



UNESP - Universidade Estadual Paulista
Faculdade de Odontologia de Araraquara



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**EFEITO DO L-NAME NA EXPRESSÃO DE iNOS, MPO E NA
PERDA ÓSSEA ALVEOLAR EM RATOS DIABÉTICOS COM
PERIODONTITE EXPERIMENTAL**

Tese apresentada ao Programa de Pós-Graduação em Periodontia da Faculdade de Odontologia de Araraquara, Universidade Estadual Paulista, para obtenção do título de Doutor, em Periodontia.

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Efeito do L-NAME na expressão de iNOS, MPO e na perda óssea alveolar em
ratos diabéticos com periodontite experimental

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RESUMO

Mediadores inflamatórios como mieloperoxidase (MPO) e óxido nítrico (NO) participam do processo inflamatório da doença periodontal e na associação ao diabetes. Inibidores da Óxido Nítrico Sintase (NOS), como o L-NAME têm sido administrados na tentativa de minimizar danos teciduais decorrentes da inflamação. O objetivo deste estudo foi avaliar o efeito do L-NAME sobre os níveis da isoforma induzível de óxido nítrico (iNOS), sobre a perda óssea alveolar e sobre os níveis de mieloperoxidase (MPO) em ratos diabéticos com periodontite induzida. Foram utilizados 192 ratos divididos em grupos de 24 animais cada: grupo C: Controle com ingestão de água; grupo C-L: Controle com ingestão de L-NAME; grupo D: Ratos diabéticos com ingestão de água; grupo D-L: Ratos diabéticos com ingestão de L-NAME; grupo P: Ratos com periodontite experimental e ingestão de água; grupo P-L: Ratos com periodontite experimental e ingestão de L-NAME; grupo DP: Ratos diabéticos com periodontite experimental e ingestão de água; e grupo DP-L: Ratos diabéticos com periodontite experimental e ingestão de L-NAME. O sacrifício dos animais foram realizados aos 3,7,15,e 30 dias após a indução da periodontite experimental. Foram realizadas análises da taxa glicêmica, da perda óssea alveolar, da atividade de mieloperoxidase e da expressão de iNOS. Os grupos com D, P e DP mostraram

níveis de iNOS estatisticamente mais altos quando comparados aos grupos D-L, P-L e DP-L que ingeriram L-NAME em todos os períodos. ($p < 0.05$). Os grupos que ingeriram L-NAME mostraram perda óssea estatisticamente menor quando comparados aos grupos que ingeriram água ($p < 0.05$). A alta expressão de MPO foi observada nos grupos com periodontite P e DP que ingeriram água. Foi verificado um pico nos níveis de MPO aos 7 dias em todos os grupos experimentais que ingeriram água. O uso do L-NAME diminuiu estatisticamente os níveis de MPO de todos os grupos com condições patológicas D, P e DP ($p < 0.05$). Dentro dos limites deste estudo, pode-se sugerir que L-NAME diminui os níveis de iNOS, MPO e consequentemente a perda óssea alveolar em ratos com periodontite, associado ou não ao diabetes.

Palavras chaves: Doença periodontal, diabetes mellitus, óxido nítrico, peroxidases, ratos.

Gomes DAS. Effect of L-NAME on iNOS, MPO expression and alveolar bone loss in diabetic rats with experimental periodontitis (Tese de Doutorado). Araraquara: Faculdade de Odontologia da UNESP; 2009.

ABSTRACT

Inflammatory mediators such as myeloperoxidase (MPO) and nitric oxide (NO) play a role in inflammatory processes related to periodontal disease and diabetes. NO-Synthase (NOS) inhibitors as L-NAME have been administered in attempts to reduce tissue damage resulting from such inflammation. The aim of this study was to evaluate the effects of L-NAME on alveolar bone loss, NO and MPO levels in diabetic rats with experimental periodontitis. A hundred ninety-two rats were divided into one of the following groups with 24 animals each: group C-W: control group with water intake; group C-L: control group with L-NAME intake; group D-W: diabetic rats with water intake; group D-L: diabetic rats with L-NAME intake; group P-W: chronic periodontitis rats with water intake; group P-L: chronic periodontitis rats with L-NAME intake; group DP-W: diabetic chronic periodontitis rats with water intake, and group DP-L: diabetic chronic periodontitis rats with L-NAME intake. The killing was performed at 3, 7, 15 and 30 days after ligature-periodontitis induction to obtain gingival specimens and to evaluate MPO and NO activity and radiographic bone loss. Groups with D-W or P-W and DP-W showed statistically higher iNOS expression compared to L-NAME groups in all periods ($p < 0.05$). L-NAME treatment (L) statistically decreased iNOS expression in all groups with inflammatory pathological

conditions, such as D, P and DP. In general, groups with L-NAME intake showed lower bone loss compared to water intake groups.

In addition, P-L or DP-L rats demonstrated statistically lower bone loss compared to diabetics and controls at 30 days ($p < 0.05$). The highest MPO expression was verified in periodontitis groups with water intake (P-W e DP-W). There was a peak in MPO levels at 7 days in all experimental groups with water intake during experimental evolution. The L-NAME statistically decreased MPO levels from all groups with pathological conditions, D, P and DP ($p < 0.05$). Within the limits of this study, it may be suggest that the L-NAME leads to a decrease the MPO and iNOS levels and alveolar bone loss in of experimental periodontitis alone and in diabetic periodontitis rats.

Key words: Periodontal disease, diabetes mellitus, nitric oxide, peroxidases, rats.

INTRODUÇÃO

O diabetes mellitus é um distúrbio crônico do metabolismo dos carboidratos, lipídios e proteínas, que pode ocorrer devido a uma disfunção das ilhotas pancreáticas (Report of Expert Committee on the Diagnosis and Classification of Diabetes Mellitus⁵⁶, 2003). Esta doença apresenta a hiperglicemia como uma das características mais comuns (Crawford, Cotran 2000¹⁰, Gunczler et al.²⁰, 2001), porém outros distúrbios podem ser verificados, tais como retinopatia, nefropatia, alterações vasculares e prejuízos no processo de cicatrização (Mealey⁴³, 1999).

Além das alterações sistêmicas decorrentes do estado diabético, algumas manifestações bucais também podem ser observadas, como o aumento de prevalência e severidade da doença periodontal e, aumento da predisposição a outras infecções (Crawford, Cotran¹⁰, 2000, Gunczler et al.²⁰, 2001). A relação entre periodontite e diabetes já está bem estabelecida na literatura (Genco et al.¹⁶, 1998, Nishimura et al.⁴⁹, 1998, Lalla et al.³², 2000, Salvi et al.⁵⁸, 2005), sendo que a doença periodontal foi reconhecida como a sexta complicação mais comum do diabetes (Löe³⁸, 1993).

No sítio infectado, o processo inflamatório é capaz de carrear numerosos componentes do sistema complemento, os quais, ativados por via clássica e/ ou alternativa podem liberar fatores quimiotáticos para leucócitos e elementos vasoativos, potencializando a resposta inflamatória que pode estar alterada no estado diabético (Salvi et al.⁵⁷, 1998, Holzhausen et al.²³, 2004). Defeitos na

função e quimiotaxia dos leucócitos polimorfonucleares, alteração da síntese e metabolismo do colágeno, aumento da atividade de collagenases e, acúmulo de produtos finais da glicosilação (AGEs) poderiam atuar sobre receptores de macrófagos e monócitos, resultando em aumento na secreção de mediadores pró-inflamatórios (Nishimura et al.⁴⁹, 1998, Salvi et al.⁵⁷, 1998, Lalla et al.³², 2000, American Academy of Periodontology Committee on Research Science and Therapy³, 2000).

Considerando que a mieloperoxidase (MPO) é uma das enzimas liberadas por células da primeira linha de defesa, principalmente nos casos de periodontites (Gomes et al.¹⁷, 2009) e, também na associação ao diabetes (Gonçalves et al.¹⁸, 2008), os mecanismos envolvidos no processo de inflamação de ambas as doenças têm sido cada vez mais investigados. A MPO apresenta-se de forma abundante em neutrófilos e monócitos, constituindo 5% e 1-2%, respectivamente, do peso seco total dessas células. Está estocada nos grânulos azurófilos primários desses leucócitos e é secretada no meio extracelular e no compartimento fagolisossomal após ativação do fagócito por uma variedade de estímulos, sendo eficiente na morte de microrganismos (Zipfel et al.⁶⁸, 1997, Buchmann et al.⁸, 2002). Por apresentar essas características, alguns estudos na literatura tem usado a quantificação da MPO tecidual, como marcador inflamatório, para estimar o acúmulo de neutrófilos (Yamalík et al.⁶⁷, 2000, Buchmann et al.⁸, 2002, Liskmann et al.³⁷, 2004, Wei et al.⁶⁶, 2004, Gomes et al.¹⁷, 2009).

Um dos principais mediadores inflamatórios que parece exercer um papel fundamental na evolução da doença periodontal e, que também está alterado em

condições de diabetes, é o óxido nítrico (NO) (Moncada, Higgs⁴⁵, 1993, Kröncke et al.³⁰, 1997, Kendall et al.²⁸, 2001). Há evidências científicas que apontam o NO como um dos fatores contribuintes na etiopatogenia da doença periodontal (Rausch-Fan, Matejka⁵³, 2001, Batista et al.⁵, 2002).

O NO é um radical livre produzido a partir da L-arginina pela ação de isoenzimas denominadas NO sintases (NOS). Existem 2 isoformas constitutivas da NOS presente no endotélio (NOSe) e no cérebro (NOSn) e, uma isoforma (NOSi) que está presente em macrófagos e polimorfonucleares, dentre outras células, e é expressa em resposta a estímulos inflamatórios tais como a IL-1, TNF- α , IFN- γ e LPS. A ativação da NOSi pode levar a produção de altas quantidades de NO por um longo período de tempo, sendo considerada de grande importância na resposta imune (Moncada et al.⁴⁶, 1991, Kendall et al.²⁸, 2001).

O NO tem sido considerado uma importante molécula sinalizadora em vários tecidos. Além disso, o NO também pode exercer um papel significativo como mediador citotóxico da resposta imune não-específica, podendo levar a efeitos benéficos ou danosos a fisiopatologia geral dos tecidos (Moncada, Higgs⁴⁵, 1993, Kröncke³⁰ 1997, Kendall et al.²⁸, 2001). Efeitos benéficos compreendem a atividade antimicrobiana e a modulação imune (Allaker et al.², 2001). Por outro lado, efeitos danosos podem incluir uma ação citotóxica contra os tecidos adjacentes do hospedeiro como o osso alveolar (Laurent et al.³⁴, 1996, Lohinai, Sgabó³⁹, 1998).

Além disso, o NO pode modular a atividade de osteoblastos e osteoclastos (Van't Hof, Ratston⁶⁵, 2001). Pesquisas *in vitro* têm demonstrado que o NO

aumenta a síntese de osteocalcina e a formação de matriz mineralizadora pelos osteoblastos enquanto que, a inibição da óxido nítrico sintase (NOS) leva a um efeito antiproliferático dos osteoblastos. Por outro lado, a liberação de grandes quantidades de NO por células estimuladas por citocinas inflamatórias pode levar a um efeito antiproliferático sobre os osteoblastos. Portanto, o NO parece exercer um efeito bifásico sobre as células formadoras de osso: em baixas concentrações ele promove a formação óssea, enquanto que em altas concentrações o NO exerce um efeito inibitório sobre os osteoblastos. De maneira similar, estes efeitos estimuladores e inibitórios têm sido descritos em osteoclastos (Van't Hof, Ratston⁶⁵, 2001).

Em vários modelos experimentais e em amostras humanas de doença periodontal tem sido encontrada uma alta produção local de NO (Lohiani, Sgabó³⁹, 1998, Lohiani et al.⁴⁰, 1998, Kendall et al.²⁸, 2001, Allaker et al.², 2001, Hirose et al.²², 2001). Alguns estudos relacionam a presença do NO com o aumento da reabsorção óssea alveolar (Chaé⁹, 1997, Lohinai, Sgabó³⁹, 1998).

Considerando a ação tecidual destrutiva do NO, alguns estudos têm sugerido o uso de inibidores da NOS na tentativa de controlar os efeitos maléficos do NO, dentre eles pode-se destacar análogos da arginina e agem como inibidores não seletivos da NOS: N-substituídos, como a N^G-monometil-L-arginina (L-NMMA), N-imino-etil-L-ornitina (L-NIO), N^G-amino-L-arginina (L-NAA), N^G-nitro-L-arginina (L-NA) e o metil éster correspondente, o N^G-nitro-L-arginina-metil-éster (L-NAME) (Moncada et al.⁴⁵, 1993, Rees et al.⁵⁴, 1990).

Neste contexto, sugere-se que os mecanismos moleculares envolvidos no processo inflamatório, precisam ser compreendidos, estudados e interligados.

PROPOSIÇÃO

O presente estudo teve como objetivos estudar o efeito do L-NAME sobre os níveis da isoforma induzível de óxido nítrico (iNOS), sobre a perda óssea alveolar induzida por ligadura e sobre os níveis de mieloperoxidase (MPO) em ratos diabéticos com periodontite induzida.

Capítulo 1- Revisão de Literatura

O papel do Óxido Nítrico na modulação do processo inflamatório da doença periodontal.

El papel del Óxido Nítrico en la modulación del proceso inflamatorio de la Enfermedad Periodontal.

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El papel del Óxido Nítrico en la modulación del proceso inflamatorio de la Enfermedad Periodontal.

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Abstract

Nitric oxide (NO) is a free radical with participation in almost all physiologic host processes; however, in high concentrations it may damage the tissues. Its immunoregulatory action is present in the inflammation and in auto-immune mechanisms, being intensively studied in the medical area. Recently, some studies have also reported that NO could play a role as etiopathogenic factor of the periodontal disease, which shows inflammatory and multifactorial course. Due to beneficial or damage effects of NO, according to its concentrations, some studies have been focused in the evaluation of inhibitor of NO-Synthase (NOS) as therapeutic agents in inflammatory processes. In this context, the aim of this study was to report the role of NO and NOS inhibition in the periodontal disease modulation process. In conclusion, it could be suggested that NO seems to play an essential role in evolution of inflammatory periodontal disease, and that the NOS inhibition may be considered as a promising therapeutic in modulation of inflammatory process. *Keywords:* nitric oxide, periodontal diseases, therapeutics.

Resumen

El óxido nítrico (NO) es un radical libre que participa de prácticamente todos los procesos fisiológicos del organismo; sin embargo, en grandes concentraciones puede actuar de forma perjudicial en los tejidos. Su acción inmuno-regulatoria está presente en la inflamación y en los mecanismos de auto inmunidad, siendo intensamente estudiada en el área médica. Recientemente, algunos estudios también han citado al NO como factor etiopatogénico de la enfermedad

periodontal, el cual posee naturaleza inflamatoria y multifactorial. La acción del NO depende de su concentración en el organismo, pudiendo ser benéfica o perjudicial y, basada en eso, investigaciones han enfocado el efecto de algunos inhibidores de NO como supuestos agentes terapéuticos en los procesos inflamatorios. A partir de ese contexto, este trabajo tuvo como objetivo relatar el papel del NO y de la inhibición de la óxido nítrico sintasa (NOS) en el proceso de la modulación de la enfermedad periodontal. En conclusión, se puede sugerir que el NO parece ejercer un papel fundamental en la evolución de la enfermedad periodontal inflamatoria y, que la inhibición de la NOS puede ser considerada una terapéutica promissora en la modulación del proceso inflamatorio. *Palabras clave:* óxido nítrico, enfermedad periodontal, terapéutica.

INTRODUCCIÓN

El óxido nítrico (NO) es un radical libre con múltiples funciones biológicas (1-3). En cantidades fisiológicas, el NO regula el tono vascular, inhibe la agregación/adhesión plaquetaria y regula intracelularmente los efectos de aminoácidos excitatorios (4). Algunos estudios relatan que el NO posee efectos importantes en la función ósea celular y, la presencia de inhibidores de la enzima óxido nítrico sintasa (NOS) estaría actuando como regulador de la remodelación ósea en procesos fisiológicos o patológicos (5, 6). La activación de NO ha demostrado ser relevante en la patogénesis de enfermedades inflamatorias, como por ejemplo, en la enfermedad periodontal. Esta importancia se da por la

ocurrencia de la activación de citocinas pro-inflamatorias, que ocasionan un aumento directo en la producción de NO (7, 8). Además de eso, el NO parece ser un importante regulador del metabolismo óseo, pues tanto los osteoblastos como los osteoclastos no sólo se producen, sino también responden a su presencia (9, 10).

El NO es considerado el principal mediador citotóxico de células inmunes efectoras activadas, constituyéndose como una de las más importantes moléculas reguladoras del sistema inmune (11, 12) y, siendo encontrado en niveles aumentados en el tejido gingival inflamado cuando es comparado al tejido sano (13, 14). Algunos autores (7, 15-17) asocian los niveles elevados de NO con la mayor pérdida ósea alveolar; de manera que este aumento de NO puede ser inhibido por el uso de inhibidores de la NOS. Algunos agentes considerados análogos de la L-arginina actúan como inhibidores de la NOS y, resultados alentadores vienen siendo demostrados, a fin de contribuir futuramente como agentes terapéuticos de varias enfermedades inflamatorias, inclusive para las patologías periodontales.

En los últimos veinte años, el NO ha sido blanco de muchos estudios y está constantemente sorprendiendo a la comunidad científica, dada la cantidad de procesos biológicos en que está envuelto. Frente a tales consideraciones, este trabajo tuvo como objetivo relatar el papel del NO y de la inhibición de la NOS en el proceso de la modulación de la enfermedad periodontal.

REVISIÓN DE LA LITERATURA Y DISCUSIÓN

1. Síntesis de Óxido Nítrico (NO) y Actuación de las isoformas de NO-Sintasa (NOS)

El óxido nítrico (NO) es un radical libre, gaseoso, inorgánico e incoloro, que constituye una de las menores y más simples moléculas bio-sintetizadas en el organismo (18, 19). La síntesis del NO resulta de la oxidación de uno de los dos nitrógenos guanidino del aminoácido L-arginina, que es convertido en L-citrulina, siendo esta reacción catalizada por la enzima NO-sintasa (NOS) (20, 21).

Una variedad de isoformas de NOS ha sido purificada en diferentes tejidos de mamíferos y muchas ya tuvieron sus genes clonados. Estudios bioquímicos y de análisis secuencial de aminoácidos revelan que estas isoformas representan una familia de proteínas y, aparentemente, son productos de genes distintos. Así, las isoformas de la NOS son agrupadas en dos categorías, la NOS constitutiva (cNOS) y la NOS inducible (iNOS). La cNOS dependiente de iones calcio (Ca^{++}) y de calmodulina, que están envueltos en la señalización celular, mientras que la iNOS es producida por macrófagos y otras células activadas por citocinas (21, 22).

La isoforma constitutiva comprende la NOS neuronal (nNOS, tipo I), presente normalmente en las neuronas (23, 24), y la NOS endotelial (eNOS, tipo III), presente normalmente en las células endoteliales vasculares (21) y en las plaquetas (25). El NO resultante de la eNOS tiene un papel crucial en la protección del vaso sanguíneo y esta acción está asociada al mantenimiento del

tono vascular (26, 27), regulación de la presión sanguínea (27), prevención de la agregación plaquetaria (28), inhibición de la adhesión de monocitos y neutrófilos al endotelio vascular (2), efecto antiproliferativo (29, 30) y efecto antioxidativo (31).

Mientras la eNOS esta expresa fisiológicamente en el organismo, la iNOS no está expresa sobre condiciones normales, siendo inducida por citocinas y/o endotoxinas como IL-1, TNF- α , IFN- γ y LPS en una variedad de células, incluyendo macrófagos, linfocitos T, células endoteliales, miocitos, hepatocitos, condrocitos, neutrófilos y plaquetas (4, 21). Esta isoforma requiere algunas horas para ser expresa, por lo tanto, una vez sintetizada, libera cantidades mayores de NO que la cNOS. Así, la producción de iNOS continúa indefinidamente hasta que la L-arginina o los co-factores sintetizantes sean cesados, o hasta que ocurra la muerte celular (32).

El NO resultante de la activación de la iNOS posee acción citotóxica y citostática, promoviendo la destrucción de microorganismos, parásitos y células tumorales (4, 5, 33). La citotoxicidad del NO resulta de su acción directa o de su reacción con otros compuestos liberados durante el proceso inflamatorio, pudiendo incluir una acción citotóxica contra los tejidos adyacentes del huésped, como por ejemplo, al hueso alveolar (34). En procesos infecciosos, las células activadas como macrófagos, neutrófilos y células endoteliales secretan, simultáneamente, NO e intermediarios reactivos del oxígeno, siendo que la acción

citotóxica indirecta del NO consiste, principalmente, en su reacción con esos intermediarios del oxígeno.

Estudios relatan que el NO es capaz de modular la actividad de osteoblastos y osteoclastos (6). Así, el NO parece ejercer un efecto bifásico sobre las células formadoras de hueso, pues en bajas concentraciones promueve la formación ósea, mientras que en altas concentraciones ejerce un efecto inhibitorio sobre los osteoblastos. Investigaciones *in vitro* (6) han demostrado que el NO aumenta la síntesis de osteocalcina y la formación de matriz mineralizada por los osteoblastos, mientras que la inhibición de la NO sintasa lleva a un efecto antiproliferativo de los osteoblastos. Por otro lado, la liberación de grandes cantidades de NO por células estimuladas por citocinas inflamatorias puede llevar a un efecto antiproliferativo sobre los osteoblastos. De manera similar, estos efectos estimuladores e inhibitorios han sido descritos en osteoclastos (6).

Evidencias han sugerido que el NO puede contribuir significativamente para algunas condiciones patológicas como asma (35), artritis reumatoide (36), lesiones ateroscleróticas (37), tuberculosis (38), esclerosis múltiple (39), Alzheimer (40), gastritis inducida por *Helicobacter pylori* (41), diabetes (8) y enfermedad periodontal (42). En la evolución de la enfermedad periodontal inflamatoria, el NO parece ser de fundamental importancia pues, su alta producción local ha sido encontrada en varios modelos experimentales y en muestras de tejidos gingivales de humanos (13, 14, 34, 43). De esa forma, algunos

estudios relacionan directamente la presencia del NO con el aumento de la reabsorción ósea alveolar (7, 34).

2. NO en la Enfermedad Periodontal

La enfermedad periodontal es caracterizada como un proceso inflamatorio de origen bacteriano primario, pero que presenta etiología multifactorial (44). La gingivitis y periodontitis son tradicionalmente las dos principales categorías de enfermedad periodontal, por ello, la gingivitis es una enfermedad reversible que acomete al periodonto de protección de los dientes (45), mientras que la periodontitis envuelve al periodonto de soporte de los dientes, tales como cemento radicular, ligamento periodontal y hueso alveolar (46). Esas enfermedades se presentan de forma muy variada, pudiendo tener períodos de actividad intensa y de estancamiento o remisión, siendo que su evolución está directamente relacionada a los tipos de agresores microbianos envueltos y a la respuesta inmunológica del individuo (47, 48).

La inflamación es un mecanismo de protección del huésped a desafíos externos, pero puede ocasionar desde una lesión periodontal hasta la pérdida de la función dental en caso de no ser controlada. Por otro lado, muchas respuestas inflamatorias son limitadas o resueltas por sí solas, sugiriendo la existencia de mediadores antiinflamatorios endógenos como IL-1 β , TNF- α , IFN- α , PGE2 durante el curso de la inflamación (14, 49). Estudios demostraron que el óxido nítrico (NO), en cantidades fisiológicas, puede ser considerado un importante mediador del proceso inflamatorio, regulando el tono vascular, inhibiendo la

agregación y adhesión plaquetaria, o mediando los efectos de aminoácidos excitatorios intracelularmente (6, 50).

En varios modelos experimentales y en muestras humanas de enfermedad periodontal ha sido encontrada una alta producción local de NO (13, 14, 34, 43, 44). Estudios experimentales mostraron que el óxido nítrico proveniente de la iNOS induce pérdida ósea por medio de diferenciación osteoclástica durante el desarrollo óseo y, también después de la infección bacteriana en periodontitis inducida por *Porphyromonas gingivalis* en ratones (51, 52). Otros autores también relacionan la presencia del NO con el aumento de la reabsorción ósea alveolar (7, 14, 34, 53).

Autores sugieren que el aumento en la producción de NO observado en el tejido gingival de humanos estaría relacionado a la elevación en las concentraciones de los aminoácidos L-arginina y L-citrulina, paralelo a un aumento de prostaciclina (54). Además, Hesse et al. (55) considera otras vías metabólicas en las cuales la L-arginina y la L-citrulina estarían envueltas, como la vía del ciclo de la urea, en la cual la expresión de la enzima arginasa se encuentra aumentada en procesos inflamatorios. Otros autores también verificaron que el aumento de NO en la periodontitis ocasiona una elevación en la concentración de iNOS en sitios con la enfermedad (44, 56-58). Hirose et al. (14) evaluaron la expresión de la isoforma iNOS por medio de RT-PCR, en muestras de tejido gingival de pacientes con periodontitis y sanos y, observaron que los niveles de iNOS estaban aumentados en muestras de tejido gingival inflamado. De esa

forma, se sugiere que la iNOS puede estar envuelta en el proceso de inflamación periodontal. Por otro lado, autores como Skaleric et al. (8) no encontraron expresión de iNOS en tejido gingival no inflamado, de pacientes sanos o diabéticos.

Además, niveles aumentados de NO también fueron verificados en la saliva de pacientes con periodontitis crónica severa, en comparación a los pacientes con periodontitis moderada o no portadores de enfermedad periodontal (59). Por el contrario, Auer et al. (42) relataron que las concentraciones de NO en la saliva de pacientes con periodontitis crónica o agresiva fueron menores en comparación a los individuos sanos.

3. Inhibidores de NOS en la enfermedad periodontal

Todas las isoformas de NOS pueden ser inhibidas por análogos de la arginina N-sustituídos, como la N^G-monometil-L-arginina (L-NMMA), N-imino-etil-L-ornitina (L-NIO), N^G-amino-L-arginina (L-NAA), N^G-nitro-L-arginina (L-NA) y el metil-éster correspondiente, y el N^G-nitro-L-arginina-metil-éster (L-NAME). Estos análogos compiten con la L-arginina y reaccionan como inhibidores estereoespecíficos de la NOS (21, 60). Además de estos inhibidores, la aminoguanidina es también capaz de inhibir la NOS y presenta una relativa selectividad para iNOS (15). Otros inhibidores considerados selectivos para nNOS e iNOS son denominados análogos de 7-nitroindazol y mercaptoetilguanidina, respectivamente (34, 61).

El uso de inhibidores de la NOS han mostrado un efecto benéfico en varias patologías. En la artritis experimentalmente inducida en animales, ocurre una atenuación de los signos clínicos e histológicos de la enfermedad (62). En la glomerulonefritis, hay una disminución de la deposición de complejos inmunes en los riñones, llevando a una considerable mejoría clínica (63). En la diabetes dependiente tipo 1, inducida inmunológicamente, estudios demostraron que la destrucción de las células β pancreáticas es mediada por el NO y las primeras tentativas de inhibir la iNOS fueron animadoras (33). En el trasplante renal, fue obtenida una mejoría substancial de los signos clínicos de rechazo después de la inhibición selectiva de la iNOS (63). En el choque séptico, la inhibición de la iNOS también fue benéfica (15).

La inhibición de la NOS ha sido estudiada para reducir la reabsorción ósea en animales y para controlar el proceso inflamatorio (16, 17). Los resultados encontrados demostraron una disminución de los niveles de pérdida ósea en animales con periodontitis inducida, (7, 34). Esos relatos sugieren que los inhibidores específicos de la NOS pueden ser utilizados como tratamiento y/o prevención de enfermedades sistémicas (64), además de participar como agentes terapéuticos en la inflamación periodontal, reduciendo la pérdida ósea alveolar (7). Frente a tales consideraciones, otros estudios experimentales y ensayos clínicos son necesarios para evidenciar claramente los beneficios y posibles efectos colaterales del uso de inhibidores selectivos de la NOS en la enfermedad periodontal.

CONCLUSIÓN

Conociendo la enfermedad periodontal como una enfermedad multifactorial y de carácter inflamatorio, se puede concluir que el NO está relacionado al mecanismo inmunológico de la respuesta del huésped, pudiendo participar directamente en el proceso de reabsorción ósea alveolar. Resultados alentadores con la utilización de inhibidores de la NOS vienen siendo estudiados, a fin de contribuir futuramente como agentes terapéuticos de varias enfermedades inflamatorias, inclusive para las patologías periodontales.

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Capítulo 2 – Artigo Experimental

Effect of Nitric Oxide Synthase inhibitor on alveolar bone loss of diabetic and non-diabetic chronic periodontitis rats.

Efeito do inibidor da óxido nítrico sintase sobre a perda óssea alveolar de ratos diabéticos e não diabéticos com periodontite crônica.

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Effect of a Nitric Oxide Synthase inhibitor on alveolar bone loss of diabetic and non-diabetic chronic periodontitis rats.

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Keywords: nitric oxide, nitric oxide synthase, diabetes mellitus, periodontal
diseases.

ABSTRACT

Background: The role of nitric oxide (NO) in the pathogenesis of periodontitis and diabetes has not been fully elucidated. The aim of this study was to evaluate the effects of L-NAME on alveolar bone loss in diabetic and non-diabetic periodontitis rats.

Methods: A hundred ninety-two rats were divided into one of the following groups with 24 animals each: group C-W: control group with water intake; group C-L: control group with L-NAME intake; group D-W: diabetic rats with water intake; group D-L: diabetic rats with L-NAME intake; group P-W: chronic periodontitis rats with water intake; group P-L: chronic periodontitis rats with L-NAME intake; group DP-W: diabetic chronic periodontitis rats with water intake, and group DP-L: diabetic chronic periodontitis rats with L-NAME intake. The sacrifices were performed at 3, 7, 15 and 30 days after ligature-induced periodontitis to assess radiographic findings and iNOS inhibition in gingival tissues.

Results: Groups with D-W or P-W and DP-W showed significantly higher iNOS expression compared to L-NAME groups in all periods. L-NAME treatment (L) significantly decreased iNOS expression in all groups with inflammatory pathological conditions ($p < 0.05$). In general, groups with L-NAME intake showed lower bone loss compared to water intake groups. In addition, P-L or DP-L rats demonstrated statistically lower bone loss compared to diabetics and controls at 30 days ($p < 0.05$).

Conclusion: It may be suggest that L-NAME leads to a decrease the iNOs levels and alveolar bone loss in of experimental periodontitis alone and in diabetic periodontitis rats.

1. Introduction

Nitric oxide (NO) is a gas with several biological activities and is produced from L-arginine by nitric oxide synthase (NOS). A major role for NO has been suggested in immune regulation and inflammation [1]. Different isoforms of NOS can be verified, such as constitutive endothelial (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). The iNOS pathway is primarily regulated at the level of transcription, and its activation produces a larger amount of NO for an extended period of time [2]. Production of a large amount of NO by iNOS has been shown to play an important role in immune reactions and inflammatory processes, including the pathogenesis of periodontal diseases [3-5].

iNOS can be expressed upon stimulation by proinflammatory cytokines such as interleukin-1 β , tumor necrosis factor - α and interferon - γ [6-9], while glucocorticoids and the anti-inflammatory cytokines interleukin-4, IL-10 and transforming growth factor- β suppress NO production [2,10]. The role of NO in the pathogenesis of periodontitis has not been fully elucidated. It has been verified that low levels of NO appear to be essential to human osteoblast metabolism [11], but an increase in NO levels during inflammatory reactions may result in tissue damage [12]. Thus, the physiological role of NO can be established by inhibition of NOS by some agents, such as L-Arginine Methyl Ester (L-NAME) [2,13].

On the other hand, NO is among the endothelial mediators that are released by endothelial cells in response to insulin stimulation, and it is the most

important vasodilator that is generated by these cells. Therefore, metabolic abnormalities, such as insulin resistance, dyslipidemia, compensatory hyperinsulinemia and overt hyperglycemia, might all contribute to impaired NO bioavailability and abnormal vasodilatation in diabetic patients [14]. Drugs that are utilized for hypertension or diabetes can already limit oxidation, reduce the formation of the advanced glycation end product (AGE), or interact with the receptor for AGE (RAGE), but new treatment by NO modulators may limit the deleterious effect of red blood cell adhesion to the endothelium [15].

Abnormal NO synthesis has been implicated in the pathogenesis of both periodontitis and diabetes mellitus. Increased iNOS expression in diabetic inflamed gingiva was correlated with NO in your gingival crevicular fluid in diabetic patients [16]. Thus, it was hypothesized that biochemical changes associated with diabetes may modulate NO expression in sites with chronic periodontitis. The aim of this study was to evaluate the inhibition of iNOS expression in gingival tissues to verify the role of NO on the severity of alveolar bone loss of diabetic and non-diabetic periodontitis rats.

2. Material and Methods

2.1. Experimental design

The protocol of this study was in accordance with guidelines approved by the Institutional Experimentation Committee of the School of Dentistry at Araraquara, São Paulo, Brazil (process 28/2003). One hundred and ninety-two

young male Wistar rats (*Rattus norvegicus albinus*), weighing approximately 140 ± 8.3 g, were used in this study. The animals were housed under a 12:12h light and dark cycle at 22-25 °C, in cages in a temperature-controlled room, were fed with a standard laboratory diet and were given tap water ad libitum or an inhibitor of NOS, L-arginine methyl ester (L-NAME).

The animals were divided into one of the following groups, with 24 animals each: 1) group C-W, control group with water intake; 2) group C-L, control group with L-NAME intake; 3) group D-W, diabetic rats with water intake; 4) group D-L, diabetic rats with L-NAME intake; 5) group P-W, chronic periodontitis rats with water intake; 6) group P-L, chronic periodontitis rats with L-NAME intake; 7) group DP-W, diabetic chronic periodontitis rats with water intake; and 8) group DP-L, diabetic chronic periodontitis rats with L-NAME intake. Twenty-four hours after the diabetic status was checked, the day of periodontitis induction was considered baseline (day 0). The killing was performed at 3, 7, 15 and 30 days after baseline by guillotine, and 6 rats per group were sacrificed in each experimental period. The chronology of the experimental period can be observed in Figure 1. Plasma glucose level was verified in each period, and hemimandibles were removed, to assess radiographic findings and iNOS inhibition by L-NAME in specimens of gingival tissues.

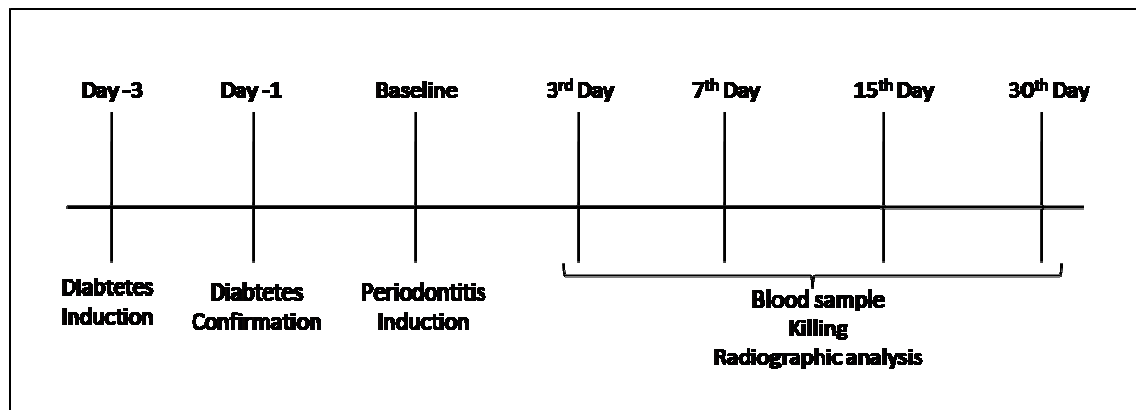


Fig. 1. Experimental chronology

2.2. Diabetes induction method

Diabetes mellitus was induced by a single injection of 50 mg/kg body weight of streptozotocin (STZ) (Sigma, St. Louis, MO, USA) dissolved in sodium citrate buffer (0.01 M; pH 4.5) into the jugular vein [17,18]. Diabetes was induced in rats of groups D-W, D-L, DP-W and DP-L, while the non-diabetic animals (C-W, C-L, P-W and P-L) were injected with a saline solution (0.15 ml) as a sham treatment. Figure 1 outlines the experimental protocol.

2.3. Blood glucose analysis

Blood samples were obtained from the tip of the tail of rats through a scissor cut made approximately 2 mm from the distal extremity. The samples were collected in Eppendorf tubes containing liquaemin sodium (Liquemine®, La Roche Ltd, Basel, Switzerland). After centrifugation, the plasma was separated to assess the glucose level by a glucose analyzer (Autoanalyzer, Technicom RA-XT,

Bayer, Dublin, Ireland). A plasma glucose level greater than 300 mg/dl confirmed the presence of diabetes [17,18].

2.4. Periodontitis induction

Animals from groups P-W and P-L received general anesthesia with an intraperitoneal injection of Ketamine 0.08 ml/100 g of body weight (Francotar®, Virbac do Brasil Ind. e Com. LTDA, São Paulo, Brazil) and 0.04 mL of Xylazine hydrochloride 0.04 ml/100 g of body weight (2% Virbaxyl, Virbac do Brasil Ind. e Com. LTDA, São Paulo, Brazil). Then a cotton ligature (corrente #24) was placed around the cervix of the left lower first molar in a submarginal position, to induce experimental periodontitis [19,20]. The procedure was performed with modified tweezers and an explorer that were sterilized.

2.5. Drug treatment

The treatment groups received L-NAME (30 mg/kg/dia) in the water provided to the animals, 1 hour before diabetes induction and then daily until sacrifice at 3, 7, 15 and 30 days. Two hundred mg of L-NAME was diluted in 1 L of water and was administered daily to the rats.

2.6. RNA Extraction and Real-time PCR

Gingival tissues were submitted to RNA extraction with Trizol reagent (Gibco BRL, USA). Then it was placed into test tubes (1 ml for 50 mg tissue), homogenized for 30 seconds and stored at room temperature for 5 minutes. For each 1 ml, 0.2 ml of chloroform was added (Sigma), and the sample was

centrifuged at 13,000 rpm for 15 minutes at 4°C. The aqueous phase was placed in another tube, isopropanol of the same volume was added, the sample was mixed by a “vortex”, and then incubated for 20 minutes at -20° to precipitate RNA. Then the tubes were centrifuged at 13,000 rpm for 15 minutes at 4°C, and the precipitated phase was washed in 100% ethanol and dried at room temperature. The RNA samples were suspended in 50 µl deionized water and placed at -70° C. Five microliters were utilized to obtain the concentration of each sample in RNA/µl, using Gene Quant (Pharmacia, USA). Complementary DNA (cDNA) was synthesized by reverse transcription (Superscript II, Gibco Life Tech.). Real-time PCR analysis was performed in GeneAmp 5700 (Applied Biosystems – USA) with the SYBR-green fluorescence quantification system (Applied Biosystems, Warrington, UK). Standard PCR conditions were 50°C (2 min.), 95°C (10 min.), 40 cycles of 95°C (15 sec.), and then at 60°C (1 min.), followed by the standard denaturation curve.

2.7. Alveolar bone loss measurement

Standardized digital radiographs were obtained with the use of a computerized imaging system, CDR® (Computed Dental Radiography for Microsoft Windows, Schick Technologies, Inc., Dialom Dental Products, USA). Electronic sensors were exposed at 70 kV and 10 mA, with an exposition of 0.2 seconds. The source-to-film distance was always set at 50 cm. The distance from the alveolar bone crest to the cemento–enamel junction (2 fixed points) was measured radiographically for each mesial surface of the left lower first molars of

each rat by Sigma-Scan 2.0 software (Jandel Corporation, San Raphael, USA). Three linear measurements (in millimeters) were obtained for each X-ray by the same examiner (blinded and gauged), based on the work of Nassar et al. [20].

2.8. Statistical Analyses

Data analyses were performed using the BioStat 5.0 software (Optical Digital Technology, Belém, PA, Brazil). All of the data were analyzed for distribution by the Shapiro-Wilk test. Comparisons among periods in each group were performed by one-way ANOVA followed by Tukey's test. Analyses among all of the groups in each experimental period were also performed by one-way ANOVA and Tukey's test. Comparisons between the groups with Water or L-NAME intake were analyzed by Mann-Whitney's test at a significance level of 5%.

3. Results

Diabetic animals (D-W, D-L, DP-W and DP-L) maintained glycemic values over 480 mg/dl up to the end of the experimental period, independently of ligature-induced periodontitis condition.

3.1. iNOS expression

The groups with diabetes (D) or periodontitis (P) alone, and diabetes combined with experimental periodontitis (DP) with water intake (W), showed significantly higher iNOS expression compared to the L-NAME groups at all of

the time periods ($p < 0.05$). During progression of periodontitis, the highest iNOS expression was statistically significant in the P-W group at 30 days (6.2 ± 0.4) compared to 3 days (4.7 ± 0.7), 7 days (5.4 ± 0.3) and 15 days (4.9 ± 0.7). The same behavior was verified in the DP-W group, which presented the highest iNOS expression at 30 days (6.8 ± 0.7), compared to 3 days (4.9 ± 0.1), 7 days (5.9 ± 0.5) and 15 days (5.5 ± 0.1) (Table 1).

The evolution of inflammatory processes in groups with L-NAME intake could be verified with statistically significant differences in the D-L, P-L and DP-L groups at 30 days (D-L: 3.5 ± 0.4 , P-L: 4.1 ± 0.6 , DP-L: 4.6 ± 0.1) compared to the initial periods of 3 days (D-L: 2.6 ± 0.4 , P-L: 2.6 ± 0.5 , DP-L: 3.4 ± 0.3) and 7 days (D-L: 2.3 ± 0.5 , P-L: 3.4 ± 0.3 , DP-L: 3.8 ± 0.6) (Table 1). The decrease in iNOS expression was statistically significant with L-NAME treatment (L) in all of the groups with inflammatory pathological conditions, such as D, P and DP ($p < 0.05$) (Table 1). This fact may suggest there is a beneficial effect of L-NAME treatment on inflammatory reduction.

Table 1

Mean $\pm$ Standard deviation of iNOS expression in gingival tissue of the control and test groups.

Groups	Periods			
	3 days	7 days	15 days	30 days
C-W	$3.5 \pm 0.3^{a\alpha}$	$3.6 \pm 0.2^{a\alpha}$	$3.4 \pm 0.4^{a\alpha}$	$3.9 \pm 0.1^{a\alpha*}$
C-L	$3.2 \pm 0.1^{a\alpha}$	$3.2 \pm 0.7^{a\alpha}$	$2.9 \pm 0.6^{a\alpha}$	$2.1 \pm 0.2^{a\beta}$
D-W	$4.1 \pm 0.6^{a\beta*}$	$4.2 \pm 0.6^{a\varphi*}$	$4.9 \pm 0.4^{a\beta*}$	$4.5 \pm 0.5^{a\gamma*}$
D-L	$2.6 \pm 0.4^{a\gamma}$	$2.3 \pm 0.5^{a\gamma}$	$3.5 \pm 0.5^{b\alpha}$	$3.5 \pm 0.4^{b\alpha}$
P-W	$4.7 \pm 0.7^{a\varphi*}$	$5.4 \pm 0.3^{a\beta*}$	$4.9 \pm 0.7^{a\beta*}$	$6.2 \pm 0.4^{b\varphi*}$
P-L	$2.6 \pm 0.5^{a\gamma}$	$3.4 \pm 0.3^{a\alpha}$	$3.6 \pm 0.3^{a\alpha}$	$4.1 \pm 0.6^{b\gamma}$
DP-W	$4.9 \pm 0.1^{a\varphi*}$	$5.9 \pm 0.5^{a\beta*}$	$5.5 \pm 0.1^{a\beta*}$	$6.8 \pm 0.7^{b\varphi*}$
DP-L	$3.4 \pm 0.3^{a\alpha}$	$3.8 \pm 0.6^{a\alpha}$	$3.7 \pm 0.4^{a\alpha}$	$4.6 \pm 0.1^{b\gamma}$

C: Control group; D: Diabetic group; P: Periodontitis group; DP: Diabetes and Periodontitis group; W: Water intake; L: L-name intake.

^{a, b} Different superscript letters in the rows indicate statistically significant differences among periods in each experimental group by one-way ANOVA's test followed by Tukey's test ($p < 0.05$).

^{α, β} Different superscript symbols in the columns indicate statistically significant differences among all of the groups by one-way ANOVA's test followed by Tukey's test ($p < 0.05$).

* Indicate statistically significant differences between each treatment group (Water or L-NAME) by Mann-Whitney's test ($p < 0.05$).

3.2. Radiographic data

Groups with periodontitis alone (P), or diabetes combined with periodontitis (DP) with water intake (W), showed higher alveolar bone loss in periods later than 15 days (P: 3.7 ± 0.3 , DP: 4.5 ± 0.4) and 30 days (P: 3.9 ± 0.1 , DP: 4.7 ± 0.2) compared to 3 days (P: 2.3 ± 0.2 , DP: 2.7 ± 0.3) and 7 days (P: 2.9 ± 0.8 , DP: $3.1 \pm$

0.5) ($p < 0.05$). Group D-W presented statistically significant alveolar bone loss at 30 days (3.2 ± 0.2) compared to the other periods (3 days: 1.8 ± 0.5 , 7 days: 2.6 ± 0.5 and 15 days: 2.9 ± 0.2). It is likely that the progressive bone loss may be caused by the severity of the diabetes condition.

Considering the treatment with the NOS inhibitor, the bone loss in the groups with periodontitis alone (P-L), or in association with diabetes (DP-L) was significantly higher than that of the groups without periodontitis (C-L and D-L) in the 15 (P-L: 3.4 ± 0.2 ; C-L: 2.8 ± 0.3 ; DP-L: 3.7 ± 0.3 ; D-L: 1.9 ± 0.1) and 30 (P-L: 3.6 ± 0.1 ; C-L: 2.9 ± 0.4 ; DP-L: 4.0 ± 0.1 ; D-L: 3.0 ± 0.4) days. It is possible to note that groups treated with L-NAME showed the same pattern which the respective water intake groups in these periods experimental. However, in general, the groups treated with L-NAME (P-L: 3.6 ± 0.1 ; DP-L: 4.0 ± 0.1) showed a significantly lower bone loss compared to the water intake groups (P-W: 3.9 ± 0.1 ; DP-W: 4.7 ± 0.2) when the periodontitis was established (at 30 days) (Table 2).

Table 2

Mean $\pm$ Standard deviation of alveolar bone loss (in millimeters) in the control and test groups.

Groups	Periods			
	3 days	7 days	15 days	30 days
C-W	$1.5 \pm 0.3^{a\alpha*}$	$2.4 \pm 0.5^{b\alpha}$	$2.6 \pm 0.4^{b\alpha}$	$3.0 \pm 0.3^{b\alpha}$
C-L	$0.9 \pm 0.35^{a\beta}$	$1.9 \pm 0.7^{b\alpha}$	$2.8 \pm 0.3^{c\alpha}$	$2.9 \pm 0.4^{c\alpha}$
D-W	$1.8 \pm 0.5^{a\alpha}$	$2.6 \pm 0.5^{a\alpha}$	$2.9 \pm 0.2^{a\alpha*}$	$3.2 \pm 0.2^{b\alpha}$
D-L	$1.5 \pm 0.4^{a\alpha}$	$1.9 \pm 0.9^{a\alpha}$	$1.9 \pm 0.1^{a\beta}$	$3.0 \pm 0.4^{b\alpha}$
P-W	$2.3 \pm 0.2^{a\gamma*}$	$2.9 \pm 0.8^{a\alpha}$	$3.7 \pm 0.3^{b\gamma}$	$3.9 \pm 0.1^{b\beta*}$
P-L	$1.45 \pm 0.8^{a\alpha}$	$2.0 \pm 0.7^{a\alpha}$	$3.4 \pm 0.2^{b\gamma}$	$3.6 \pm 0.1^{b\gamma}$
DP-W	$2.7 \pm 0.3^{a\gamma*}$	$3.1 \pm 0.5^{a\alpha}$	$4.5 \pm 0.4^{b\varphi*}$	$4.7 \pm 0.2^{b\varphi*}$
DP-L	$1.7 \pm 0.1^{a\alpha}$	$2.6 \pm 0.5^{b\alpha}$	$3.7 \pm 0.3^{c\gamma}$	$4.0 \pm 0.1^{c\beta}$

C: Control group; D: Diabetic group; P: Periodontitis group; DP: Diabetes and Periodontitis group; W: Water intake; L: L-name intake.

^{a, b} Different superscript letters in the rows indicate statistically significant differences among periods in each experimental group by one-way ANOVA's test followed by Tukey's test ($p < 0.05$).

^{α, β} Different superscript symbols in the columns indicate statistically significant differences among all of the groups by one-way ANOVA's test followed by Tukey's test ($p < 0.05$).

* Indicate statistically significant differences between each treatment group (Water or L-NAME) by Mann-Whitney's test ($p < 0.05$).

4. Discussion

Diabetes induction by streptozotocin promoted a selective destruction of pancreatic β cells, thereby decreasing insulin secretion and increasing the glucose concentration in the total blood [21-23].

Previous authors have verified that NO plays an active role in the pathogenesis of inflammatory processes, such as periodontitis [4, 24-28] and diabetes [28-32]. In addition, Lohinai et al. [12] showed that an increase in NO levels during inflammatory reactions may result in tissue damage. Work by Pan et al. [28] demonstrated that iNOS expression was more prominent in the gingiva of diabetic chronic periodontitis patients, and our results also show higher iNOS expression in diabetic chronic periodontitis rats.

These results may be a result of the chronic periodontitis condition, which may promote inflammatory processes and exacerbate secretion of pro-inflammatory mediators that play a role in tissue destruction [33-36]. In addition, the pro-inflammatory cytokines, such as interleukin-1 β , tumor necrosis factor alpha and interferon gamma, may be involved on the exacerbated expression of iNOS [6-8].

Previous authors have suggested that the elevated NOS activity observed in diabetic patients was due to the diabetes [37]. Similarly, Honing et al. [38] suggested that high oxidative stress was present in diabetic patients. The authors explained that this fact may be due hyperglycemia, which stimulates production of advanced glycosylated end products and consequently activates protein kinase C.

This fact may explain the high iNOS expression seen in groups with diabetes (D-W) compared to the control group (C-W) and also seen principally in groups with an association between diabetes and periodontal disease (DP-W), which also showed higher bone loss.

In an attempt to reduce the malicious effects of NO, some studies have suggested the use of NO-Synthase (NOS) inhibitors [2,13,39]. Moreover, NO inhibition may decrease tissue and systemic reactions related to periodontitis [13]. Other authors have suggested that lower levels of NO appear to be essential to the metabolism of human osteoblasts and osteoclasts [11,40]. Along these same lines, our findings demonstrated a reduction of alveolar bone loss during periodontitis progression in groups treated with the NOS inhibitor (L-NAME). In addition, L-NAME treatment significantly decreased iNOS expression in diabetes (D-L) or periodontitis alone (P-L), and in diabetic periodontitis rats (DP-L). Similarly, Leitão et al. [13] suggested that the nitric oxide synthase inhibitor, L-NAME, reduced alveolar bone loss and prevented inflammatory bone resorption in experimental periodontitis.

Within the limits of this study, the L-NAME treatments lead to a decrease in iNOS levels and a consequent reduction on alveolar bone loss in rats with experimental periodontitis alone and in association with diabetes. Therefore, we suggest that NOS inhibition could be used as a possible therapeutic agent to prevent tissue destruction. However, future studies must be performed to better elucidate the inflammatory host-defense mechanisms.

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Capítulo 3 – Artigo Experimental

Effects of L-NAME on tissue myeloperoxidase levels in rats with periodontitis and/or diabetic inflammatory disease

Efeito do L-NAME sobre os níveis de mieloperoxidase tecidual em ratos com doenças inflamatórias periodontite e ou diabetes

Artigo será enviado para publicação

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Effects of L-NAME on tissue myeloperoxidase levels in rats with periodontitis and/or diabetic inflammatory disease

Running title: NOS inhibition on myeloperoxidase levels.

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Summary

Inflammatory mediators such as myeloperoxidase (MPO) and nitric oxide (NO) play a role in inflammatory processes related to periodontal disease and diabetes. NO-Synthase (NOS) inhibitors have been administered in attempts to reduce tissue damage resulting from such inflammation. The aim of this study was to evaluate the effect of L-NAME on the levels of myeloperoxidase in tissues of diabetic rats with ligature-induced periodontitis. In total, 192 rats were divided into 8 groups of 24 animals each with Water intake (W) or L-NAME intake (L), as follows: group C-W, control group; group C-L, control group; group D-W, diabetic rats; group D-L, diabetic rats; group P-W, rats with chronic periodontitis; group P-L, rats with chronic periodontitis, group DP-W: diabetic chronic periodontitis rats; group DP-L: diabetic chronic periodontitis rats. The animals were sacrificed at 3, 7, 15 or 30 days after ligature-periodontitis induction to obtain gingival specimens for evaluation of MPO activity. The highest MPO levels were present in rats with periodontitis with water intake (P-W and DP-W). In all experimental groups with water intake, there was a peak in MPO levels at 7 days. The DP-W group showed significantly higher MPO expression than the other water-intake groups at 7 days ($4.8 \text{ U/mg} \pm 0.3$). L-NAME treatment significantly decreased MPO levels in all groups with pathological conditions ($p < 0.05$). Within the limits of this study, it may be suggested that the L-NAME leads to a decrease in tissue MPO activity in inflammatory pathological conditions such as diabetes and periodontitis.

Keywords: peroxidase, enzymes, nitric oxide synthase, inflammation, diabetes mellitus, periodontal diseases.

Introduction

The role of neutrophils in areas with tissular lesions is complex and includes the endocytosis of antigens, which can stimulate the secretion of intracellular enzymes including elastases, endopeptidases and myeloperoxidases (Arnhold, 2004). Myeloperoxidase (MPO) is a cationic protein thought to be the main constituent of the azurophilic granules in neutrophils, monocytes and some subtypes of tissue macrophages. Following inflammatory stimuli, myeloperoxidase is quickly liberated and participates in the innate immune host response (Dileep *et al.*, 2006). Some studies have used quantification of myeloperoxidase to estimate the accumulation of neutrophils in inflammatory processes, such as those occurring in periodontal diseases (Yamalík *et al.*, 2000; Buchmann *et al.*, 2002; Wei *et al.*, 2004; Gomes *et al.*, 2009), including that associated with diabetes mellitus (Gonçalves *et al.*, 2008) and in cardiovascular disease (Brennan *et al.*, 2003).

MPO liberates free radicals such as nitric oxide (NO) and other oxidants through reaction with hydrogen peroxide; at low concentrations, these oxidants promote antimicrobial effects (Rimele *et al.*, 1988; Kaplan *et al.*, 1989; Salvemini *et al.*, 1989; Van Dervort *et al.*, 1994). However, MPO-activated liberation of free radicals may, at high concentrations, result in oxidative tissue damage (Kendall *et al.*, 2000; Hirose *et al.*, 2001; Arnhold, 2004). As a result, the adherence of leucocytes to endothelial cells on blood vessel walls could be inhibited, impeding or delaying the delivery of defense cells from the blood to the injured tissue (Kubes *et al.*, 1991).

Just as high concentrations of MPO represent an exacerbated inflammatory process (Yamalík *et al.*, 2000; Buchmann *et al.*, 2002; Wei *et al.*, 2004; Gomes *et al.*, 2009), high NO levels can also lead to inflammatory tissue damage (Lappin *et al.*, 2000; Kendall *et al.*, 2000; Lohinai *et al.*, 2001; Irer *et al.*, 2007; Reher *et al.*, 2007; Pan *et al.*, 2009). Excessive production of NO or of oxidants derived from NO has been related to the pathophysiology of many inflammatory diseases, including gastrointestinal diseases (Boughton-Smith *et al.*, 1993; Szabó, 1995). Recently, some studies have also suggested that NO may play a role in the etiopathogeny of periodontal disease, a disease of inflammatory and multifactorial nature (Pig *et al.*, 2005; Skaleric *et al.*, 2006).

NO not only exerts regulatory actions during inflammatory processes and in autoimmunity mechanisms (Hibbs *et al.*, 1989; Marletta *et al.*, 1988) but also is an important inflammatory mediator released by stimulation of cells with pro-inflammatory cytokines (Kubes *et al.*, 1991; Bejjamim *et al.*, 2000). Considering the malicious effect of NO excess, some authors suggested the use of agents that inhibit NO synthase (NOS) to reduce NO to physiologically tolerable levels (Lohinai *et al.*, 1998; Pig *et al.*, 2005; Skaleric *et al.*, 2006). Indeed, experimental studies have shown that the administration of NOS inhibitors decreased alveolar bone loss (Pig *et al.*, 2005) and the expression of local inflammatory parameters, such as nitration of proteins, lipid peroxidation and leukocyte infiltration (Di Paola *et al.*, 2004).

There are few published studies evaluating the influence of treatment with NOS inhibitors on tissue levels of myeloperoxidase. Considering that leukocyte

infiltration can be reduced by administration of NOS inhibitors, as cited above, and that epidemiological research has demonstrated a positive correlation between diabetes and the prevalence and severity of periodontal disease (Grossi, 2001; Benatti *et al.*, 2003; Holzhausen *et al.*, 2004), the aim of this study was to evaluate the effect of L-NAME on the expression of MPO in diabetic rats with induced periodontitis.

Materials and Methods

Experimental design

The protocol of this study adhered to guidelines approved by the Institutional Experimentation Committee of the School of Dentistry at Araraquara, São Paulo, Brazil (process 28/2003). One hundred ninety-two young male Wistar rats (*Rattus norvegicus albinus*) weighing approximately 140 ± 8.3 g were used in this study. The animals were housed in cages in a temperature-controlled room, fed with a standard laboratory diet, and given tap water *ad libitum*. In some experimental groups, an inhibitor of NOS, L-arginine methyl ester (L-NAME, 30 mg/kg), was included in the water provided to the animals.

Animals were divided into 8 groups of 24 animals each: group C-W, control group with water intake; group C-L, control group with L-NAME intake; group D-W, diabetic rats with water intake; group D-L, diabetic rats with L-NAME intake; group P-W, chronic periodontitis rats with water intake; group P-L, chronic periodontitis rats with L-NAME intake; group DP-W, diabetic chronic

periodontitis rats with water intake; and group DP-L, diabetic chronic periodontitis rats with L-NAME intake. Twenty-four hours after diabetic status was checked, the day of periodontitis induction was considered as baseline (day 0). Animals were sacrificed by guillotine at 3, 7, 15 or 30 days after baseline; 6 rats per group were sacrificed in each experimental period. The experimental chronology is shown in Figure 1.

Plasma glucose level was verified in each period. Hemimandibles were removed to obtain gingival specimens and to evaluate MPO activity.

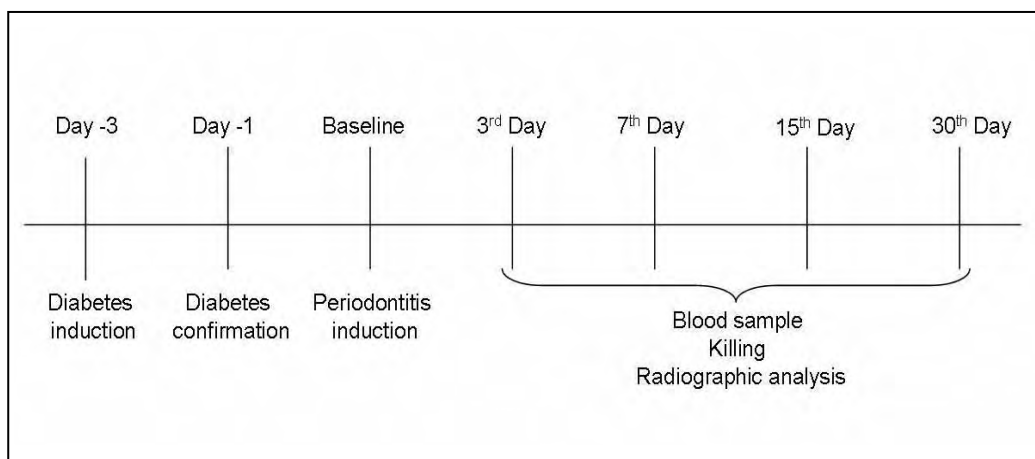


Figure 1 Experimental chronology.

Diabetes induction method

Diabetes mellitus was induced by a single injection of 50 mg/kg body weight streptozotocin (STZ) (Sigma, St. Louis, MO, USA) dissolved in sodium citrate buffer (0.01 M; pH 4.5) into the jugular vein (Pepato *et al.*, 1993; Pepato *et al.*, 1999; Holzhausen *et al.*, 2004). Diabetes was induced in rats of groups D-W,

D-L, DP-W and DP-L, while the non-diabetic animals (C-W, C-L, P-W and P-L) were injected with saline solution (0.15 ml) as sham treatment.

Blood glucose analysis

Blood samples were obtained from the tip of the tail of rats through a scissor cut made approximately 2 mm from the distal extremity. The samples were collected in Eppendorf tubes containing liquaemin sodium (Liquemine®, La Roche Ltd, Basel, Switzerland). After centrifugation, plasma was separated for assessment of glucose level by a glucose analyzer (Autoanalyzer, Technicom RA-XT, Bayer, Dublin, Ireland). Plasma glucose level greater than 280 mg/dl confirmed the presence of diabetic status (Pepato *et al.*, 1993; Pepato *et al.*, 1999).

Periodontitis induction

Animals from groups P-W and P-L received general anesthesia with intraperitoneal injection of ketamine 0.08 ml/100 g body weight (Francotar®, Virbac do Brasil Ind. e Com. LTDA, São Paulo, Brazil) and 0.04 mL of xylazine hydrochloride 0.04 ml/100 g body weight (2% Virbaxyl, Virbac do Brasil Ind. e Com. LTDA, São Paulo, Brazil). Then, a cotton ligature (corrente #24) was placed around the cervix of the left lower first molar in a submarginal position to induce experimental periodontitis (Holzhausen *et al.*, 2002; Nassar *et al.*, 2004). The procedure was performed with a modified tweezer and an explorer, both sterilized.

Drug treatment

Treatment groups received L-NAME (30 mg/kg/dia) one hour before diabetes induction and daily until sacrifice at 3, 7, 15 or 30 days. Two hundred mg of L-NAME was diluted in 1 L of water and administered daily to the rats.

MPO expression

Gingival samples were weighed; then 1 ml of hexadecyltrimethylammonium bromide (HTAB, Sigma Chem. Co., St. Louis, USA) was added for each 50 mg of tissue, followed by homogenization (Heidolph DiAx 900, Schwabach, Alemanha). Homogenates were heated for two hours at 60°C and then centrifuged at 10,000g for 5 minutes. The supernatants were used to measure MPO expression. Fifty microliters of homogenate were added to 200 µl of potassium phosphate buffer (pH 6.0) containing 0.164 mg/ml of o-dianisidine dihydrochloride (Sigma Chemical Co., St. Louis, USA) and 0.0005% of hydrogen peroxide (Merck, Darmstadt, Alemanha). Absorbance was measured in an ELISA scanner (SpectraMax Plus 384, Sunnyvale, USA) at 460 nm for 20 minutes, with recordings made at intervals of 20 seconds. Graphs showing the absorbance variation as a function of time were obtained and the Vmax/sec was calculated from these graphs. The time interval where the measurement of absorbance variation (optical density or absorbance) showed linearity (corresponding to the value of r^2 closer to 1) was used to obtain the value Vmax/sec. The mean of duplicate measurements from each gingival sample was calculated and was divided by the amount of tissue in each plate well (each 10 µl

of homogenate was equivalent to 0.5 mg of tissue). One unit of MPO activity was defined as the degradation of one micromole (μmol) of H_2O_2 per minute (Bradley *et al.*, 1982). Considering that 1 μmol of H_2O_2 corresponds to a variation of 0.0113 AU (absorbance units), the activity of MPO was expressed in MPO units and corrected by the amount of tissue (mg) added to the assay (U/mg).

Statistical Analyses

Data analyses were performed using BioStat 5.0 software (Optical Digital Technology, Belém, PA, Brazil). All data were analyzed for distribution by Shapiro-Wilk test. Comparisons among periods in each group were performed by one-way ANOVA followed by Tukey's test. Analyses among all groups in each experimental period were also performed by one-way ANOVA (Tukey's test). Comparisons between groups with water or L-NAME intake were analyzed by Mann-Whitney's test at a significance level of 5%.

Results

The highest MPO expression was seen in periodontitis groups with water intake (P-W and DP-W). Over the course of the experiment, there was a peak in MPO levels at 7 days in all experimental groups with water intake. In addition, animals with both diabetes and periodontitis (DP-W) showed statistically higher MPO expression (4.8 ± 0.3) at 7 days compared to other groups with water intake

at 7 days, represented by C-W (1.3 ± 0.2), D-W (2.9 ± 0.3) and P-W (4.1 ± 0.4). (Table 1)

L-NAME treatment significantly decreased MPO levels in all groups with pathological conditions, including diabetes (D) and periodontitis (P) alone and periodontitis in association with diabetes (DP) ($p < 0.05$). In addition, reductions in MPO levels at all time points were observed for all groups of animals receiving L-NAME as compared to the corresponding groups with water intake: at 3 days (D: 1.4 to 0.6, P: 3.0 to 1.8, DP: 3.7 to 2.7), 7 days (D: 2.9 to 1.2, P: 4.1 to 2.6, DP: 4.8 to 3.5), 15 days (D: 1.3 to 0.9, P: 2.6 to 1.3, DP: 3.8 to 2.8) and 30 days (D: 2.1 to 1.1, P: 3.2 to 2.5, DP: 4.5 to 3.3).

Table 1 Mean $\pm$ Standard deviation of the MPO activity in the gingival tissue (U/mg) during experimental periodontitis.

GROUPS	PERIODS			
	3 DAYS	7 DAYS	15 DAYS	30 DAYS
C-W	1.2 $\pm$ 0.1 ^{a α *}	1.3 $\pm$ 0.2 ^{a α *}	0.8 $\pm$ 0.1 ^{b α}	1.1 $\pm$ 0.1 ^{a α}
C-L	0.5 $\pm$ 0.1 ^{a β}	0.6 $\pm$ 0.2 ^{a β}	0.6 $\pm$ 0.1 ^{a α}	0.8 $\pm$ 0.2 ^{a α}
D-W	1.4 $\pm$ 0.2 ^{a α *}	2.9 $\pm$ 0.3 ^{b γ *}	1.3 $\pm$ 0.1 ^{a β *}	2.1 $\pm$ 0.6 ^{b β *}
D-L	0.6 $\pm$ 0.1 ^{a β}	1.2 $\pm$ 0.3 ^{b α}	0.9 $\pm$ 0.1 ^{b α}	1.1 $\pm$ 0.3 ^{b α}
P-W	3.0 $\pm$ 0.1 ^{a γ *}	4.1 $\pm$ 0.4 ^{b δ *}	2.6 $\pm$ 0.6 ^{a γ *}	3.2 $\pm$ 0.3 ^{a γ *}
P-L	1.8 $\pm$ 0.1 ^{a δ}	2.6 $\pm$ 0.4 ^{b γ}	1.3 $\pm$ 0.4 ^{a β}	2.5 $\pm$ 0.3 ^{b β}
DP-W	3.7 $\pm$ 0.4 ^{a ε *}	4.8 $\pm$ 0.3 ^{b ε *}	3.8 $\pm$ 0.1 ^{a δ *}	4.5 $\pm$ 0.2 ^{c δ *}
DP-L	2.7 $\pm$ 0.1 ^{a ζ}	3.5 $\pm$ 0.2 ^{b ζ}	2.8 $\pm$ 0.1 ^{a γ}	3.3 $\pm$ 0.7 ^{b β}

C: control group; D: diabetic rats; P: periodontitis rats; DP: rats with diabetes and periodontitis; W: water intake; L: L-NAME intake.

^{a, b} Different superscript letters in the rows indicate statistically significant differences among periods in each experimental group by one-way ANOVA's test followed by Tukey ($p < 0.05$).

^{α, β} Different superscript symbols in the columns indicate statistically significant differences among all groups by one-way ANOVA's test followed by Tukey ($p < 0.05$).

* Indicates statistically significant difference between each treatment group (water or L-NAME) by Mann-Whitney's test ($p < 0.05$).

Discussion

Higher levels of myeloperoxidase (MPO) were observed in tissues of animals with experimental periodontitis (P-W and DP-W), in agreement with previous findings by Gomes *et al.* (2009); the concordance of these results suggests that measurement of MPO activity can be used as a marker of inflammation due to periodontitis. The increased levels of myeloperoxidase can

be explained by the local migration of neutrophils to the inflamed area and increased secretion of intracellular enzymes in response to periodontal infection (Bradley *et al.*, 1982; Arnhold, 2004). Moreover, the inflammatory process that occurs as a result of periodontitis involves numerous components of the complement system that could be activated by classic or alternative mechanisms resulting in the release of chemotactic factors for leukocytes and vasoactive components (Stashenko *et al.*, 1991). The peak of MPO expression that occurred at 7 days in all the experimental groups emphatically demonstrates the role of the innate immune response and its ability to activate host defenses at the cellular level (Kantarci *et al.*, 2002).

When periodontitis was associated with diabetes, our results showed an exacerbation of the levels of MPO, suggesting a superimposed response of the two inflammatory conditions. According to Grossi *et al.* (1997), a synergistic effect could be verified in diabetes and periodontitis because both are chronic diseases that may activate innate immunity and result in the establishment of chronic inflammation. Moreover, some studies have suggested that the chemotactic activity of neutrophils is impaired in diabetic animals (PMNs) (Ramamurthy *et al.*, 1979; Sawant, 1993; Inoue *et al.*, 1997; Uchimura *et al.*, 1999); however, the perpetuation of the inflammatory process may activate immune response mechanisms (Dennison & Van Dyke, 1997; Nassar *et al.*, 2002). In addition, interactions between glycation end products (AGE) and RAGE receptors can lead to neutrophil activation and to increases in oxidative stress (Karima *et al.*, 2005). Some authors have suggested that superoxides released by

neutrophils could enhance the severity of periodontitis in diabetics (Karima *et al.*, 2005).

Taken together with the observed high expression of tissular MPO in the groups with diabetes and periodontitis alone or in combination, *in vitro* findings of others (Shishihbor *et al.*, 2003) suggest that MPO could provide an alternative pathway for generation of oxidants such as nitrogen dioxide (NO₂) and nitrotyrosine from NO; such free radicals are cytotoxic to nearby tissues and can promote degranulation of neutrophils with peroxidase accumulation (Beckman & Koppenol, 1996; Lappin *et al.*, 2000; Kendall *et al.*, 2000; Lohinai *et al.*, 2001; Irer *et al.*, 2007; Reher *et al.*, 2007; Pan *et al.*, 2009). In this context, as previously mentioned, some studies have shown that NO may play a role in the pathogenesis of periodontal diseases (Lohinai *et al.*, 1998; Hirose *et al.*, 2001; Shibata *et al.*, 2001; Pan *et al.*, 2009) and of diabetes (Skaleric *et al.*, 2006; Pan *et al.*, 2009).

Our results showed a reduction in tissue MPO levels when L-NAME was used as an adjunct therapeutic for inhibition of NO synthases (NOS), in agreement with studies by Moncada *et al.* (1991), Lohinai *et al.* (1998) and Pig *et al.* (2005). The decreased levels of MPO in animals with periodontitis treated with L-NAME could hypothetically be explained because this nonselective inhibitor may have inhibited the activity of lympho-monocytes and consequently reduced the migration of host defense cells to the injured tissue (Dal Secco *et al.*, 2004). Moreover, the administration of NO inhibitors has been shown to cause decreases in local inflammatory parameters such as the nitration of proteins, lipid peroxidation and leukocyte infiltration related to periodontitis (Di Paola *et al.*,

2004; Pig *et al.*, 2005). Other studies have demonstrated reduction in bone reabsorption and control of the inflammatory process in animals with experimental periodontitis treated with a NOS inhibitor (Broulík *et al.*, 2003; Fan *et al.*, 2004).

Based on our findings, it is suggested that further studies to better elucidate the mechanisms involved in the inflammatory process related to MPO and NO, mainly focusing on the basic areas of biological science, may have clinical application resulting in improved strategies for diagnosis and treatment of inflammatory disease. Within the limits of this study, it is suggested that treatment with L-NAME leads to a decrease in tissue MPO activity and reduces the deleterious effects of products derived from oxidative stress.

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CONSIDERAÇÕES FINAIS

Evidências suportam a idéia de que a hiperglicemia é um dos possíveis fatores responsáveis pelo desencadeamento das complicações diabéticas, como por exemplo, a doença periodontal ^{32,24}. No presente estudo foi utilizado a droga estreptozotocina (STZ) para a indução do diabetes. A STZ é um antibiótico capaz de promover destruição seletiva das células β do pâncreas e consequente diminuição da secreção de insulina, ocasionando assim, um acúmulo de glicose no sangue ^{51,52,42}, fato este que justifica a metodologia empregada.

O modelo experimental de doença periodontal associado ao diabetes tem sido foco de estudos em animais com intuito de elucidar o efeito do diabetes sobre o periodonto, o que ocasiona uma exacerbação no processo inflamatório da doença periodontal ^{14,32}. Tal fato justifica o interesse do presente estudo em demonstrar o efeito da administração de um inibidor da síntese de (NOS) em ratos diabéticos com doença periodontal induzida.

O óxido nítrico (NO) é um radical livre, gasoso, inorgânico e incolor, que constitui uma das menores e mais simples moléculas bio-sintetizadas no organismo ^{47,6}. Assim, as isoformas da síntese de óxido nítrico (NOS) são agrupadas em duas categorias, a NOS constitutiva (cNOS) e a NOS induzível (iNOS). A cNOS depende de íons de cálcio (Ca^{++}) e de calmodulina, que estão envolvidos na sinalização celular, enquanto que a iNOS é produzida por macrófagos e outras células ativadas por citocinas ^{46,41}. Esta isoforma requer algumas horas para ser expressa, no entanto, uma vez sintetizada, libera

quantidades maiores de NO que a cNOS. Assim, a produção de iNOS continua indefinidamente até que a L-arginina e os fatores sintetizantes sejam cessados, ou até que ocorra a morte celular ¹⁵.

O NO resultante da ativação da iNOS possui ação citotóxica e citostática, promovendo a destruição de microrganismos, parasitas e células tumorais ^{28,43,30}. A citotoxicidade do NO resulta de sua ação direta ou de sua reação com outros compostos liberados durante o processo inflamatório, podendo incluir uma ação citotóxica contra os tecidos adjacentes do hospedeiro, como por exemplo, ao osso alveolar ³⁹. Em processos infecciosos, as células ativadas como macrófagos, neutrófilos e células endoteliais secretam, simultaneamente, NO e intermediários reativos de oxigênio, sendo que a ação citotóxica indireta do NO consiste, principalmente, em sua reação com esses intermediários de oxigênio.

Lohinai et al.⁴⁰ (1998) demonstraram que um aumento nos níveis de NO durante as reações inflamatórias pode resultar em dano tecidual. Neste contexto, Pan et al.⁵⁰ (2009) demonstraram que a expressão de iNOS no tecido gengival, foi proeminente em pacientes diabéticos com periodontite crônica. No presente estudo foi observado um aumento significativo na expressão de NO nos casos com periodontite (P), diabetes (D) e na associação das duas condições patológicas. Relacionado a este fato foi observado maior perda óssea em ratos com periodontite (P) e periodontite associado ao diabetes (DP).

Estes resultados podem ser explicados pelo fato da periodontite promover um processo inflamatório exacerbado por neutrófilos, secreção de mediadores pró-inflamatórios responsáveis por um dano tecidual ^{31,49,3}. Adicionalmente,

citocinas pró-inflamatórias, como interleucina 1 β , fator de necrose tumoral e interferon- γ , pode estar envolvido na exacerbação da expressão de iNOS^{62,21,36}.

Neste contexto, quando os neutrófilos migram para os sítios inflamados, são liberados componentes celulares e enzimas que atuam nos tecidos adjacentes sobre outras células e microrganismos na tentativa de controlar a inflamação⁷. A mieloperoxidase (MPO) é uma dessas enzimas liberadas principalmente pelos neutrófilos que tem demonstrado propriedades antimicrobianas e citotóxicas, bem como habilidade de inativar fatores quimiotáticos que podem limitar a migração de neutrófilos^{29,4}.

Nossos resultados demonstraram uma maior expressão de MPO nos grupos com periodontite experimental (P e DP), este fato já foi estudado anteriormente por Gomes et al.¹⁷ (2009), sugerindo a medição da atividade de mieloperoxidase como marcador inflamatório. Além disso, no sítio infectado pela periodontite experimental, o processo inflamatório é capaz de carrear numerosos componentes do sistema complemento, os quais, ativados por via clássica e/ ou alternativa podem liberar fatores quimiotáticos para leucócitos e elementos vasoativos, potencializando a resposta inflamatória⁵⁷.

Quando a periodontite esteve associada ao diabetes (DP), nossos resultados não só demonstraram elevação nos níveis de iNOS e maior perda óssea mas também demonstraram uma exacerbação na expressão de MPO, sugerindo uma potencialização da resposta inflamatória. De acordo com GROSSI et al.¹⁹ (2001), um efeito sinérgico pode ser verificado no diabetes e na periodontite porque ambas são doenças crônicas que pode ativar a resposta imune-inata estabelecendo dessa forma condições inflamatórias crônicas.

A literatura sugere que o diabetes impede a atividade quimiotática dos neutrófilos (PMNs) ^{59,25,64}. Entretanto, a perpetuação da inflamação ativa, mecanismos da resposta imune ^{12,48}, e interações entre AGEs e RAGEs pode ocasionar uma ativação de neutrófilos e aumento do estresse oxidativo ²⁷. Alguns autores relatam que o estresse oxidativo (liberação de NO e seus produtos) causado por neutrófilos pode aumentar a severidade da doença periodontal em pacientes diabéticos ²⁷.

Considerando ainda a elevada expressão de MPO tecidual nos grupos com as patologias (D, P e DP), evidências *in vitro* ⁶⁰, sugerem a MPO como uma rota alternativa para geração de oxidantes derivados de NO (radicais livres), como o dióxido de nitrogênio (NO₂) e a nitrotirosina, os quais podem ocasionar uma ação citotóxica aos tecidos e maior degranulação de neutrófilos e acúmulo das peroxidases ^{6,33,28,26,55,50}.

Neste contexto, o efeito maléfico do excesso de NO têm sido minimizado com a utilização de inibidores da óxido nítrico sintase (NOS), no intuito de reduzir o NO a níveis toleráveis fisiologicamente ^{39,35,61}. Estudos experimentais relataram que a administração de inibidores de NOS ocasionou menor perda óssea alveolar ³⁵, e diminuição da expressão dos parâmetros inflamatórios locais, tais como, nitratação de proteínas, peroxidação lipídica e infiltrado leucocitário ¹³.

Tais estudos podem ser utilizados para justificar nossos resultados que mostraram uma diminuição significativa nos níveis de iNOS, na perda óssea alveolar e nos níveis de MPO nos grupos com condições patológicas (D, P e DP) que foram tratados com L-NAME. Além disso, a diminuição da expressão de

MPO observada nos grupos com periodontite (P-L e DP-L) tratados com L-NAME também pode ser hipoteticamente explicada pelo fato deste inibidor não-seletivo (L-NAME) inibir a linfomonocitose, e consequentemente, reduzir a migração de células de defesa do organismo para o tecido lesado ¹¹.

É importante relatar que o uso de inibidores da NOS tem mostrado um efeito benéfico em várias patologias. Na artrite experimental induzida em animais, ocorre uma atenuação nos sinais clínicos e histológicos da doença ⁴⁴. Na glomerulonefrites ocorre uma diminuição da deposição de complexos imunes nos rins, levando a uma considerável melhoria clínica ¹. No diabetes dependente tipo 1, induzido imunologicamente, estudos demonstraram que a destruição das células β pancreáticas é mediada por NO e as primeiras tentativas de inibir a NOS foram animadoras ³⁰. No transplante renal, foi obtida uma melhoria substancial dos sinais clínicos de inchaço depois da inibição seletiva da iNOS ¹. No choque séptico, a inibição da iNOS também foi benéfica ⁶³.

De acordo com os resultados obtidos e dentro dos limites deste estudo, o tratamento com L-NAME diminuiu os níveis de iNOS, MPO e consequentemente a perda óssea alveolar em ratos com periodontite associada ou não ao diabetes. Tal fato pode sugerir que o L-NAME pode ser usado como um possível agente terapêutico na diminuição do dano tecidual experimental. Entretanto, estudos futuros devem ser realizados na tentativa de melhor elucidar os mecanismos inflamatórios de defesa do hospedeiro, enfocando principalmente áreas básicas da ciência biológica, a fim de trazer ao cenário clínico, mudanças nas estratégias de diagnóstico e tratamento das doenças inflamatórias.

CONCLUSÃO

Dentro dos limites deste estudo, o tratamento com L-NAME diminuiu os níveis de iNOS, MPO e consequentemente a perda óssea alveolar em ratos com periodontite associada ou não ao diabetes. Tal fato pode sugerir o uso do inibidor da síntese de óxido nítrico (L-NAME) como um possível agente terapêutico na diminuição do dano tecidual.

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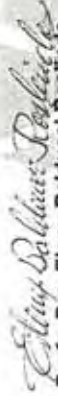
CERTIFICADO

Certificamos que o protocolo nr. 28/2003 referente à pesquisa "Influência do diabetes mellitus na microbiota e na dinâmica do processo inflamatório da doença periodontal induzida em ratos" sob a responsabilidade da Profª Drª Denise Madalena Palomari Spolidório e a/c Juliana Rico Pires está de acordo com os Princípios Éticos em Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pelo Comitê de Ética em Experimentação Animal (CEEA) da Faculdade de Odontologia de Araraquara-UNESP em reunião de 06/outubro/2008.

CERTIFICATE

We certify that the protocol 28/2003 referring to the research "Influence of diabetes mellitus in the microbiota and in the pathogenesis of periodontal disease in rats" under responsibility of Profª Drª Denise Madalena Palomari Spolidório and a/c Juliana Rico Pires is in agreement with the Ethical Principles in Animal Research adopted by Brazilian College of Animal Experimentation (COBEA) and was approved by the Araraquara Dental School-UNESP Ethical Committee for Animal Research (CEEA) in October 06, 2008.

Araraquara, 07 de Outubro de 2008


Prof. Dra. Eleny Balducci Roslindo
Coordenadora do CEEA/FOAr/UNESP

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Araraquara, 05 de janeiro de 2010.

DEBORA ALINE SILVA GOMES