterol (mg/dl)	106 (73.0-159.0)	111 (70.0-164.0)	108 (76.0-204.0)	0.80[1]	0.53[4]	0.59[4]
Glucose (mg/dl)	86 (69.0-101.0)	88.5 (72.0-141.0)	88.5 (69.0-108.0)	0.93[2]	0.12[3]	0.19[3]
Eosinophils	136 (0.0-388.0)	192 (0.0-763.0)	148 (42.0-386.0)	0.12[1]	0.05[3]	0.23[4]
Basophils	0.0 (0.0-109.0)	0.0 (0.0-69.0)	0.0 (0.0-61.0)	0.21[1]	0.50[3]	0.65[3]
Lymphocytes	2094 (1287-3263)	2176 (118 -3330)	2183 (1196-3434)	0.37[2]	0.45[4]	0.37[4]
Monocytes	438 (200-756)	359 (181-923)	383 (0.0-557)	0.53[2]	0.32[4]	0.13[4]
Total Leukocytes	6110 (4060-9950)	6285 (3360-9700)	6140 (2720-9820)	0.25[2]	0.70[4]	0.70[4]

[1] Wilcoxon test, [2] Paired t test, [3] Mann Whitney test, [4] t test.

Results of Neutrophil Phagocytosis Assays

Prior to periodontal treatment, peripheral blood neutrophils from subjects with periodontitis exhibited significantly lower phagocytic indices and lower median number of ingested or adhered *S. cerevisiae* cells per neutrophil than neutrophils from control subjects with healthy periodontium (Table 3 and 4). This difference was observed with both pre-sensitized and non-sensitized yeast cells. Following completion of periodontal therapy, neutrophil phagocytic indices among periodontitis subjects were no longer significantly lower than those of control subjects. Periodontal treatment resulted in significantly increased phagocytosis of both pre-sensitized and non-sensitized *S. cerevisiae*.

In assays using non-sensitized *S. cerevisiae*, improvements in the median phagocytic index following periodontal treatment (from 1.5 pre- to 3.5 post-treatment,

P = 0.001, Paired t test) resulted from higher percent of neutrophils engaged in phagocytosis (from 0.5% pre- to 2.25% post-treatment, *P* = 0.0007, Wilcoxon test), suggesting that periodontal therapy restored the phagocytic function. The average number of non-sensitized yeasts cells ingested by or adhering to neutrophils did not increase significantly with periodontal treatment.

In assays using pre-sensitized S. cerevisiae, periodontal treatment resulted in increased percentages of neutrophils engaged in phagocytosis (from 65.0 pre- to 79.0 post-treatment, *P* = 0.02, Paired t test), as well as higher mean number of yeast cells ingested by or adhering to neutrophils (from 2.10 pre- to 2.27 post-treatment, *P* = 0.04, Paired t test).

Table 3 Phagocytosis of killed *S. cerevisiae* for non-sensitized neutrophils by peripheral blood from patients at baseline and post-supportive therapy compared to control subjects.

	Control (n=27)	Pre-treatment (n=28)	Post-treatment (n=23)	Control x Pre	Pre x Post	Control x Post
	Median (minimum – maximum)					
Phagocytic index	2.25 (0.0-19.5)	1.5 (0.0-13.5)	3.5 (0.0-14.5)	P = 0.03[2]	P = 0.001[3]	P = 0.34[2]
Average number of ingested/ attached cells per neutrophil	1.04 (0.0-2.5)	1.00 (0.0-4.0)	1.20 (0.0-2.41)	P = 0.99[2]	P = 0.73[1]	P = 0.43[2]
% of cells involved in Phagocytosis	1.75 (0.0-14)	0.50 (0.0-6.0)	2.25 (0.0-9.50)	P = 0.007[2]	P = 0.0007[1]	P = 0.35[2]

[1] Wilcoxon test, [2] Mann Whitney test, [3] Paired t test.

Table 4 – Phagocytosis of killed *S. cerevisiae* for sensitized neutrophils by peripheral blood from patients at baseline and post-supportive therapy compared to control subjects.

	Control (n=27)	Pre-treatment (n=28)	Post-treatment (n=23)	Control x Pre	Pre x Post	Control x Post
	Median (minimum – maximum)					
Phagocytic index	157.00 (28.8-338.0)	113.00 (12.5-277.5)	157.00 (93.0-299.0)	P = 0.05[1]	P = 0.01[2]	P = 0.52[1]
Average number of ingested/ attached cells per neutrophil	2.28 (1.34-5.58)	2.10 (0.5-3.1)	2.27 (1.5-3.3)	P = 0.11[3]	P = 0.04[2]	P = 0.88[3]
% of cells involved in Phagocytosis	72.00 (25-98)	65. 0 (10.5-90-5)	79.00 (46.5-94.5)	P = 0.03[1]	P = 0.02[2]	P = 0.48[1]

[1] t test, [2]Paired t test, [3]Mann Whitney test.

DISCUSSION

The current study contributes to the literature by comparing a basic immune function (phagocytic activity of neutrophils) in individuals with and without periodontal disease, as well as the effect of periodontal treatment on immune function. A strength of this study was the use of defined periodontal clinical criteria for the inclusion of subjects in supportive therapy and to determine when treatment was complete. The improvement in phagocytic activity of peripheral blood neutrophils observed in this study is consistent with observed associations between periodontitis and overall health indicators (Nicu et al, 2009; Bizzarro et al, 2010). Intrinsic defects in neutrophils could result in compromising the immune condition, as found in some syndromes (Hart et al, 1994). However, subjects with periodontitis in this study were otherwise healthy and did not have recurrent infections. Ours result suggest that the decreased function of phagocytes observed is a consequence and not the cause of periodontitis.

Both environmental and genetic factors have been implicated in the etiology of periodontal disease. Genotypic differences (Kobayashi et al 2000; Quappe et al, 2004; Nicu et al, 2007) and/or bacterial factors, either by direct action (Eick et al 2000; Johansson et al, 2000; Burns et al, 2006) or indirect activation associated with changes of inflammatory mediators, influence on hyperactive/ primed (Wright et al, 2008; Dias et al, 2011) or reduced function of neutrophils (Agarwal et al, 1994; Hidalgo et al, 1997). Studies have found that in subjects with more severe forms of periodontal disease, the increase in inflammatory cytokines in serum is responsible for neutrophil priming and hyperactivity (Dias et al, 2011; Wright et al, 2008) or decreased neutrophil function (Agarwal et al, 1994; Hidalgo et al, 1997). Agarwal et al. (1994) suggested that the depression found in the chemotactic response in PMN might not be an abnormality associated with the cell but rather the result of high systemic level of quantitative cytokine produced during the host-pathogen interaction. The increased interleukin-1 β concentration in gingival crevicular fluid as a characteristic of periodontitis was confirmed in the study by Figueredo et al (1999), and decreased after periodontal therapy (Hou et al, 1995; Toker et al 2008). Various cells secrete cytokines in response to injury or stimulation. These proteins are

biologically active in even femtomolar concentrations and form a complex network that controls the inflammatory and immune responses (Whicher and Evans, 1990).

Some studies were not able to establish the effect of therapy on levels of systemic inflammatory mediators (Ide et al, 2003; Radvar et al, 2008; Behle et al 2009), while others found reduction of these molecules after periodontal therapy (Nakajima et al, 2010; Duarte et al, 2010). These contradictory results are most likely associated with different periods of time used to evaluate the inflammatory mediators after therapy, as well as the different responses to the therapy. Fokkema et al (2003) observed changes in monocytes and decreased serum levels of IL-8 and macrophage chemoattractant protein-1 following therapy involving full-mouth tooth extraction in a case with generalized terminal adult periodontitis. Similarly, Papapanou et al (2007) observed that after periodontal therapy, changes occurred in the biological functions of monocytes, related to innate immunity, apoptosis and signs of cellular transduction in 1/3 of the subjects with severe periodontitis. Periodontal infection may cause alterations in serum levels of chemokines (Agarwal et al, 1994; Dias et al, 2011) Our findings are consistent with alterations in neutrophil functions in periodontitis, since periodontal therapy increased involvement of neutrophils in phagocytosis, suggesting enhanced mobility of these cells.

According to Loesche et al (1988), PMN collected from diseased sites may be exhausted by the large numbers of bacteria present in these sites or may be specifically inhibited by these bacteria. The study by Carvalho et al (2009) found a high frequency of *P. gingivalis, T. forsythia* and *A. actinomycetemcomitans* in subjects with periodontitis. They observed that in these subjects the frequency of *P. gingivalis* and *T. forsythia* showed a negative correlation with phagocytosis in PMNs from peripheral blood. Barnett and Baker, 1983, suggest that *in vitro* assays of crevicular neutrophil function may exaggerate the extent of impairment of phagocytosis because of cellular activity that took place prior to the time of sampling. Interestingly, in the periodontal pocket, most studies have found reduced phagocytic activity locally (Newman et al, 1982; Barnett et al, 1983; Sigusch et al, 1992; Eick et al, 2000; Asif and Kothiwale, 2010). However, in peripheral blood, some studies have shown decreased phagocytic activity (Cogen et al, 1986; Van Dyke et al, 1986; Kimura et al, 1992; Gomez et al, 1994; Asif and Kothiwale, 2010), while others have shown increased activity of peripheral blood neutrophils (Johnstone et al, 2007; Nicu et al 2007; Guentsch et al, 2009; Nibali et al, 2010).

Our results show that subjects that underwent non-surgical periodontal treatment and strict supportive therapy for 6 months had improved phagocytic activity in peripheral blood neutrophils. More research is needed to investigate the immunoinflammatory events implicated in periodontitis and their effects on the immune system. Phagocytosis is considered a key element in the defense against infectious agents. Several factors are involved in the pathogenesis of periodontal disease and genetic defects may be related to increased susceptibility to periodontal disease (Meng et al, 2007). The relationship between changes in neutrophils function due to elevation of inflammatory mediators (Wright et al, 2008; Dias et al, 2011) and oxygen radicals (Gustafsson et al, 2006; Matthews et al, 2007; Nibali et al, 2010) in more severe forms of periodontal disease and systemic phagocytic activity deserves greater attention.

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ARTIGO CIENTÍFICO 3

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Periodontitis and phagocitic cells: from the focal infection to the periodontal medicine

Valéria Martins Araújo CARNEIRO[a], Ana Cristina Barreto BEZERRA[b], Maria do Carmo Machado GUIMARÃES[a], Maria Imaculada MUNIZ-JUNQUEIRA[c].

a- Periodontics Division, University of Brasilia, DF, Brazil.

b- Pediatric Dentistry Division, University of Brasilia, DF, Brazil.

c- Laboratory of Cellular Immunology, Faculty of Medicine, University of Brasilia, DF, Brazil.

Corresponding address: Valéria Martins Araújo Carneiro, Universidade de Brasília-UnB-Departamento de Odontologia, Campus Universitário Darcy Ribeiro, Brasilia-70910-900, DF-Brasil- Phone:+55 61 3307 2514, Fax number: +55 61 3107 1974 e-mail: valeriamartins@unb.br.

Corresponding address: Ana Cristina Barreto Bezerra, Universidade de Brasília-UnB-Departamento de Odontologia, Campus Universitário Darcy Ribeiro, Brasilia-70910-900, DF-Brasil- Phone:+55 61 3307 2514, Fax number: +55 61 3107 1974 e-mail: acrisbarreto@gmail.com.

Corresponding address: Maria do Carmo Machado Guimarães, Universidade de Brasília-UnB-Departamento de Odontologia, Campus Universitário Darcy Ribeiro, Brasilia-70910-900, DF-Brasil- Phone:+55 61 3307 2514, Fax number: +55 61 3107 1974 e-mail: mmgcarmo@gmail.com.

Corresponding address: Maria Imaculada Muniz-Junqueira, Universidade de Brasília-UnB- Faculdade de Medicina, Patologia, Laboratório de Imunologia Celular, Campus Universitário Darcy Ribeiro, Brasilia-70910-900, DF-Brasil- Phone:+55 61 3307 2273, Fax number: +55 61 3273-3907 e-mail: mimjunqueira@unb.br.

Periodontitis and phagocitic cells: from the focal infection to the periodontal medicine

This study evaluates the phagocytosis by monocytes and the production of superoxide before and after periodontal therapy. The sample included 28 individuals with periodontitis and 27 controls without periodontal disease. The oxidative capacity of phagocytes was evaluated by the nitroblue tetrazolium test (NBT). The phagocytic index (PhI) by monocytes was assessed multiplying the average number of *Saccharomyces cerevisiae* adhered to / ingested by phagocytes by the percentage of phagocytes involved in phagocytosis. Additionally, the same tests were reassessed at the end of supportive therapy. After supportive therapy, the periodontitis group showed significant improvement in clinical periodontal status(P < 0,0002). The phagocytosis observed in the periodontitis group post-supportive therapy was significantly higher than those in control subjects. By contrast, before therapy, the phagocytosis was significantly lower in periodontitis patients than in control subjects. Such effect was observed in both analyses, when using non sensitized and sensitized *S. cerevisiae*. There was a significant difference in NBT analyses before and after therapy. Periodontal disease caused a weakening in monocytes function, while the clinically successful non-surgical periodontal therapy increased the phagocytic function in monocytes, however the increase was higher than the control group, suggesting hyperactivity for both PhI as in the production of superoxide.

Key Words: Periodontitis, Monocytes. Phagocytosis, Periodontal Therapy, Superoxide

INTRODUCTION

Periodontitis is initiated by oral pathogens that induce an inflammatory cascade, which stimulates host-mediated tissue destruction. In many aspects periodontal disease is similar to rheumatoid arthritis, whit the main diference being that the etiology is infectious rather than autoimmune (Smolik et al 2009). Additionally to the local effects, the bacterial virulence factors and inflammatory mediators arising from this parasite-host interaction may create and sustain a chronic systemic

inflammatory process in the bloodstream (Loss et al. 2000). There is an increase in data observed in current literature about with the potential impact of periodontal infections on overall general health (Bizzaro et al. 2010; Somma et al. 2010). Various hypotheses, including common susceptibility, systemic inflammation, direct bacterial infection and cross-reactivity, or molecular mimicry, between bacterial antigens and self-antigens, have been postulated to explain these relationships (Pizzo et al. 2010).

In the most aggressive forms of periodontal disease, the role played by the elevated serum levels of TNF- α and IL-1 has been considered partly responsible for altering the functions of PMN (Agarwal et al. 1994; Dias et al. 2011). In regards to phagocytosis by monocytes in these patients, studies are still scarce. Additionally, interventional clinical trials in periodontitis fail to include endpoint criteria that can be used to assess the resolution of periodontal disease. In fact, if periodontitis truly have measurable effects on general health, treatment of these infections should reduce the severity of the outcomes, including phagocytosis.

The objective of this interventional clinical trial was to evaluate the impact of periodontal disease in phagocytosis by monocytes and the effect of periodontal therapy on phagocytosis and production of oxygen radical. Additionally, the study design included pre-determined outcome criteria for the inclusion of subjects in a periodontal maintenance program with the goal to provide to all patients a similar level of resolution of inflammation.

MATERIAL AND METHODS

The protocol used in this study was approved by the Research Ethics Committee of the Health Science Faculty – (University of Brasilia 045/2008). The subjects were evaluated and selected for inclusion in the present study at the Periodontal Clinic of the University Hospital of (Brasilia), from August 2008 until August 2010. The sample included 55 systemically healthy, non-smokers: 28 in the periodontitis group and 27 in the control group. The periodontitis group consisted of 19 women and 9 men (ages ranging from 20-45, median 34.5), with the presence at least 18 teeth. The diagnosis of Generalized Chronic Periodontitis (n=14), Generalized Aggressive Periodontitis (n= 8) and Localized Aggressive Periodontitis (n= 6) was according to the classification by Armitage (1999) and Armitage and

Cullinan (2010). The control group consisted of 18 women and 9 men (ages ranging from 21-44, median 34), with clinical probing depths (PD) ≤ 3mm and clinical attachment level (CAL) ≤ 3 mm, ≤ 10% sites with bleeding on probing and no radiographic evidence of bone loss. The following conditions were considered exclusion criteria: prior periodontal therapy, antimicrobial therapy for systemic conditions or topical oral use in the last 12 months, pregnant or lactating women, diabetes, 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) (WHO, 2000), and any other clinical situation that might alter the function of the immune system, use of medications as corticoid and immunosuppressive therapy, that could alter the level of inflammatory mediators.

Clinical examination

The clinical examinations were performed by a single experienced examiner, and included visible plaque accumulation (PI), without the use of any disclosing agent, bleeding on probing (BOP), probing depth (PD) and clinical attachment level (CAL). The measurements were assessed at four sites around each tooth, buccal, lingual and proximal sites (for each proximal surface the greatest depth was recorded) using a manual probe (Michigan O probe with Williams markings), excluding third molars. The calibration and measurements of PD and CAL were repeated within 24 hours and demonstrated agreement over 80%. The BOP was calculated by the Kappa coefficients and the intraexaminer agreement was >0.85. The clinical examinations were assessed at baseline and at the end of the period of 6 months of the supportive therapy.

Treatment Protocol

The subjects of the periodontitis group were treated in three stages: 1- mechanical periodontal therapy, 2- reinstrumentation of sites, 3- supportive periodontal therapy. Stage 1 was performed in ≤ 14 days. One month later, stage 2, was performed in all patients for treatment of persistent deep pockets, bleeding on probing and calculus. In this stage, meticulous scaling and root planing was done until one of the following predetermined periodontal conditions were met: probing depth up to 4mm at three or fewer sites, probing depth up to 5 mm at two or fewer two sites, plaque index ≤15% and bleeding on probing ≤10%. At stage 3, the subjects

were scheduled biweekly or monthly depending on need for plaque control. Supportive periodontal therapy was performed for 6 months.

Phagocytosis test

The phagocytosis of *Saccharomyces cerevisiae* was adapted from a previously described technique (Muniz-Junqueira et al. 2003). Briefly, samples of 40 µL per marked area of heparinized whole peripheral blood obtained by means of venopuncture from each subject were placed on clean glass slides containing 8 marked areas of 7mm diameter each, in duplicate preparations, and incubated in a wet chamber for 45 min at 37°C. The slides were then rinsed with 0.15M phosphate-buffered saline (PBS) pH 7.2 at 37°C to remove non-adherent cells. After washing, monocytes remained adhered onto the slide approximately in the same proportion as they were in the whole blood. Adherent cells (12,534 ± 5,050 cells/marked area; 5.63 ± 0.85% monocytes were incubated with a suspension of 2.5×10^5 *S. cerevisiae* in 20 µL Hanks-tris (Sigma, St Louis, MO, USA) pH 7.2, with 10% heat-inactivated foetal calf serum (FCS) (Gibco) for 30 min in a wet chamber at 37°C. To evaluate the influence of complement molecules on phagocytosis in periodontitis, the *S. cerevisiae* were incubated at 37° for 30 min with 10% fresh serum from the donor in Hanks-Tris solution. Slides were then rinsed with 0.15M PBS at 37°C to eliminate nonphagocytosed *S. cerevisiae* and the final washing was done with 30% FCS in Hanks-tris. The slides were fixed with absolute methanol and stained with 10% Giemsa solution. The number of *S. cerevisiae* phagocytosed by 200 monocytes in individual preparations was assessed by optical microscopy. Microscopic fields distributed throughout the slide were randomly selected and all monocytes in each particular field were examined. The phagocytic index was calculated as the average number of phagocytosed *S. cerevisiae* per phagocytosing monocytes, multiplied by the percentage of these cells engaged in phagocytosis (Muniz-Junqueira et al. 1992).

Baking yeast (*Saccharomyces cerevisiae*) was prepared according to a technique previously described (Muniz-Junqueira et al. 2003). In short, 50 g of fresh live yeast (Fleischmann, Brazil) were suspended in 220mL of PBS, pH 7.2, autoclaved at 120°C for 30 min, washed in PBS until obtaining a clear supernatant and the sediment was suspended in 28mL of a 0.1M 2-mercaptoethanol solution in PBS. After 2 hours incubation with stirring, yeasts were washed again and suspended in 55mL of 0.02M iodoacetamide in PBS. After 2 hours incubation with

stirring at room temperature, they were washed 3 times and suspended in 220mL of PBS, pH 7.2. Yeasts were again autoclaved, washed and suspended in 110ml of veronal buffered saline, pH 7.2, containing sodium azide, and stored at 4°C until use. Before each experiment, dead *S. cerevisiae* were washed in PBS, quantified and suspended in Hanks-tris solution.

NBT test

Nitro blue tetrazolium (NBT) test was adapted from a technique previously described by Campbell and Douglas (1997). 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 is identified under optical microscopy by a blue color in the cytoplasm of the cell (Berridge et al 2005). The amount of NBT reduced is directly proportional to the amount of oxygen radicals produced by phagocytes, and these molecules are among the main microbicidal agents produced by phagocytes. Briefly, phagocytes adhered on slide, as previously described, were incubated with 0.05% NBT solution in Hanks-tris (Sigma, St Louis, MO, USA) for 20 min 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 distillate water. The percent phagocytes with NBT reduced in the cytoplasm was assessed by optical microscopy

Statistical Analysis

Statistical analysis was performed using the Sigma Stat 32.Ink® software and Prism® software (Graphpad, USA, 2005). Sample size was determined for a desired power of 90% and an alpha level of significance of 0.05, which showed a minimum number of 25 individuals by group, by employing the Sigma Stat software. In order to apply the statistical test, the variables in the samples were previously verified for normality, using the Skewness and Kurtosis and Kolmogorov-Smirnov tests. For comparison between two independent variables with normal distribution, the t-test was used, and for those that did not present normal distribution, the Mann Whitney test was used. For comparison between two paired variables with normal distribution, the paired t-test was used, and for those that did not present normal distribution, the Wicoxon test was used. The differences among the compared variables were

considered statistically significant when the bi-caudal probability of their occurrence due to chance (error type I) was lower than 5% ($p<0.05$).

RESULTS

Retention

Of the 28 subjects in periodontitis group, 23 concluded the 3 stages of the periodontal protocol. The phagocytosis by monocytes and NBT test was analyzed in 28 subjects at baseline and 23 subjects at the end of this study, for a total of 52 samples. The subjects in the periodontitis group reported no adverse effects after treatment.

Clinical and Demographical Characteristics

The demographical and clinical characteristics of control and periodontitis groups (pre and post-therapy) are summarized in Table 1 and 2. No significant differences were observed between groups regarding age and gender. Statistically significant differences between control and periodontotis groups were noted for body mass index ($P = 0.001$, Mann Whitney test). Statistically significant decreases in all clinical parameters were observed for periodontitis groups at the end of supportive therapy ($P < 0.0002$, Wilcoxon test). The hematological characteristics of all groups are summarized in Table 3. No statistically significant difference was observed between control and periodontitis groups as well as pre and post-therapy, except for the increase in HDL in the periodontitis group post-therapy ($P = 0.003$, Wilcoxon test).

Table 1 – Demographic characteristics at baseline and post-supportive therapy

Characteristics / Parameters	Control (n=27)	Pre (n=28)	Post (n=23)	Test (p) Pre x Post	Test (p) Control x Pre	Test (p) Control x Post
Age – years – median (extremes)	34.0 (21.0-44.0)	34.5 (20.0-45.0)	35.5 (21.0-45.0)	0.47[4]	0.84[4]	0.37[4]
Gender (males/females: n)	9/18	9/19		-	1.0000[5]	-
Numbers of teeth median (extremes)	28 (26.0-32.0)	29 (18.0-32.0)	25 (12.0-32.0)	0.0009[1]	0.74[3]	0.03[3]
BMI	22.0 (18.0-27.0)	26.0 (19.0-39.0)	25.5 (18.0-37.5)	0.40[1]	0.001[3]	0.0086[3]
Systolic Blood Pressure (mmHg)	120 (110.0-132.0)	120 (98.0 -165.0)	116 (97.0-140.0)	0.07[1]	0.92[3]	0.11[3]
Diastolic Blood Pressure (mmHg)	80 (76.0-86.0)	79 (65.0-115.0)	76 (62.0-102.0)	0.48[1]	0.02[3]	0.002[3]

Pre = Periodontitis group Pre-therapy, Post = Periodontitis group Post-supportive therapy, BMI (body mass index) , [1] Wilcoxon test, [2] Paired t test, [3] Mann Whitney test, [4] t test, [5] Chi square test.- CAL post-supportive therapy-not applicable.

Table 2 – Clinical parameters at baseline and post-supportive therapy

Characteristics / Parameters	Control (n=27)	Pre (n=28)	Post (n=23)	Test (p) Pre x Post	Test (p) Control x Pre	Test (p) Control x Post
PI (%; median - extremes)	4.0 (2.0-10.0)	63 (10.0-100.0)	2.0 (0.0-22.0)	0.00011	0.0013	0.073
BOP (%; median - extremes)	2.0 (1.0-7.0)	37.5 (2.0-100.0)	0.0 (0.0-10.0)	0.00011	0.00013	0.00013
PD (mm; median - extremes)						
≤3mm	100.0	77.5 (29.0-113.0)	99.9 (94.4-100.0)	0.00022	-	-
4 mm	0.0	4.0 (0.0-12.0)	0.0 (0.0-2.8)	0.00021	-	-
5-6 mm	0.0	16.5 (2.0-40.0)	0.0 (0.0-4.0)	0.00011	-	-
≥ 7 mm	0.0	9.0 (1.0-34.0)	0.0 (0.0-2.6)	0.00011	-	-
CAL (mm; median - extremes)						
≤3mm	100.0	73.0 (10.0-111.0)	-	-	-	-
4 mm	0.0	4.0 (0.0-15.0)	-	-	-	-
5-6 mm	0.0	17.0 (4.0-36.0)	-	-	-	-
≥ 7 mm	0.0	11.0 (1.0-42.0)	-	-	-	-

Pre = Periodontitis group Pre-therapy, Post = Periodontitis group Post-supportive therapy, PI (plaque index), BOP (bleeding on probing). PD (probing depth), CAL (clinical attachment level), [1] Wilcoxon test, [2] Paired t test, 3 Mann Whitney test, 4 t test, 5 Chi square test.- CAL post-supportive therapy-not applicable.

Table 3 – Hematological characteristics at baseline and 6 months post-supportive therapy.						
Characteristics / Parameters	**Control (n=27)**	**Pre-treatment (n=28)**	**Post-treatment (n=23)**	**Test (p) Pre x Post**	**Test (p) Control x Pre**	**Test (p) Control x Post**
			Median (minimum – maximum)			
Triglycerides (mg/dl)	77. (47.0-190.0)	96.5 (31.0-240.0)	102.5 (30.0-183.0)	0.89[2]	0.29[4]	0.12[4]
Total Cholesterol (mg/dl)	168 (124.0-242.0)	169 (112.0-231.0)	186 (128.0-280.0)	0.08[2]	0.82[3]	0.32[3]
HDL-cholesterol (mg/dl)	44 (30.0-72.0)	39 (24.0-74.0)	42.5 (29.0-112.0)	0.003[1]	0.11[3]	0.66[4]
LDL-cholesterol (mg/dl)	106 (73.0-159.0)	111 (70.0-164.0)	108 (76.0-204.0)	0.80[1]	0.53[4]	0.59[4]
Glucose (mg/dl)	86 (69.0-101.0)	88.5 (72.0-141.0)	88.5 (69.0-108.0)	0.93[2]	0.12[3]	0.19[3]
Eosinophils	136 (0.0-388.0)	192 (0.0-763.0)	148 (42.0-386.0)	0.12[1]	0.05[3]	0.23[4]
Basophils	0.0 (0.0-109.0)	0.0 (0.0-69.0)	0.0 (0.0-61.0)	0.21[1]	0.50[3]	0.65[3]
Lymphocytes	2094 (1287-3263)	2176 (118 -3330)	2183 (1196-3434)	0.37[2]	0.45[4]	0.37[4]
Monocytes	438 (200-756)	359 (181-923)	383 (0.0-557)	0.53[2]	0.32[4]	0.13[4]
Total Leukocytes	6110 (4060-9950)	6285 (3360-9700)	6140 (2720-9820)	0.25[2]	0.70[4]	0.70[4]

[1] Wilcoxon test, [2] Paired t test, [3] Mann Whitney test, [4] t test.

Figure 1: Mean percentage of bleeding on probing and probing depth at control group and periodontits group (Pre-therapy and Post-supportive therapy).

Phagocytic Test Results

In the analysis of phagocytosis by monocytes when using non-sensitized *S. cerevisiae*, we found that periodontal therapy increased the phagocytic function. This occurred because treatment caused an increment in the percentage of cells involved in phagocytosis. Treatment caused an increment of 30.0 fold in phagocytic index that changed from 13.25 before treatment to 43.28 after treatment (p= 0.01, Wilcoxon test). This increase was caused by an increment in the percentage of cells engaged in phagocytosis that changed from 8.5% before treatment to 20.5% after treatment (p=0.0006, Wilcoxon test), because the therapy did not influence the average number of yeasts adhered to/ingested by monocytes. The increase achieved in the phagocytic index after therapy (43.27) is greatest to the control group (27.39), however, the results were not statistically significant, (p=0.45, Mann Whitney test).The data are summarized in Table 4.

Similar results were observed when using sensitized *S. cerevisiae*. We found that periodontal therapy increased the phagocytic function. However, this occurred because treatment caused a increase in the percentage of cells engaged in phagocytosis, which changed from 36.75 before treatment to 63.50 after treatment, (p=0.0009, Wilcoxon test), as in the average number of particles ingested by monocytes, which increased from 1.59 to 1.86 (p=0.09, wilcoxon test). Resulting in an increase in the phagocytic index from 61.49 before treatment to 110.0 after treatment (p=0.005 paired t test). However, the increase in the phagocytic index after therapy (110.0) was higher than the control group 98.00 (p= 0.04, Mann Whitney test).The data are summarized in Table 5.

Table 4 Median of phagocytosis test by monocytes for non-sensitized *S. cerevisiae*

	Control	Pre-therapy	Post-therapy	Control x Pre	Pre x Post	Control x Post
PhI	27.39 (4.97-86.00)	13.25 (0.0-68.25)	43.28 (4.0-63.00)	P = 0.01[2]	P = 0.01[1]	P = 0.45[2]
Yeasts adhered to/ ingested	1.41 (1.0-2.02)	1.50 (0.0-2.47)	1.63 (1.1-1.90)	P = 0.77[4]	P = 0.68[3]	P = 0.03[4]
% Cells in Ph	18.75 (3.5-49.00)	8.5 (0.0-37.50)	20.5 (2.5-37.50)	P = 0.04[2]	P = 0.006[1]	P = 0.82[2]

Pre = Periodontitis group pre-therapy, Post = Periodontitis group post- supportive therapy, PhI = Phagocytic Index, Yeasts adhered to/ingested = Average number of yeasts adhered to/ingested by monocytes, % Cells in Ph = Percentage of cells involved in phagocytosis, [1] Wilcoxon test, [2], Mann Whitney test. [3] Paired t test, [4] t test

Table 5 – Median of phagocytosis test by monocytes for sensitized *S. cerevisiae.*						
	Control	**Pre-therapy**	**Post-therapy**	**Control x Pre**	**Pre x Post**	**Control x Post**
PhI	98.00 (36.99-176.50)	61.49 (12.48-156.50)	110.0 (61.50-223.00)	P = 0.005[1]	P = 0.005²	P = 0.04[3]
Yeasts adhered to/ ingested	1.8 (1.27-2.28)	1.59 (1.04-2.62)	1.86 (1.40-2.65)	P = 0.09[3]	P = 0.16[4]	P = 0.43[3]
% Cells in Ph	51.75 (27.00-78.50)	36.75 (12.00-24-00)	63.50 (32.0-99.0)	P = 0.01[3]	P = 0.0009[4]	P = 0.007[1]

Pre = Periodontitis group Pre-therapy, Post = Periodontitis group Post-supportive therapy, PhI = Phagocytis Index, Yeasts adhered to/ingested = Yeasts adhered to/ingested by neutrophils, % Cells in Ph = Percentage of cells involved in phagocytosis, [1] t test., [2]Paired t test, [3]Mann Whitney test,
[4] wilcoxon test.

Superoxide Anion Production

The capacity of monocytes and neutrophils to produce oxygen radicals was evaluated by the percentage of phagocytes that reduced the NBT. For the percentage of basal NBT reduction, there was a statistically significant difference between the groups. The median values of the periodontitis group pre-therapy (76.25%) and post-therapy (84.50%), (p=0.02, t-test). The increase in the NBT post-therapy was higher to the control group for both analyses(p=0009, t test) and (p=003, t test). Table 6

Table 6– Median of Nitro blue Tetrazoium test						
	Control	**Pre-therapy**	**Post-therapy**	**Control x Pre**	**Pre x Post**	**Control x Post**
Basal NBT	76.25 (16.00-92.00)	76.25 (39.50-99.00)	84.50 (53.00-97.50)	P = 0.78[1]	P = 0.02²	P = 0.009[1]
Stimulated NBT	69.5 (33.50-90.50)	75.25 24.00-97.00	80.75 (54.00.97.00)	P = 0.24[1]	P = 0.25[2]	P = 0.003[1]

Pre = Periodontitis group Pre-therapy, Post = Periodontitis group Post-supportive therapy, [1] t test., [2]Paired t test, [3]Mann Whitney test.

DISCUSSION

The emergence of periodontal medicine increased interest in defining the behaviour of peripheral blood cells in periodontitis. The current work is the first longitudinal intervention study analyzing the impact of periodontal treatment on phagocytosis by monocytes in peripheral blood. Furthermore, we investigate the effects of therapy in oxygen radical production in periodontitis. It is important to emphasize that the clinical protocol allowed that all patients, at the end of supportive therapy, had a similar level of resolution of inflammation. Analysis of the data presented demonstrates that the improvement of periodontal inflammation, confirmed by the reduction of pockets, bleeding on probing, and plaque (table 2 and Fig.1), resulted in increase of the phagocytic index of monocytes both when yeasts were incubated with or without fresh plasma from the donor (Table 4 and 5). This was caused by a higher percentage of monocytes engaged in phagocytosis. By contrast, before periodontal treatment, the phagocytosis was significantly lower compared to the control group (Tables 4 and 5). Probably, the observed decrease in phagocytosis of monocytes before therapy may be due to the development of particular strategies used by pathogens to subvert the mechanisms of phagocytosis observed in neutrophils (Eick et al. 2000; Johansson et al. 2000). Carvalho et al (2009) found high frequency of *P. gingivalis, T. forsythia* and *A actinomycetemcomitans* in individuals with aggressive periodontitis. They observed that in these individuals the frequency and quantity of *P. gingivalis* and *T. forsythia* presented negative correlation with phagocytosis in PMN. Another possibility is the high systemic level of quantitative cytokine produced during the host-pathogen interaction. These regulatory factors in serum may modulate reduced function in PMN (Agarwal et al. 1994; Hidalgo et al. 1997) and probably, in monocytes. Although, most current studies consider that the increase in inflammatory mediators contribute to cell hyperactivity (Wright et al. 2008; Dias et al. 2011), phagocytosis in this context is not yet elucidated. Fokkema et al. (2003) found that the changes in monocytes are acquired, in that after therapy that included full-mouth tooth extraction in a case with generalized terminal adult periodontitis, the levels of IL-8 decreased. Similarly, Papapanou et al. (2007) observed that after periodontal therapy, changes occurred in the biological functions of monocytes, related to innate immunity, apoptosis and signs

of cellular transduction in 1/3 of the patients with severe periodontitis al. Our findings are consistent with the hypothesis that periodontal infection may cause alterations in serum levels of chemokines (Nakajima et al. 2010, Duarte et al. 2010) and consequently alterations in monocytes functions related to phagocytosis, since the periodontal therapy increased the phagocytic function as a consequence of the increased involvement of monocytes in phagocytosis.(table 4 and 5).

Nonpathogenic *Saccharomyces cerevisiae* are used because receptors involved in their uptake are involved in phagocytosis by neutrophils of pathogenic bacteria present in periodontal disease (Underhill and Ozinsky, 2002).

Periodontal disease did not influence the production of oxygen radical compared to the control group, however, periodontal therapy caused increased in superoxide production (table 6). Other studies also reported hyperactivity on the production of oxygen radicals in periodontal treated patients (Asman et al. 1988; Fredriksson et al. 2003, Matthews et al. 2007). The increase observed in the oxygen radicals production and phagocytosis of periodontitis group post-supportive therapy compared to control group suggest intrinsic cell hyperactivity (Table 4 and 5). The hyperactivity found after therapy can be explain by the presence of genetic polymorphism mainly for molecules involved in inflammatory and innate immune response, such as Interleukin-1, tumor necrosis factor-α, Interleukin-10 (Meng et al, 2007). We hypothesize that during periodontal infection frequent bacteraemic episodes causes excessive cell overload and consequently reduction in the phagocytosis subsequent in vitro test. Another possibility is the potent immunosuppressant factors of periodontal pathogens (Getka et al. 1996). In fact, periodontal infection either leads to a reduction in phagocytosis or it is so exaggeratedly increased that the cell is unable to phagocytose the subsequent particle in vitro test.

More research must be done in order to investigate the immunoinflamatory events implicated in phagocytosis by monocytes in periodontitis. Phagocytosis is considered a key element in the defense against infectious agents, thus, the effect of periodontal infection on phagocytosis must be better understood since studies that implemented periodontal therapy to assess phagocytosis in monocytes are scarce. Based on these results we conclude that mechanical periodontal therapy increased the monocytes function related to phagocytosis, however the observed increase of phagocytosis, when analysed with fresh plasma from the donor and the production of

oxygen radical suggests an intrinsic cellular hyperactivity. Hyperactivity monocytes in periodontitis group may contribute to accelerated breakdown of tooth-supportive tissue. These results reinforce the need for periodic controls in this group of patients in order to prevent recurrence of periodontal disease.

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DISCUSSÃO COMPLEMENTAR

DISCUSSÃO

As mudanças de paradigmas nas últimas décadas a respeito dos mecanismos patogênicos de uma variedade de doenças se estendem também à Periodontia. Um entendimento inovador e mais amplo da etiopatogenia das periodontites fez emergir um novo conceito de risco, no qual fatores imunológicos e genéticos foram identificados como componentes importantes para o desenvolvimento da doença. Estes fatores conferem à etiologia da doença, primariamente de natureza microbiana, diferentes graus de patogenicidade, que se diferenciam pelo caráter inflamatório. O polimorfismo genético no qual a expressão de determinados genes resulta em monócitos predispostos a produzirem quantidades elevadas de mediadores inflamatórios contribui, em grande parte, para o caráter inflamatório exacerbado das periodontites severas [31,32]. Outro responsável por este caráter constitui o neutrófilo alterado por fatores intrínsecos e ou extrínsecos[33]. Hiperatividade relacionada ao aumento da adesão neutrofílica, liberação de enzimas, aumento do surto oxidativo e produção elevada de mediadores inflamatórios são observados no neutrófilo alterado e também explicam a intensidade do quadro inflamatório das doenças severas agressivas e crônicas. Nas últimas décadas surgiu o conceito de "*primed*" ou hiperatividade tanto em neutrófilo como em monócito e o papel central das citocinas neste contexto.

Entretanto, ainda não está esclarecido se o neutrófilo e monócito de indivíduos com periodontite são intrinsicamente diferentes daquele de indivíduos sem doença periodontal e se as alterações observadas nesses fagócitos são consequência da infecção periodontal. Adicionalmente, não há relatos na literatura sobre a fagocitose por monócitos em indivíduos com doença periodontal, uma vez que os estudos sobre o papel destas células na periodontite se concentram em avaliar a produção elevada de mediadores inflamatórios.

A microbiota do biofilme da bolsa periodontal está em constante alteração, de forma que, espécies que são relevantes em um estágio da doença periodontal podem não ser em outros. Com isto, a destruição tecidual pode resultar, em parte, da combinação de fatores bacterianos que sofrem variações com o tempo. Isto contrasta a periodontite da maioria das outras doenças infecciosas clássicas como, por exemplo, tuberculose, sífilis, em que o agente etiológico se restringe a um único microrganismo e o diagnóstico da doença se baseia na presença deste. Nas doenças periodontais, principalmente, nas

formas mais severas, a patogenicidade dos microrganismos se relaciona mais com a capacidade inata/ e ou inflamatória e/ ou imune individual do hospedeiro do que com a virulência propriamente dita das bactérias[34,35]. Porém, não se pode desprezar a capacidade da virulência bacteriana inteferir na atuação dos fagócitos. O *A.a. é* capaz de alterar a quiomataxia do neutrófilo, bem como resistir à fagocitose[36]. As bactérias do complexo vermelho podem inibir as funções de defesa inata do hospedeiro por produzirem uma estrutura de lípide A que atua como antagonista ao receptor *Toll-like* 4 (TLR4)[37]. Além disso, não está totalmente compreendida a exata relação do biofilme com a inflamação. Uma concepção ainda mais recente sobre a etiologia das periodontites propõe uma nova sequência do desenvolvimento da doença[38,39]. Nela, a resposta inflamatória do hospedeiro dita a composição do biofilme, ou seja, altera o microambiente e leva à seleção de microrganismos específicos. De acordo com esta ideia, a inflamação precede o crescimento microbiano e propicia, na bolsa periodontal, a elevação do número de *P. g.* e *T. f.*

A associação de fatores microbianos, genéticos e imunológicos conferem, portanto, complexidade às doenças periodontais severas. Há, ainda, a influência ambiental sobre o caráter poligênico (interação gene-ambiente)[40,41], de forma que o controle e a expressão de alguns genes em tecidos específicos podem ser substancialmente modificados pelo estilo de vida, incluindo dieta, estresse e acúmulo de bactérias[42].

A compreensão de cada um destes fatores tem sido alvo de diversas pesquisas. Os estudos sobre neutrófilos se fundamentam no seu reconhecido papel como célula de primeira linha de defesa. O sistema de resposta imune inata do hospedeiro é altamente ativo em tecidos saudáveis, entretanto um desequilíbrio ou ruptura na expressão de mediadores inflamatórios contribui grandemente para a destruição dos tecidos e estruturas de suporte dentário[42].

A atuação eficaz do neutrófilo, mediante o estímulo bacteriano na área subgengival, requer que estejam intactas as funções de sua capacidade de defesa, incluindo migração transendotelial, quimiotaxia, migração transepitelial, opsonização, fagocitose e morte intrafagolisossomal[43]. A ocorrência de defeitos intrínsicos ou extrínsicos de alguma destas funções compromete, substancialmente, o seu desempenho e, por esta razão, têm sido um dos principais objetos de pesquisa.

Os trabalhos pioneiros indicaram enfraquecimento da função neutrofílica. Muitos desses estudos avaliaram a quimiotaxia em resposta ao estímulo bacteriano derivado de FMLP (N-formil-metionil leucil-fenilanina), um peptídeo presente em bactérias Gram-

positivas. A redução da quimiotaxia se deveu a defeitos na migração direta em concentrações de 10^{-9} para $^{10-7}$ e em proporções significantemente elevadas (72-86%). Entretanto, quanto à migração aleatória, os resultados se mostraram inalterados ou até mesmo aumentados[44,45].

Similarmente, outros estudos encontraram defeitos na quimiotaxia do neutrófilo, tanto na bolsa periodontal, como no sangue periférico[46,47]. Além disso, pacientes que apresentam alterações na resposta quimiotática ao FMLP também apresentam enfraquecimento na quimiotaxia ao leucotrieno B4[48].

Os defeitos descritos na quimiotaxia podem estar relacionados à redução no número de receptores na membrana da célula, seja ao receptor FMLP ou ao co-receptor glicoproteína 110 (GP 110) ou ainda ao CD38, responsável por facilitar e intensificar a resposta quimiotática. Estes defeitos são descritos como sendo adquiridos ou inerentes[49]. Hurttia et al.[50] (1998) observaram que a expressão de FMLP se mostrou reduzida em pacientes com formas severas de doença periodontal, entretanto, as integrinas que medeiam adesão celular mostraram-se elevadas.

Evidências indicam que em muitos pacientes com formas severas de doença periodontal, defeitos quimiotáticos são geneticamente inerentes e não podem ser revertidos pelo tratamento[10,51,52]. Em outros pacientes, porém, esses defeitos foram adquiridos por meio da exposição aos mediadores inflamatórios presentes no soro [53]. Em estudo subsequente, Agarwall; Suzuki [54](1991); Agarwall et al.[55] (1994) observaram que o soro de pacientes apresentava regulação e expressão de FMLP diminuídas. Tais efeitos foram eliminados por anticorpo para TNF-α e IL-1. Estas citocinas inflamatórias modulam a expressão de moléculas de adesão (CD11 / CD18) em células fagocitárias.

Os genes que codificam a IL-1 parecem influenciar a severidade da periodontite. O polimorfismo leva os macrófagos a produzirem elevadas quantidades de IL-1 em resposta aos LPS de bactérias[31,32]. Embora os resultados sejam contraditórios, inúmeros outros polimorfismos genéticos foram relacionados com formas mais severas de doença periodontal, como os polimorfismos para interleucina 4, interleucina 10 e fator de necrose tumoral alfa[10]. Adicionalmente, a interação de LPS com macrófagos estimula também a produção de prostanoides, principalmente PGE_2, detectada em altas concentrações no fluido gengival de sítios com perda de inserção[56]. A PGE_2 é um lípide inflamatório implicado na reabsorção óssea e, estudos feitos em modelos animal e clínico, demonstraram que sua produção e a perda óssea alveolar foram substancialmente atenuados por uso local e sistêmico de antiinflamatórios não esteroidais[39,57].

Em modelo de indução de periodontite em ratos houve decréscimo da perda óssea quando se administrou receptores solúveis para IL-1α, IL-1β e TNF-α, inibindo estas proteínas de se ligarem a seus receptores[58]. Em ratos deficientes de E-selectina, P-selectina e IL-10, observou-se desenvolvimento de doença periodontal espontânea[59,60]. Contudo, naqueles animais em que se administrou antibiótico, não houve desenvolvimento da doença periodontal, confirmando o papel das bactérias bucais na periodontite[59]. Contrariamente, no estudo de Dayan et al.[61] (2004), a superprodução de IL-1α em ratos transgênicos levou ao desenvolvimento de periodontite, mesmo com contínua administração de antibiótico. Outros estudos de indução da doença em modelos animais mostraram que diferentes mediadores inflamatórios também contribuem para a perda óssea. A IL-10 previne a superexpressão de componentes inatos do hospedeiro em resposta ao biofilme bacteriano. Tal fato demonstra a importância do equilíbrio entre mediadores pró-inflamatórios e anti-inflamatórios como necessários para manter a saúde periodontal[62].

No estudo de Hurttia et al.[50](1998), as alterações de adesão do neutrófilo às células do endotélio capilar, em indivíduos com periodontite “juvenil”, foram caracterizadas por alta aderência à placa de cultura, quando estes foram comparados aos controles saudáveis. Contrariamente, outro estudo que examinou, em indivíduos com periodontite agressiva localizada e periodontite crônica, a expressão de CD 18 / CD11a e CD18/ CD11b, no sangue periférico e no fluido gengival, não demonstrou nenhuma diferença entre os grupos, assim como, nenhuma deficiência na expressão dessas integrinas de adesão[63].

Aumento da atividade enzimática intracelular em indivíduos com periodontite severa estão descritas em diferentes estudos. Pippin et al.[64] (2000) observaram aumento da enzima beta-glucuronidase, característica dos grânulos azurófilos. Albandar et al.[65] (1998) observaram que esta enzima se apresentou elevada no fluido gengival na periodontite agressiva, contribuindo para o aumento da destruição tecidual, enquanto, Kaner et al.[66] (2006) encontraram aumento da enzima mieloperoxidase no fluido gengival, correlacionada à presença de sangramento e supuração nas áreas de bolsa.

O evento que mais caracteriza a hiperatividade neutrofílica em formas severas de doença periodontal é o aumento da síntese e liberação de produtos relacionados ao surto oxidativo; ânion superóxido, radical hidroxilado e peróxido de hidrogênio. Estes produtos são provenientes dos mecanismos oxidativos na lise dos PMN durante a morte das bactérias[33].

Paralelamente, à realização dos estudos sobre fatores genéticos e imunidade inata/adquirida acima destacados, outras pesquisas focam sua investigação nos efeitos da terapia periodontal sobre estes fatores. O maior impacto dos avanços, tanto na compreensão da patogenia, como dos efeitos do tratamento sobre a ação imuno genética, será, sem dúvida, a condução de novas estratégias terapêuticas baseadas nesta ação. Isto significa conduta adequada aos diferentes graus de suscetibilidade à doença, em outras palavras, graus de risco, implicando, consequentemente, em formas individualizadas de prevenir e de tratar a doença.

Os estudos que implementaram terapia periodontal objetivando avaliar os efeitos no surto oxidativo, encontraram atividade elevada mesmo após o tratamento [30,67,68].

Em relação à atividade de surto oxidativo, nosso estudo utilizou o NBT para avaliar a produção intrínseca da célula e NBT com levedura opsonizada para avaliar a produção de radical de oxigênio mediante estímulo da célula. Em estudos prévios há diferentes respostas, tanto de hipoatividade [69,70] como de hiperatividade[28,50,67,68,71,72,73,74,75], assim como respostas semelhantes entre indivíduos com doença periodontal e controles[76,77]. Contudo, a maioria dos estudos relataram hiperatividade na produção de radicais de oxigênio.

Dados sobre a fagocitose por neutrófilo do sangue periférico de indivíduos com periodontite são controversos. Há relatos tanto de redução[27,69,77,78,79,80], assim como de aumento da fagocitose do neutrófilo [28,75,81,82]. Da mesma forma, os nossos resultados foram conflitantes em relação aos estudos sobre efeitos da terapia periodontal na fagocitose. A redução na fagocitose foi demonstrada no estudo de Kimura et al.[27] (1992) e não sofreu alteração pela terapia periodontal, implicando em um defeito inerente. Já no estudo de Johnstone et al.[28] (2007), observou-se fagocitose aumentada, mesmo após terapia periodontal. Entretanto, os pacientes do estudo foram refratários ao tratamento.

Percebe-se nos estudos que utilizaram bactérias periodontais como estímulo, fagocitose periférica aumentada nos indivíduos com periodontite[75,81,82] enquanto nos estudos com levedura [80] e outros estímulos, fagocitose periférica reduzida [70,76]. Respostas divergentes nos faz pressupor que patógenos periodontais causariam uma exaustiva função celular impedindo uma fagocitose eficiente, comparada a outros estímulos, o que sugere certo grau de imunodeficiência na periodontite. É importante ressaltar que independente do estímulo, a análise da capacidade fagocitária na bolsa periodontal descrita em diferentes estudos é, notadamente, reduzida [70,80, 83,84,85,86,87,88].

Neste estudo, a técnica utilizada para avaliar a capacidade fagocitária do neutrófilo e monócito foi a aderência destas células à lâmina de microscopia, seguida pela fagocitose de *S. Cerevisiae,* sensibilizada ou não com o soro fresco do indivíduo. Trata-se de um método de baixo custo em que se utiliza pouca quantidade de sangue. Outra vantagem é que não necessita de meio estéril, além da contagem simultânea de neutrófilo e monócito[89].

Pesquisadores observaram que a capacidade fagocitária obtida diretamente por fagocitose em lâmina é maior, comparada à técnica de sedimentação por dextran e técnica de *percoll* por gradiente de centrifugação[89]. Adicionalmente, a variação individual dos resultados encontrados pelo teste de aderência em lâmina não é diferente de outros testes funcionais que avaliam o sistema imune[90]. Embora *S. Cerevisia* não seja um patógeno relevante na periodontite, dá-nos uma indicação da função celular. Adicionalmente, os fagócitos utilizam os mesmos receptores para fagocitar tanto leveduras como bactérias relacionadas com a doença periodontal[5]. Utilizando-se leveduras sensibilizadas e não sensibilizadas, foi possível avaliar a fagocitose via diferentes receptores, tais como receptores para imunoglobulina, componentes do complemento e padrões moleculares. Testamos a fagocitose via reconhecimento padrão avaliada com partículas de leveduras não sensibilizadas. Neste caso, resíduos de manose na superfície da levedura são o maior ligante. Nesta análise, observou-se menor índice fagocitário no grupo periodontite tanto em neutrófilo, como em monócito.

A fagocitose via opsonina também foi testada, incubando fagócitos com leveduras previamente sensibilizadas com soro humano fresco (imunoglobulina e complemento). Essas opsoninas causaram aumento no índice fagocitário de monócito e neutrófilo tanto no grupo controle como no grupo periodontite. No entanto, nesta primeira etapa do estudo (análise transversal), nossos dados indicaram que a função de ambos neutrófilo e monócito foi intrinsicamente afetada, uma vez que o nível de fagocitose foi menor no grupo periodontite na ausência de componentes do soro. Na análise por opsoninas, a função do neutrófilo no grupo periodontite não foi afetada, embora tenha apresentado tendência de redução do índice fagocitário. Tal fato indica que em neutrófilo, não houve diminuição de receptores para complemento e imunoglobulina na superfície da célula.

A razão do nível reduzido de índice fagocitário no grupo periodontite ainda não está bem estabelecida. Possivelmente, a redução no índice fagocitário deste grupo reflete uma sobrecarga da célula, o que contribuiria para uma menor sensibilidade aos novos desafios, subsequentemente, introduzidos durante o teste *in vitro.* Charon et al.[84](1982)

observaram que neutrófilos coletados na bolsa periodontal apresentaram enfraquecimento na atividade quimiotática quando submetidos a vários estímulos e redução na capacidade fagocitária, quando estimulados por *Candida albicans*. Loesche[86] (1988) também observou que neutrófilos coletados na bolsa periodontal, estimulados por Porbol Miristato Acetato (PMA), apresentaram enfraquecimento na capacidade oxidativa e redução na fagocitose, comparados aos neutrófilos de sítios saudáveis no mesmo paciente.

Outro possível fator que pode explicar a diminuição da fagocitose está na capacidade da doença periodontal modificar a produção de várias moléculas que influenciam na função do sistema imune, como PGE e citocinas. Agarwal, Suzuki[54] (1991); Agarwall et al.[55] (1994) demonstraram que a diminuição na resposta quimiotática em pacientes com formas severas de doença periodontal pode não ser uma anormalidade associada à célula, mas, antes uma consequência da elevação quantitativa do nível sistêmico de citocinas produzidas durante a interação hospedeiro-parasita.

Adicionalmente, diferenças no genótipo do receptor FC tem sido relacionadas com alterações na função de PMN. Kobayashi et al.[91] (2000) demonstraram que indivíduos com periodontite crônica possuem alelo Fcy R IIIb- NA2, levando a uma fagocitose ou surto oxidativo menos eficiente, sob estímulo por bactéria opsonizada.

A frequência e nível de *A.a.*, *P.g. e T.f.* apresentaram correlação negativa com fagocitose e surto oxidativo em PMN de indivíduos com periodontite agressiva[77]. A ação imunossupressora de periodontopatógenos como *A.a., P.g.* e algumas bactérias do complexo vermelho é conhecida pela sua interferência no mecanismo protetor de defesa do hospedeiro e pelos inúmeros fatores de virulência descritos [92,93,94,95].

Nesta primeira etapa da nossa pesquisa, foi observado diminuição na capacidade fagocitária apenas para o neutrófilo não sensibilizado, enquanto que a redução em monócito ocorreu em ambas as análises. Tal diminuição sugeriu uma provável imunodeficiência na periodontite.

A etapa seguinte (segunda etapa) referiu-se ao estudo longitudinal da análise da fagocitose por neutrófilo antes e após terapia periodontal. Os resultados encontrados de aumento da fagocitose por neutrófilo após a terapia periodontal sugerem que a diminuição da capacidade fagocitária nos indivíduos com periodontite parece ser de natureza extrínseca, visto que o tratamento restabeleceu a função fagocitária. O restabecimento da capacidade fagocitária pós-terapia reforça algumas hipóteses por nós levantadas: houve uma “sobrecarga” nas células fagocitárias decorrente da infecção periodontal, ou seja,

uma fagocitose tão intensa, que dificultou a fagocitose por neutrófilo subsequente ao teste *in vitro;* ou ainda, uma fagocitose de fato reduzida pela presença de fatores sorológicos ou até mesmo enfraquecida pelos mecanismos de virulência das bactérias periodontopatogênicas.

Nesta etapa do estudo, a terminologia periodontite severa foi substituída pela classificação de Armitage et al.[96] (1999). Com base nas características clínicas, idade e história familial, esta classificação orientou a divisão da amostra nos seguintes grupos: oito indivíduos com periodontite agressiva generalizada, seis indivíduos com periodontite agressiva localizada, e quatorze indivíduos com periodontite crônica generalizada. Dois indivíduos foram classificados como periodontite crônica localizada avançada. Como não se enquadravam nas terminologias acima descritas (porém se enquadravam em uma terminologia mais abrangente, periodontite severa), foram excluídos do estudo. O total da amostra compreendeu 28 pacientes. Em recente estudo de revisão, Ford et al.[97] (2010) concluíram que sob o aspecto imunopatológico não é possível identificar a real diferença entre periodontite crônica e periodontite agressiva.

Uma vez que o objetivo do tratamento periodontal consiste em restaurar a relação homeostática entre o tecido periodontal inflamado e as bactérias presentes no biofilme dentário, a remoção mecânica do biofilme por meio de raspagem e alisamento radicular é capaz de restaurar a homeostasia tecidual e promover a recolonização de bactérias comensais, assim como desenvolver a cura dos tecidos periodontais. O ganho clínico de inserção, ausência de sangramento à sondagem e eliminação ou redução da profundidade de sondagem são sinais clínicos que evidenciam a cura dos tecidos periodontais[98]. Um critério metodológico importante deste estudo foi o desenho de um protocolo clínico de tratamento bem definido, ainda não descrito na literatura. O estabelecimento de parâmetros periodontais, previamente estabelecidos, permitiu a inclusão dos indivíduos na terapia periodontal de suporte. Nela, o controle da inflamação foi alcançado e as diferenças quanto ao nível de resolução da inflamação foram minimizadas até a conclusão do tratamento. Desta forma, no momento final da coleta do sangue periférico, os pacientes apresentavam nível similar de resolução clínica da inflamação periodontal. Para alcançar o protocolo proposto, considerou-se o processo individual de cura das bolsas periodontais. Assim, o tempo de conclusão do protocolo clínico foi diferente entre os indivíduos. Este protocolo incluiu raspagem, obtenção de uma condição periodontal mínima previamente estabelecida e manutenção por seis meses.

Dos 28 indivíduos tratados, 23 concluíram o tratamento periodontal, sendo que 10 (43%) concluíram em 9 meses, 10 (43%) em 10 meses e 3 (14%) em12 meses.

A terceira etapa do presente estudo compreendeu a avaliação longitudinal da capacidade fagocitária por monócito e produção de radical de oxigênio antes e após terapia periodontal. Os resultados encontrados sugerem que a infecção causou diminuição da capacidade fagocitária por monócito e nenhuma mudança na produção de radical de oxigênio. Diferentemente, após o tratamento, observou-se aumento da fagocitose, assim como aumento na produção de radical de oxigênio, a níveis superiores aos encontrados no grupo controle, sugerindo hiperatividade celular intrínseca. Os resultados de hiperatividade na fagocitose e na produção de radical de oxigênio pós-terapia periodontal sugerem um fenótipo intrinsicamente hiperativo. O fenótipo hiperativo descrito em muitos estudos foram relacionados ao aumento da destruição tecidual em formas severas de doença periodontal[31,32]. Os resultados encontrados reforçam a necessidade de controles periodontais periódicos neste grupo de pacientes com o objetivo de prevenir a recorrência da doença periodontal.

Dados conflitantes entre nossos achados e outros estudos certamente resultam das diferenças na população estudada, indivíduos em diferentes estágios da mesma doença ou, ainda, múltiplas subformas de doença periodontal que, provavelmente, levam a combinações de diferentes mecanismos patogênicos de destruição tecidual. Os estudos diferem também quanto às formas de ativação celular e metodologia. É possível que a inconsistência dos resultados nos diferentes estudos seja também resultado da natureza complexa e heterogênea da influência genética sobre a periodontite. Entretanto, nossos achados sugerem que a terapia periodontal afetou a magnitude da resposta immune representada pela fagocitose, tanto por neutrófilo como por monócito, caracterizando a influência da infecção periodontal nestas células. Assim, demonstrou-se o papel modulador da infecção (provavelmente interação gene-ambiente) na resposta imunológica dos pacientes e que o tratamento desta infecção local pode exercer efeitos sistêmicos na fagocitose tanto em neutrófilo como em monócito.

CONSIDERAÇÕES FINAIS S E PERSPECTIVAS FUTURAS

CONSIDERAÇÕES FINAIS

O principal objetivo da pesquisa clínica é gerar conhecimento generalizável que propicie melhor compreensão de uma determinada condição ou doença e ofereça bem estar aos indivíduos.

As pesquisas clínicas sobre doença peridontal, especialmente das últimas décadas, têm contribuído significativamente para o entendimento mais amplo da sua etiopatogenia e, a partir deste, maiores subsídios à proposição de novas estratégias terapêuticas. O conhecimento da etiopatogenia tem seu grande valor na sua aplicabilidade como base para o diagnóstico e condução clínica.

As inúmeras dificuldades que os estudos clínicos que envolvem tratamento periodontal apresentam se tornam mínimas mediante os benefícios que proporcionam, primeiramente, à amostra do estudo e, por extensão, à população em geral. Seu valor científico está no objetivo que encerram de desenvolverem uma intervenção ou comprovarem uma hipótese que possa gerar informações importantes sobre a doença e formas mais adequadas de prevenir ou tratá-la.

Não obstante, a maior evidência científica dos estudos clínicos longitudinais, seu delienamento é complexo e circunstâncias relativas ao atendimento clínico dificultam, muitas vezes, o estabelecimento de critérios ideais.

Um dos aspectos falhos observados nos estudos que incluem terapia periodontal, destacados na revisão apresentada, é que o tempo de avaliação pós-terapia é o mesmo para todos os pacientes, não sendo consideradas as diferenças de resposta alcançadas por cada um.

Este estudo estabeleceu uma condição periodontal mínima que cada paciente deveria alcançar após terapia periodontal mecânica. Após a obtenção desta condição, o paciente foi inserido na terapia de suporte por um período de seis meses. Somente ao final deste período, fez-se nova coleta de sangue. Desta forma, dentro do modelo proposto, obteve-se similaridade da condição clínica entre os pacientes no tempo da coleta. Com isto, as diferenças individuais relacionadas ao processo de cura foram minimizadas.

Outro destaque do estudo foi a investigação sobre fagocitose por monócito, até então não explorada nas pesquisas anteriores.

O tratamento periodontal restabeleceu a função fagocitária em neutrófilos a níveis similares aos do grupo controle. O estudo mostrou que com o tratamento, por outro lado, a função fagocitária em monócito e a produção de radicais de oxigênio alcançaram valores superiores aos encontrados no grupo controle. Estes dados sugerem hiperatividade intrínseca e reforçam a possibilidade de suscetibilidade no grupo periodontite.

Além disso, os dados demonstraram o papel modulador da doença periodontal na resposta imunológica sistêmica relativa à fagocitose quando se observou redução da capacidade fagocitária tanto em neutrófilo como em monócito. A este fato foi atribuído certo grau de imunossupressão, o que nos leva a pressupor que a periodontite pode constituir um fator de risco para agravamento de algumas doenças sistêmicas, ou perpetuar o estado inflamatório sistêmico crônico, como descrito em alguns estudos.

PERSPECTIVAS FUTURAS

Os dados da função do neutrófilo extraídos deste estudo vêm ao encontro dos resultados de pesquisas anteriores e, com isto, reforçam as evidências sobre atuação desta célula em indivíduos com periodontite. Apesar das importantes informações publicadas anteriormente ao nosso trabalho, em recente estudo revisional, Nussbaum; Shapira[99] (2011) concluíram que ainda não é possível definir se a modulação da resposta neutrofílica é de supressão ou de ativação e, portanto, novas investigações poderão responder a esta questão.

A relevância dos resultados do nosso estudo sobre fagocitose por neutrófilo e por monócito deverá, ainda, ser complementada por pesquisas adicionais que afiram os efeitos da infecção nestas células. A análise da influência dos mediadores inflamatórios na fagocitose e de polimorfismos genéticos deverão ser melhor exploradas, como parte dos fatores implicados no desenvolvimento da doença.

A reunião de indivíduos com características clínicas de doença periodontal severa crônica ou agressiva no grupo periodontite deste estudo se baseou na observação de que as duas categorias de doenças apresentam o mesmo quadro imunopatológico e, consequentemente, similaridades na atuação das células da imunidade inata[97]. No entanto, se a fagocitose e produção de radical de oxigênio apresentam alguma peculiaridade em cada uma delas, somente por meio da divisão da amostra em grupos distintos de periodontite crônica e periodontite agressiva poder-se-ia detectá-la.

Outro ponto importante é que, até o momento, não sabemos como seria a resposta fagocitária em neutrófilo e monócito, mediante um quadro de bacteriemia (p.ex. logo após a raspagem periodontal de toda a boca). A utilização tanto de leveduras como periodontopatógenos seriam importantes nesta análise.

Na avaliação do papel microbiano, pode-se ainda verificar a fagocitose utilizando bactérias periodontopatogênicas como estímulo, seguindo o mesmo protocolo clínico de tratamento do nosso estudo.

O conhecimento da exata relação entre o agente microbiano e a inflamação poderá resultar na implementação de formas de tratamento que tenham como alvo não o microrganismo e, sim, a redução da resposta inflamatória. Esta proposta

recente [38,39,57]considera que a efetiva eliminação do neutrófilo do tecido inflamado é pré- requisito para a completa resolução da resposta inflamatória[100]. Este princípio é suportado pelos estudos que demonstraram que a PGE_2 e LTB4 aumentam a resposta inflamatória local, levando ao recrutamento do neutrófilo e aumento do dano tecidual por ele mediado[101]. Dentro deste conceito, propostas terapêuticas atuais visam bloquear a ativação da inflamação por meio da aplicação de drogas anti-inflamatórias (não esteroidais ou inibidores do TNF) ou visam a cura com agentes como TGF-1β, moléculas de união e receptores para fagócitos[101,102]. O emprego de moléculas de resolvina (RvE1), como proposto por Hasturk (2007)[39], poderia barrar a infiltração de neutrófilos e dirigi-los à apoptose, ao mesmo tempo que atrairiam monócitos para a lesão. Os monócitos recrutados por RvE1 não são flogísticos e fagocitam neutrófilos apoptóticos sem contribuir para uma maior inflamação ou lesão tecidual. A RvE1 funcionaria, portanto, como um modulador da resposta inflamatória mudando-a para uma resolução mais rápida e eficaz, evitando a fase crônica[39].

Tais propostas terapêuticas poderão representar, se confirmada a sua aplicabilidade, um novo paradigma na Periodontia. Todavia, cabe acrescentar que a importância de se desorganizar o biofilme é um princípio claramente estabelecido e o sucesso destas propostas requer, imprescindivelmente, sua associação à terapia periodontal mecânica.

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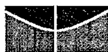
ANEXO A

Comprovante de aprovação do Comitê de Ética



Universidade de Brasília
Faculdade de Ciências da Saúde
Comitê de Ética em Pesquisa – CEP/FS

PROCESSO DE ANÁLISE DE PROJETO DE PESQUISA

Registro do Projeto no CEP: 045/2008

CAAE: 0067.0.012.012-08

Título do Projeto: "Mediadores inflamatórios e funções fagocitárias local e sistêmica na Periodontite Agressiva Localizada e Periodontite Agressiva Generalizada antes e após terapia periodontal".

Pesquisadora Responsável: Valéria Martins de Araújo

Data de entrada: 10/03/2008

Com base nas Resoluções 196/96, do CNS/MS, que regulamenta a ética da pesquisa em seres humanos, o Comitê de Ética em Pesquisa com Seres Humanos da Faculdade de Ciências da Saúde da Universidade de Brasília, após análise dos aspectos éticos e do contexto técnico-científico, resolveu **APROVAR** o projeto 045/2008 com o título: "Mediadores inflamatórios e funções fagocitárias local e sistêmica na Periodontite Agressiva Localizada e Periodontite Agressiva Generalizada antes e após terapia periodontal", analisado na 5ª Reunião Ordinária, realizada no dia 10 de Junho de 2008.

A pesquisadora responsável fica, desde já, notificada da obrigatoriedade da apresentação de um relatório semestral e relatório final sucinto e objetivo sobre o desenvolvimento do Projeto, no prazo de 1 (um) ano a contar da presente data (item VII.13 da Resolução 196/96).

Brasília, 11 de Junho de 2008.

Prof. Volnei Garrafa
Coordenador do CEP-FS/UnB

ANEXO B

Termo de consentimento livre e esclarecido

UNIVERSIDADE DE BRASÍLIA / FACULDADE DE CIÊNCIAS DA SAÚDE
DEPARTAMENTO DE ODONTOLOGIA

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

A Faculdade de Saúde, através do curso de Doutorado em Ciências da Saúde, está realizando um estudo, juntamente com o Laboratório de Imunologia Celular da Faculdade de Medicina- UnB, sobre o funcionamento de algumas células presentes no sangue e na bolsa periodontal. O estudo fará a análise sistêmica da função fagocitária e de radicais de oxigênio, antes e após terapia periodontal, em pacientes com periodontite severa.

Este termo de consentimento informa sobre o estudo o qual o (a) senhor (a) está convidado a participar, e da sua colaboração em responder um questionário, que nos fornecerá seu histórico médico e familiar. O (a) senhor (a) pode decidir não participar deste estudo, assim como, retirar seu consentimento em qualquer momento da pesquisa sem que isto lhe acarrete qualquer prejuízo ou punição.

As informações obtidas deste estudo serão publicadas, porém a identidade do (a) senhor (a) será mantida em sigilo sempre, bem como em qualquer publicação futura que vier a resultar deste estudo.

Os procedimentos que o (a) senhor (a) será submetido são os seguintes:

1-Exame clínico de sua cavidade bucal, para análise da condição de sua saúde dentária e gingival;

2-Exame radiográfico;

3-Coleta de amostras de sangue para avaliar funcionamento de algumas células, antes e seis meses após tratamento de raspagem periodontal;

4- Coleta de amostras de sangue para avaliar o estado de sua saúde geral, antes e seis meses após tratamento de raspagem periodontal;

5-Coleta de fluido gengival na área da gengiva inflamada;

6-Tratamento periodontal de raspagem:

7-Acompanhamentos a cada dois meses até o sexto mês para avaliar a escovação.

Brasília-------de ------------------------ de --

Nome Voluntário --

Nome do Pesquisador --Tel: 3307 1110

Com cópia para o voluntário

ANEXO C

Ficha de anamnese e exame clínico periodontal

PRONTUÁRIO CLÍNICO

Identificação do Paciente					
Nome:					
Data de Nascimento	RG	Expedição			Gênero: **M / F**
Pai					
Mãe					
Endereço					
Cidade		UF	CEP	Naturalidade	
Ocupação			Telefones para Contato	Nacionalidade	
Responsável					

Exame clínico: anamnese e exame físico

1. Queixa Principal
2. História da Doença Atual
3. Antecedentes Familiares

4. Questionário de Saúde	**Sim**	**Não**	***Discriminação***
Está sob tratamento médico?			
Está tomando algum medicamento?			
Já apresentou alguma reação à penicilina?			
Tem história de alergia?			
Tem ou teve problemas respiratórios?			
Tem ou teve doença articular? Artrite, febre reumática			
4. Questionário de Saúde (continuação)	**Sim**	**Não**	***Discriminação***
Tem ou teve distúrbio sangüíneo? anemia, hemorragia, leucemia?			
Tem diabetes?			
Tem dores de cabeça freqüentemente?			
Tem ou teve doença cardiovascular?hipertensão? infarto?			
Tem ou teve hepatite A? B? C?			
Tomou vacina contra hepatite?			
Está grávida? em qual período?			
Algum problema renal/hepático?			
Já recebeu transfusão de sangue?			
Pressão arterial : / mmHg			

Álcool	Fumo	Outros Hábitos
Alguma condição não questionada:		

Complementação das informações sobre alguma condição relevante:

5. Exame Físico (sinais vitais, ectoscopia e oroscopia)

ATM	Linfonodos	Pele	
Simetria facial	Lábios	Glândulas	
Mucosa labial	Mucosa jugal	Região retromolar	Gengiva
Palato	Língua	Assoalho	Orofaringe

6. Exames complementares

Radiográficos
Anatomopatológicos
Clínicos-laboratoriais

7. Registro das Necessidades Terapêuticas do Paciente

( ) Controle de placa	( ) Fluorterapia	Dentística ( ) Adequação do meio ( ) simples ( ) complexa	Periodontia ( ) periodontite leve ( ) periodontite moderada ( ) periodontite avançada ( ) cirurgia periodontal
Diagnóstico ( ) biópsia ( ) exames laboratoriais	( ) Ortodontia ____________________ ____________________		
Cirurgia ( )exodontia simples ( )exodontia de dente incluso ( )outros ____________________ ______________________________	Endodontia ( ) uni-radicular ( ) bi-radicular ( ) tri-radicular ( ) retratamento	Prótese ( ) removível ( ) fixa unitária ( ) parcial fixa ( ) total	Outros ( ) ____________________ ( ) ____________________ ( ) ____________________

Descrição do Plano de Tratamento:

FICHA CLÍNICA PERIODONTAL – Diagnóstico e Prognóstico

Nome:________________________ Idade:_______Data:____/____/__________

Dente	Profundidade de bolsa (Recessão- valor entre parênteses)				Lesão de furca	Mobilidade	Gengivite	Periodontite			Prognóstico			Índice gengival	Índice de placa
	D	V	M	L				Leve	Grave	Complicada	Bom	Duvidoso	Ruim		
18															
17															
16															
15															
14															
13															
12															
11															
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47															
48															

Cálculo índice Gengival e índice de placa= $\dfrac{N^o. \text{ total de faces com sangramento/ placa}}{n^o. \text{ total de faces}} \times 100 = \%$

ANEXO D

Fotografia mostrando a fagocitose por neutrófilo, monócito e produção de radical de oxigênio

Figura 1: Fotografia mostrando a fagocitose por neutrófilo e monócito de *Saccharomyces Cerevisial* incubadas com soro bovino inativado.

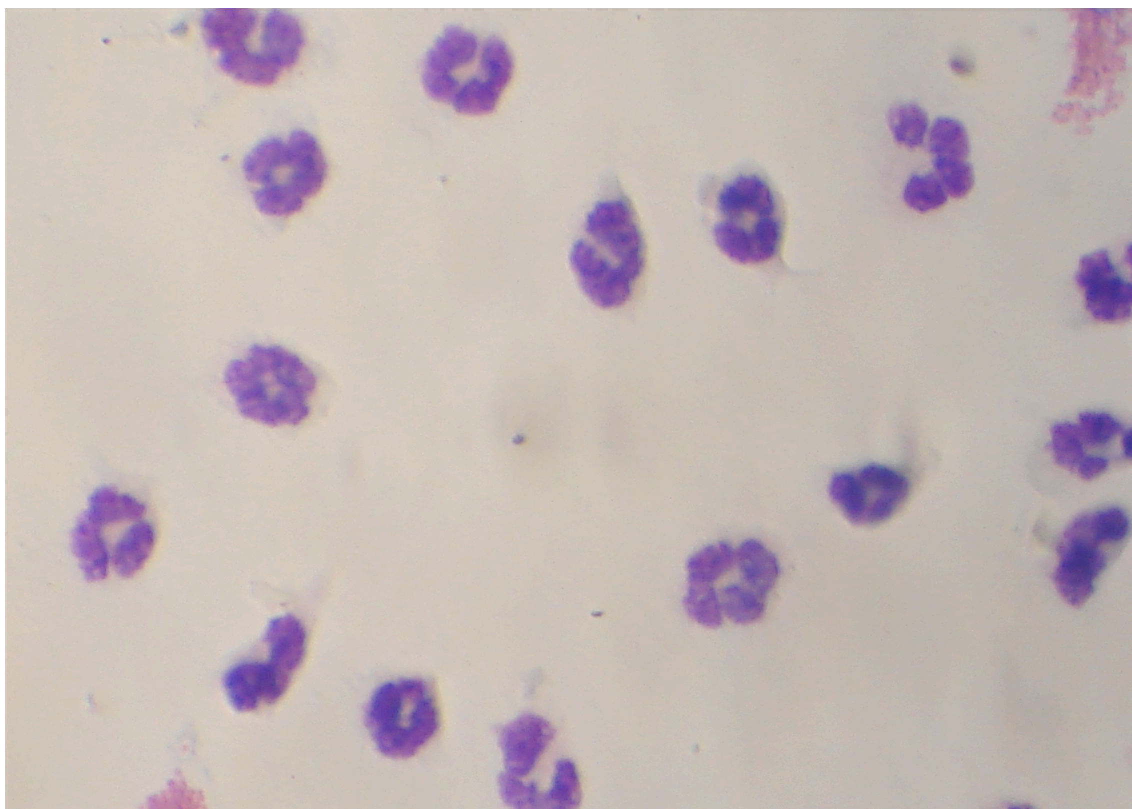


Figura 2: Fotografia mostrando a fagocitose por neutrofilo e monócito de *Saccharomyces Cerevisial* incubadas com soro do indivíduo.

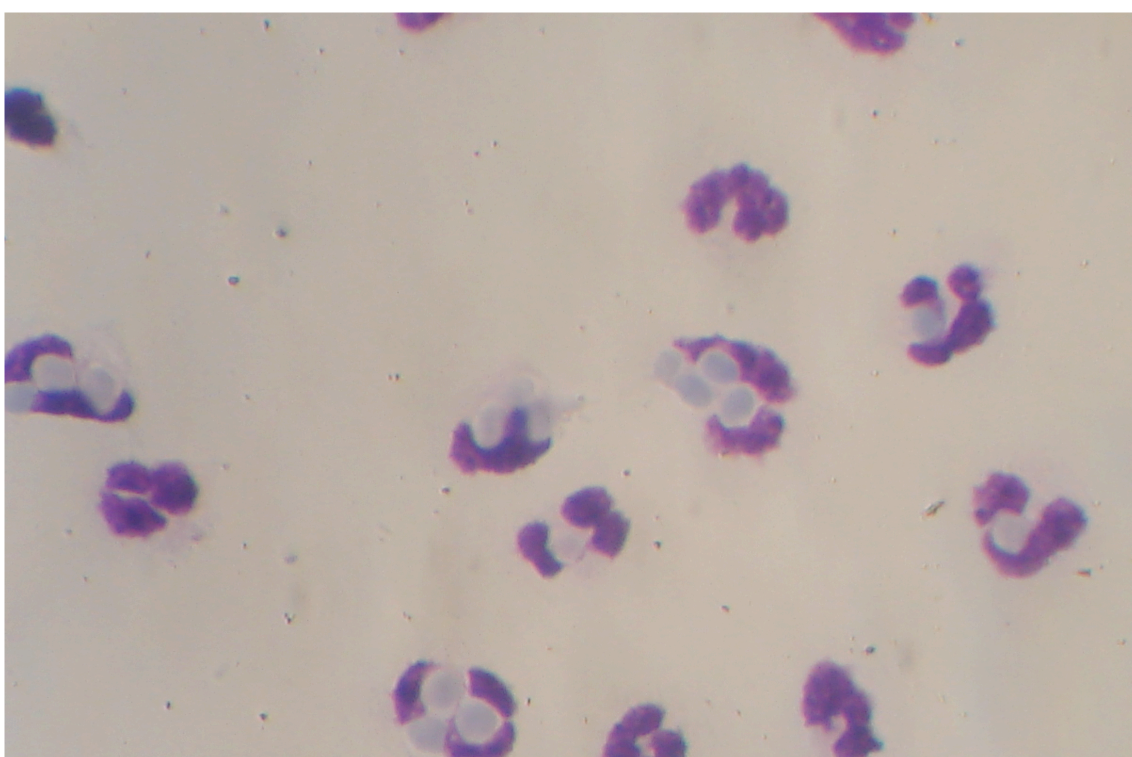


Figura 3: Fotografia mostrando a produção indireta de anion supereróxido. O processo de redução converte o NBT de um composto solúvel em um material insolúvel de coloração azul, visível no citoplasma do fagócito.

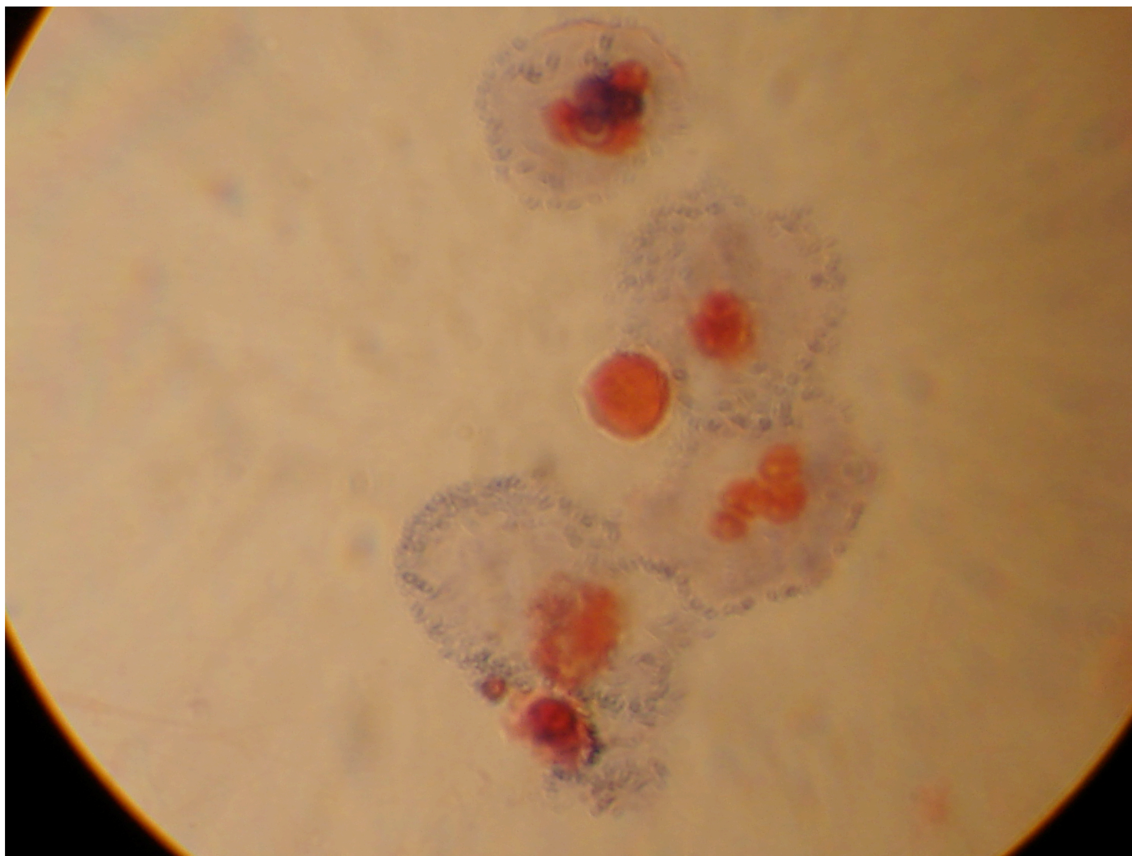
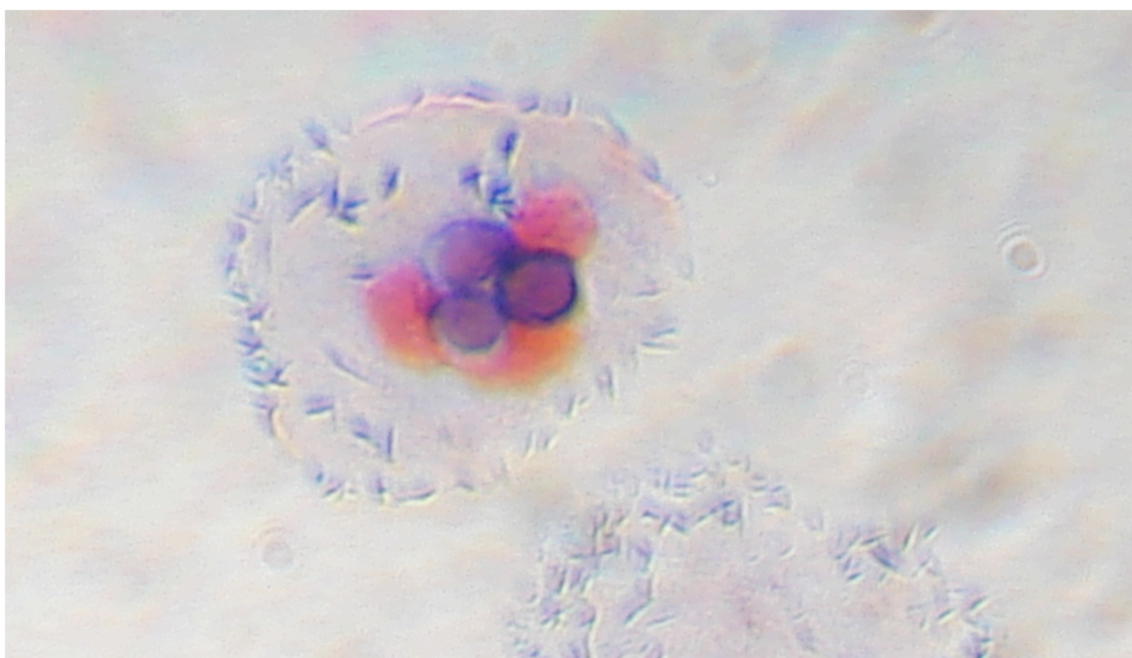


Figura 4: NBT estimulado com *Saccharomyces cerevisial* incubadas com o plasma do indivíduo.



ANEXO E

Carta de Aprovação do Artigo Científico 1Pelo Periódico Journal of Applied Oral Science

Journal of Applied Oral Science

SUMMARY **REVIEW** EDITING

Submission

Authors	Valéria Martins Araújo Carneiro, Ana Cristina Barreto Bezerra, Maria do Carmo Machado Guimarães, Maria Imaculada Muniz-Junqueira
Title	Decreased phagocytic function in neutrophils and monocytes from peripheral blood in periodontal disease
Section	Original Articles
Editor	Carlos Santos

Peer Review

Round 1

Review Version	JAOS-2081-45751-234339-2-RV.DOC 2010-12-17
Initiated	2011-06-06
Last modified	2011-06-21
Uploaded file	None

Editor Decision

Decision	Resubmit for Review 2011-06-12
Notify Editor	Editor/Author Email Record 2011-06-12
Editor Version	JAOS-2081-45751-240460-1-ED.DOC 2010-12-17
Author Version	JAOS-2081-45751-320190-1-ED.DOC 2011-07-01
Upload Author Version	

ANEXO F

Comprovante de resubmissão do Artigo Científico 2 ao Periódico Oral Health and Preventive Dentistry

Oral Health and Preventive Dentistry <ohpd@manuscriptmanager.com>

Assunto: **manuscript - Submission confirmation**

Data: 24 de junho de 2011 17:07:24 BRT

Para: Valeria Araujo <valeriamartins@unb.br>Manuscript title: Effects of Periodontal Therapy on Phagocytic Activity of Peripheral Blood Neutrophils - Evidence of an Extrinsic Cellular Defect

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The above manuscript has been successfully resubmitted online. You will receive a further receipt email directly from the journals editorial office when your submission has been checked and your manuscript files verified.

Oral Health and Preventive Dentistry

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