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ALTERAÇÃO DE VIRULÊNCIA DE *Candida albicans* INDUZIDA POR CÁTIONS METÁLICOS EM BIOFILMES DINÂMICOS

CURITIBA
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Dissertação apresentada ao Programa de Pós-Graduação em Odontologia da Pontifícia Universidade Católica do Paraná como requisito à obtenção do Título de Mestre em Odontologia, Área de Concentração de Ortodontia.

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SUMÁRIO

1 ARTIGO EM PORTUGUÊS

PÁGINA TÍTULO

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RESUMO

Introdução: As condições bucais associadas às forças exercidas sobre os aparelhos ortodônticos fixos podem incorrer em corrosão das ligas metálicas com liberação de íons. Esses íons, podem afetar a composição, a atividade metabólica e a patogenicidade da microbiota bucal. O objetivo deste estudo foi avaliar a influência dos íons comumente liberados dos aparelhos ortodônticos fixos na atividade proteolítica de biofilmes de *Candida albicans* formados em condições dinâmicas. **Métodos:** Foram preparados meios de cultura contendo Ni^{2+}, Fe^{3+}, Cr^{3+}, Co^{2+} e mistura desses, em concentrações similares às liberadas em saliva. Biofilmes de *Candida albicans* SC5314 (n=35) foram crescidos de forma dinâmica em papel filtro por 72 h e suas biomassas e atividades proteolíticas específicas foram determinadas. **Resultados:** Todos os cátions promoveram incrementos significativos na biomassa ($p < 0.05$). A Atividade Proteolítica Nominal foi incrementada somente na presença de Ni^{2+} ($p < 0.05$), enquanto que o Cr^{3+} e Co^{2+} promoveram reduções ($p < 0.05$). Em relação à Atividade Proteolítica Específica, foi verificado que todos os íons induziram modulação negativa ($p < 0.05$). **Conclusão:** Embora ocorram elevações nas biomassas de biofilmes dinâmicos de *C. albicans* expostos aos cátions estudados, esses se comportam de maneira menos agressiva em termos de capacidade proteolítica.

Descritores: *Candida albicans,* aparelhos ortodônticos, íons metálicos, biofilmes, proteases.

INTRODUÇÃO

Em 2000, 5,8 milhões de norte-americanos gastaram US$ 11,8 bilhões com tratamentos ortodônticos[1] e, de acordo com a American Association of Orthodontists (AAO), cerca de 4,5 milhões de americanos estavam sob tratamento ortodôntica com aparelhos metálicos, quatro anos depois. [2]

As variadas condições ambientais na boca (temperatura, umidade, dieta, microbiota), bem como as forças exercidas sobre os aparelhos ortodônticos incorrem em corrosão das ligas metálicas que os compõem com liberação de cátions ferro, cromo, níquel, titânio, etc. [3]

Níquel, cromo e cobalto estão relacionados com citotoxicidade e genotoxidade[4]. Além disso, esses íons podem afetar a composição, a atividade metabólica e a patogenicidade da microbiota bucal[5]. As *Candida* spp. são membros constantes dessa microbiota para a maioria da população,[6] podendo ou não estarem relacionadas com doença. Contudo, a exposição de *C. albicans* a determinados íons metálicos interfere na transição levedura-hifa, que pode ser suprimida ou aumentada por diferentes íons metálicos[7]. Além disso, tais mudanças morfológicas incorrem em variações de resistência aos antifúngicos[8] e co-regulação de mecanismos de virulência.[9,10] Essa evidência, associada com outros fatores de predisposição do paciente, pode ser importante no estabelecimento de um quadro de candidose mais severa em pacientes sob tratamento ortodônticos.

O objetivo do presente estudo foi avaliar a influência dos íons comumente liberados dos aparelhos ortodônticos fixos na atividade proteolítica de biofilmes *Candida albicans* SC5314 formados em condições dinâmicas.

MATERIAL E MÉTODOS

Preparo das soluções de íons metálicos

Caldo Base de Nitrogênio para Leveduras (Yeast Nitrogen Base® - YNB, Difco Co., Detroit, MI), por ser um meio insento de metais, contendo diferentes cátions metálicos foi preparado de acordo com as quantidades liberadas de aparelhos ortodônticos para saliva.[3,11] Os íons elencados e suas respectivas concentrações foram níquel divalente (Ni^{2+}) 10 $ng.mL^{-1}$ (170 nM), ferro trivalente (Fe^{3+}) 6,77 $ng.mL^{-1}$ (121 nM), cromo trivalente (Cr^{3+}) 4,5 $ng.mL^{-1}$ (86,5 nM) e cobalto divalente (Co^{2+}) 0,44 $ng.mL^{-1}$ (7,46 nM). O fungo foi também desafiado com mistura dos íons que foram dissolvidos em YNB, nas concentrações acima. Em função de suas maiores solubilidades, foram utilizados nitratos metálicos de alto grau de pureza (Merck KGaA, Darmstadt, Alemanha). O controle negativo continha meio de cultura sem a adição de qualquer íon metálico. O solvente empregado para a confecção dos meios de cultura e soluções foi a água reagente tipo II com resistividade específica maior que 2 $Mohm.cm^{-1}$.[12,13]

Paper-Embedded Biofilm Reactor (PEBR)

O PEBR foi montado como descrito na figura 1. Ele consiste de uma secção de tubo de aço inoxidável com 100 mm (Ø) × 70 mm (altura) × 1 mm (espessura) soldado excentricamente a uma placa de mesmo material com dimensões 150 mm × 120 mm × 2 mm.

Uma placa circular de vidro [90 mm (Ø) × 5 mm (espessura)] foi adaptada dentro do PEBR de forma a não tocar em suas paredes internas. Sobre essa placa foi depositado um disco de papel de filtro Whatman™ Qualitative Grade 3 (General Electric Co., Kent, UK) com 90 mm Ø. Esse disco serviu como uma matriz para difusão de nutriente. Trinta e cinco discos de papel de filtro para

antibiograma com 6 mm Ø (Cecon Ltd., São Paulo, Brazil) foram distribuídos na periferia com distância de 3-4 mm entre si.

Um retentor de borracha butílica foi adaptado à borda livre do PEBR. Uma placa de vidro de 120 mm × 120 mm × 4 mm, com orifício central (25 mm Ø), cobria o vaso. Um sistema de admissão de caldo nutritivo foi construído com rolha de borracha e uma agulha 121-D™ 100×20 (Höppner Produtos Veterinários Ltda, São Paulo, Brasil).

O PEBR foi fechado com a placa de vidro e esterilizado a 121 °C, 1 atm.$(cm^2)^{-1}$, e 15 min.

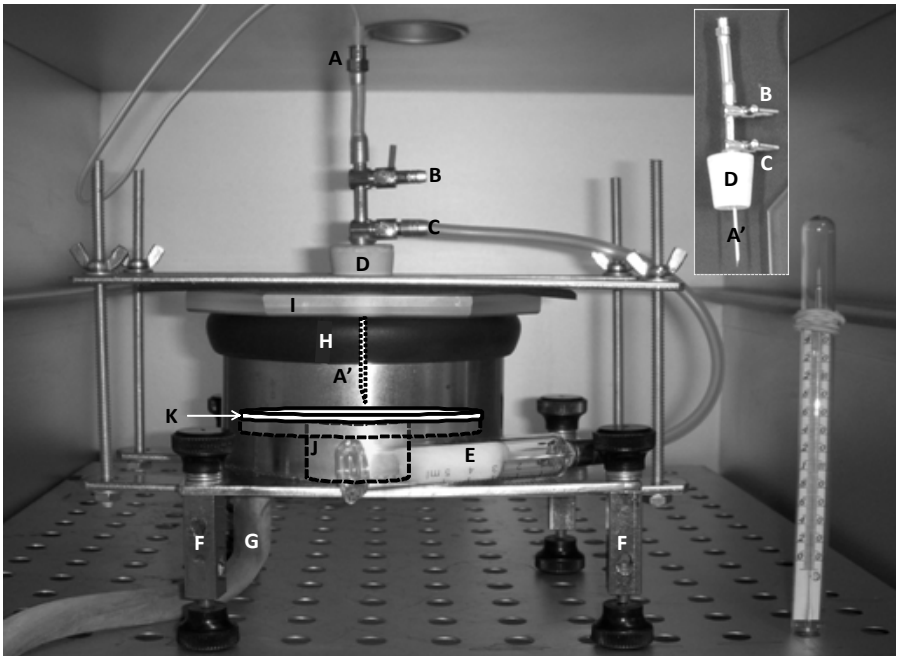

Figura 1. PEBR montado.
A, A' = agulha de admissão; B, C = portas de gás; D = rolha de borracha; E = camisa de seringa hipodérmica *Luer lock* preenchida com algodão estéril; F = niveladores; G = dreno; H = perfil de borracha; I = tampa de vidro com furo central de 20 mm; J = mesa de vidro de 90 mm (dentro do PEBR); K = folha de papel-filtro usado para difusão.

Microrganismo e crescimento em biofilme

A cepa *C. albicans* SC5314, gentilmente cedida pela Universidade de Hong Kong, foi escolhida para a condução do estudo devido ao fato de que ela é uma boa formadora de biofilmes, inclusive em condições adversas.[14] A cepa foi crescida aerobicamente em tubos contendo YNB a 37 °C, para adaptação ao

meio. Após 24 h, as células foram centrifugadas e lavadas com NaCl 145 mM estéril. As células foram resuspendidas em NaCl 145 mM até atingirem a concentração de *c.a.* 3×10^7 blastoporos.mL^{-1} (OD_{600} de 0,5), para padronizar o número de células em cada amostra.

Alíquotas de 10 µL dessa suspensão celular foram inoculadas assepticamente para os discos de antibiograma estéreis no PEBR. O bioreator foi novamente montado e conectado a reservatórios de Yeast Nitrogen Base® (YNB) contendo os cátions metálicos nas concentrações acima descritas. Os caldos foram admitidos com fluxo de 200 µL.min^{-1} e os biofilmes foram crescidos aerobicamente a 37 °C.

Atividade Proteásica Nominal (APN)

Decorridas 72 h de admissão continua de caldo, o PEBR foi desmontado e os discos de antibiograma foram cuidadosamente recolhidos com pinça metálica de ponta fina estéril. Esses foram transferidos para placas de poliestireno com fundo “U”. Os poços foram preenchidos com 200 µL de solução contendo albumina bovina fração V (BSA) 0,5 mg.mL^{-1}, citrato de sódio 10 mM e ácido cítrico 10 mM (pH 5,0), e foram incubados a 37 °C, por 4 h e 100 rpm. Decorrido o tempo de digestão, alíquotas de 100 µL de sobrenadantes foram combinados com volumes de 100 µL de solução de Coomassie (Coomassie brilliant blue G-250 0,025%, etanol 11,75%, ácido fosfórico 21,25%) em placas de microtitulação de fundo chato. Após 5 min, as densidades ópticas das misturas foram lidas em leitor de placas, determinadas em 600 nm. Como controles, foram utilizadas repetições de solução de Coomassie com solução salina (*blank*) e repetições da solução de BSA com solução de Coomassie (*basal concentration*). Uma unidade enzimática

foi arbitrariamente determinada como sendo a quantidade de enzima capaz de promover a digestão de um miligrama de BSA, por hora.

Estimativa da biomassa

Os discos de papel-filtro (N = 35) contendo os biofilmes e que digeriram a albumina foram cuidadosamente lavados por imersão em PBS 150 mM e transferidos para placas de poliestireno com fundo “U”. Os poços foram preenchidos com 200 µL de MTT 1 $mg.mL^{-1}$ (em PBS 150 mM). (Hawser & Douglas, 1994) Após incubação por 4 h a 37°C e 100 rpm, a solução de MTT foi removida por aspiração e os discos foram cuidadosamente lavados por imersão em PBS 150 mM. Isopropanol absoluto (1 mL) foi adicionado para solubilizar a formazana de MTT produzida. Essa formazana teve sua densidade óptica medida em 540 nm.

Controles foram realizados com 35 discos de antibiograma não inoculados e umedecidos com YNB (72 h, 37 °C). O processamento do MTT foi conduzido conforme acima.

Atividade Proteásica Específica (APE)

O quociente da divisão dos valores de APN por biomassa normalizada para 10^6 células representa a Atividade Proteásica Específica (APE) para cada grupo experimental. Assim, a APE é a quantidade de enzima capaz de promover a digestão de um miligrama de BSA, por 10^6 células, por hora.

Estatística

Todos os dados foram avaliados em relação à homogeneidade de variância pelo Teste de Levene e analisados pelos testes ANOVA a um critério e teste de comparações múltiplas de Games-Howell para variâncias heterogêneas,

utilizando o pacote estatístico SPSS 18.0 (SPSS Inc., Chicago, IL). O valor de $p < 0,05$ foi assumido como o ponto limítrofe para estabelecimento de diferenças.

RESULTADOS

Estimativa de biomassa

Sobre os discos de papel de filtro (n = 35) foram formados biofilmes consistentes. Entretanto, suas biomassas se mostraram heterogêneas quanto às variâncias inter-grupos. Diferenças entre os grupos foram determinadas por comparações múltiplas paramétricas de Games-Howell.

A figura 2, setor inferior, mostra que a biomassa do biofilme "Controle" foi estimada em $4,97\times10^6$ ($\pm2,67\times10^5$) células. Todos os cátions promoveram incrementos significativos na biomassa, que variou de $6,41\times10^6$ ($\pm3,53\times10^5$) células (Fe^{2+}; $p \leq 0.0215$) até $15,6\times10^7$ ($\pm1,34\times10^6$) células (Cr^{3+}; $p \leq 1.62E\text{-}07$). A mistura de cátions elevou a biomassa estimada até $9,65\times10^6$ ($\pm5,86\times10^6$) células ($p \leq 5.06E\text{-}11$).

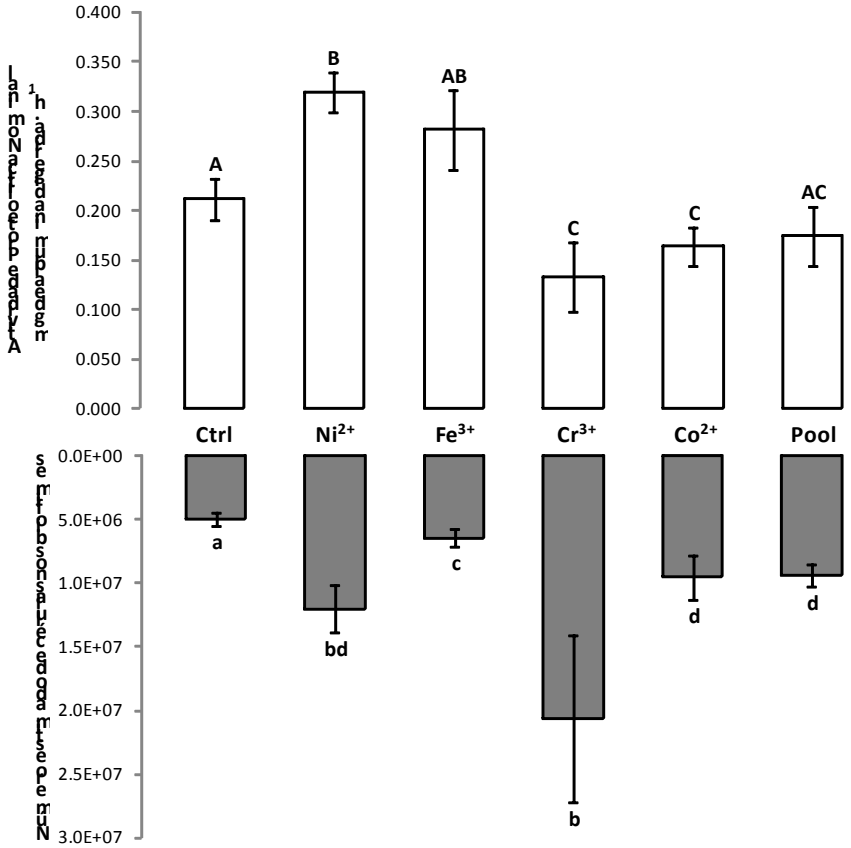

Figura 2. Atividade Proteolítica Nominal e Biomassa dos biofilmes crescidos em presença de cátions metálicos. Letras diferentes sobre as barras indicam diferenças significativas entre as médias dos grupos.

Atividade proteolítica nominal (APN)

A exposição dos biofilmes a albumina revelou atividade proteolítica variável (Figura 2, setor superior). O biofilme "Controle" secretou proteases que digeriram o substrato em velocidade de 0,21 (±0,06) $mg.h^{-1}$. Somente biofilmes formados sob influência de Ni^{2+} apresentaram incremento proteolítico [0,31 (±0,05) $mg.h^{-1}$; p < 6.36E-09]. Por outro lado, Cr^{3+} [0,12 (±0,08) $mg.h^{-1}$] e Co^{2+} [0,16 (±0,06) $mg.h^{-1}$] induziram reduções de atividade proteolítica ($p \leq 0,016$). O Fe^{3+} [0,27 (±0,10) $mg.h^{-1}$] e a mistura de cátions [0,17 (±0,08) $mg.h^{-1}$] não foram capazes de promover oscilações significativas nessa propriedade ($p > 0,069$).

Atividade proteolítica específica (APE)

A figura 3 mostra que os dados de atividade proteolítica nominal, quando normalizados por 10^6 células, revelaram que os cátions metálicos ensaiados tendem a induzir uma modulação negativa na atividade proteolítica específica ($p \leq 0,020$), a exceção do Fe^{3+} ($p = 0,921$).

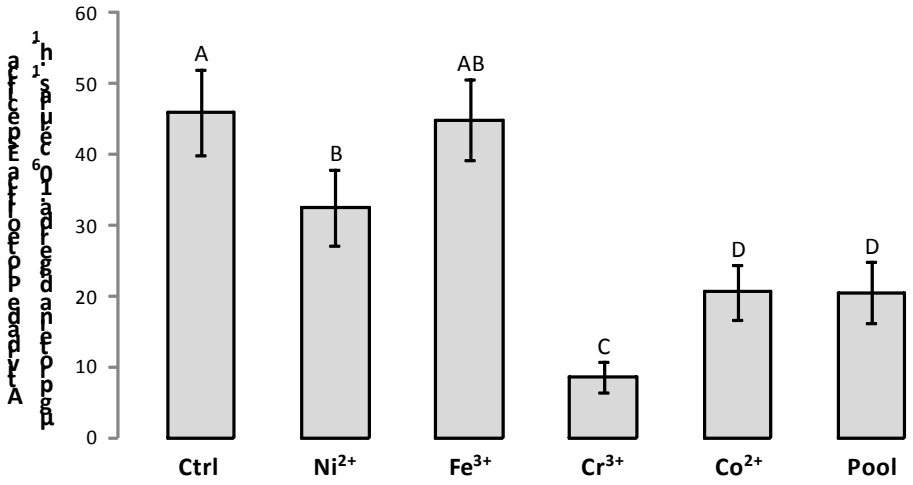

Figura 3. Atividade Proteolítica Específica dos biofilmes.
Letras diferentes sobre as barras indicam diferenças significativas entre as médias dos grupos.

Correlações entre variáveis

Quando aplicado o coeficiente de Pearson (r_P) às variáveis biomassa, APN e APE, é possível verificar que a última deriva com igual proporção da redução de biomassa e aumento da APN, independentemente do tratamento ao qual o biofilme estivesse sido submetido (Tabela 1).

Tabela 1. Coeficientes de correlação de Pearson (rP) para váriáveis Biomassa, Atividade Proteolítica Nominal (APN) e Atividade Proteolítica Específica (APE)

	Biomassa	APN	APE
Biomassa	1	negativo 0.0521	negativo 0,600**
APN		1	positivo 0,651**
APE			1

** Correlações significantes com nível de 0,01 (2-*tailed*)

DISCUSSÃO

A instalação de aparelhos ortodônticos fixos ou removíveis altera o ambiente bucal com aumento de nichos retentivos, o que favorece, em tese, a proliferação de microrganismos. Contudo, tal proposição é insuficiente para suportar a hipótese de que a simples presença de aparelhos ortodônticos instalados implique necessariamente em aumentos na frequência de candidose em pacientes carreadores do fungo.[15] Logo, outras variáveis decorrentes da instalação de aparelhos ortodônticos devem ser consideradas.

Embora a degradação passiva dos aparelhos ortodônticos seja motivo de preocupação em questões de alergia,[16,17] o impacto exercido pelos cátions metálicos na formação de biofilmes é um tópico que só recentemente foi considerado.[10]

Neste estudo, os nutrientes e os cátions metálicos alcançaram os biofilmes em formação por difusão capilar em papel de filtro qualitativo com fluxo constante e baixo *shear stress*. Os biofilmes foram formados sobre um substrato de papel

de filtro (discos para antibiograma) em contato com um *headspace* atmosférico sem imersão em fase líquida. No nosso entender, essa abordagem inovadora mimetiza as condições de formação de biofilmes bucais *bona fide* em contraste com aquelas utilizadas por outros com biofilmes formados imersos em fase líquida estática.[18-20] Em favor desse ponto de vista, obtivemos biofilmes consistentes, com coeficientes de variação entre 0,452 e 0,925.

Não nos causou surpresa o fato das biomassas obtidas se mostrarem aumentadas quando da presença dos cátions metálicos. Nosso grupo já havia demonstrado que os cátions avaliados tendem a incrementar biomassas de biofilmes de *C. albicans*.[10] Entretanto, neste estudo, os biofilmes foram crescidos em condição dinâmica na qual havia oferta de nutrientes e eliminação de metabólitos tóxicos de forma continuada e não-intermitente. Além disso, a exposição ao oxigênio molecular atmosférico presente no *headspace* ocorre de maneira mais próxima daquela que ocorre nas mucosas bucais. Essas características, quando analisadas conjuntamente, reforçam a tese de que os conteúdos celulares dos biofilmes de *C. albicans* são aumentados na presença de cátions metálicos liberados de aparelhos ortodônticos, em concentrações nanomolares.

A despeito dos incrementos nas biomassas dos biofilmes expostos aos cátions, as APEs se mostraram invariáveis (Fe^{3+}) ou, inesperadamente, reduzidas (demais cátions e *pool*) em relação ao controle. Do ponto de vista algébrico, isso é devido à falta de proporcionalidade na APN (Ni^{2+}) ou a sua redução (Cr^{3+} e Co^{2+}). Sob a óptica biológica, esses fenômenos podem decorrer de inibição na transição morfológica levedura-hifa provocada por diversos metais de transição externa, com consequente arrestamento do fungo na forma de levedura.[7] É

sabido que células leveduriformes tendem a secretar menos proteases que células micelianas.[21,22] Com base nessas constatações e pressupostos, é plausível conjecturar que os cátions metálicos, nas concentrações aqui ensaiadas, tenham induzido uma retenção celular na forma de levedura levando a menor secreção de proteases de ação extracelular. Tal fenômeno é interessante, pois, mesmo com um aumento no número de células induzido pelos cátions, essas células passam a secretar menos proteases. Proteases de ação extra-celular figuram entre os principais fatores de virulência de fungo diretamente associados ao processo de invasão epitelial.[23-25] Essa é uma característica apreciável, principalmente quando da instalação recente de aparelhos ortodônticos que podem lesionar mucosas intra-bucais [26,27] e predispor o paciente a candidoses.

CONCLUSÃO

O modelo experimental aqui apresentado mostrou que biofilmes de *C. albicans* tem sua virulência influenciada por cátions metálicos comumente encontrados na saliva de usuários de aparelhos ortodônticos metálicos. Enquanto a biomassa tende a ser aumentada, a atividade proteolítica tende a ser reduzida (exceção, Ni^{2+}). O coeficiente proteolítico (APE) se mostrou diminuído, o que pode contribuir para a redução a predisposição à candidose nos pacientes sob intervenção ortodôntica.

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2 ARTIGO EM INGLÊS

RESUM

Introduction: The oral conditions associated with forces on orthodontic appliances may incur corrosion of mettalic alloys with ion release. These ions can affect the composition, metabolic activity and pathogenicity of the oral microbiota. The aim of this study was to evaluate the influence of ions commonly released from orthodontic appliances in the proteolytic activity of Candida albicans biofilms formed under dynamic conditions. **Methods:** The prepared culture media containing Ni^{2+}, Fe^{3+}, Cr^{3+}, Co^{2+} and mixtures of these in concentrations similar to those released in the saliva. Biofilms of *Candida albicans* SC5314 (n = 35) were grown dynamically on filter paper for 72 h and their biomass and specific proteolytic activities were determined. **Results:** All cations significant increases in biomass ($p < 0.05$). Nominal proteolytic activity was increased in the presence of only Ni^{2+} ($p < 0.05$), whereas Cr^{3+} and Co^{2+} promoted reductions ($p < 0.05$). Regarding Specific Proteolytic Activity, it was found that all ions induced downregulation ($p < 0.05$). **Conclusion:** Although there increases in biomass of biofilm dynamic *C. albicans* exposed to cations studied, these behave less aggressive in terms of proteolytic ability.

Keywords: *Candida albicans,* orthodontic appliances, metal ions, biofilms proteases.

INTRODUCTION

In 2000, 5.8 million Americans spent $ 11.8 billion on orthodontic treatment[1] and, according to the American Association of Orthodontists (AAO), about 4.5 million Americans were under orthodontic treatment with metallic appliances four years later. [2]

The different environmental conditions in the mouth (temperature, humidity, diet, microbiota) and the forces exerted on braces incur corrosion of metal alloys that compose release of cations with iron, chromium, nickel, titanium, etc.. [3]

Nickel, chrome and cobalt are related to cytotoxicity and genotoxidade.[4] Moreover, these ions can affect the composition, metabolic activity and pathogenicity of microorganisms bucal.[5] The *Candida spp.* are members of this constant microbiota for most part of the population,[6] may or may not be related to disease. However, exposure of C. albicans certain transition metal ions interfere in yeast, hyphae, which can be suppressed or enhanced by various mettalic ions.[7] Moreover, such variations morphological changes incur antifungical resistance[8] and co-regulation virulence mechanisms.[9,10] This evidence, associated with other factors of the patient's predisposition may be important in establishing a frame candidosis in more severe orthodontic patients.

The aim of this study was to evaluate the influence of ions commonly released from orthodontic appliances in the proteolytic activity of *Candida albicans* SC5314 biofilms formed under dynamic conditions.

MATERIAL AND METHODS

Preparation of the solutions of metal ions

Broth Yeast Nitrogen Base (Yeast Nitrogen Base ® - YNB, Difco Co., Detroit, MI) containing different metal ions was prepared according to the quantities released from orthodontic appliances to saliva.[3,11] Ions listed and their respective concentrations were divalent nickel (Ni^{2+}) 10 ng.mL-1 (170 nM), trivalent iron (Fe^{3+}) 6.77 ng.mL-1 (121 nM), trivalent chromium (Cr^{3+}) 4,5 ng.mL-1 (86 , 5 nM) and divalent cobalt (Co^{2+}) 0.44 ng.mL-1 (7.46 nM). The fungus was also challenged with mixture of ions which were dissolved in YNB at concentrations above. Because of their higher solubilities, metal nitrates were used high purity (Merck KGaA, Darmstadt, Germany). The negative control contained medium without the addition of any metal ion. The solvent employed for the preparation of culture media and reagent solutions was water with type II specific resistivity greater than 2 Mohm.cm^{-1}.[12,13]

Paper-Embedded Biofilm Reactor (PEBR)

The PEBR was assembled as described in figure 1. It consisted of a section of stainless steel tube 100 mm (ø) × 70 mm (height) × 1 mm (thickness) eccentrically welded to a plate of the same material with dimensions 150 mm × 120 mm × 2 mm.

A circular glass plate [90 mm (ø) × 5 mm (thickness)] was adapted in the PEBR so as not touching the inner walls. On this plate was deposited a disk of filter paper Whatman Qualitative ™ Grade 3 (General Electric Co., Kent, UK) with a 90 mm Ø. This disc served as a matrix for nutrient diffusion. Thirty-five discs of filter paper for antibiogram with 6 mm Ø (CECON Ltd., São Paulo, Brazil) were distributed in the periphery with a distance of 3-4 mm apart.

A retainer of butyl rubber was adapted to the free edge of PEBR. A glass plate of 120 mm × 120 mm × 4 mm, central hole (Ø 25 mm), covered the vessel. An intake system of nutrient broth was built with a rubber stopper and a needle 121-D ™ 100 × 20 (Höppner Veterinary Products Ltd., São Paulo, Brazil).

The PEBR was closed with a glass plate and sterilized at 121 ° C, 1 atm. (Cm2) -1 and 15 min.

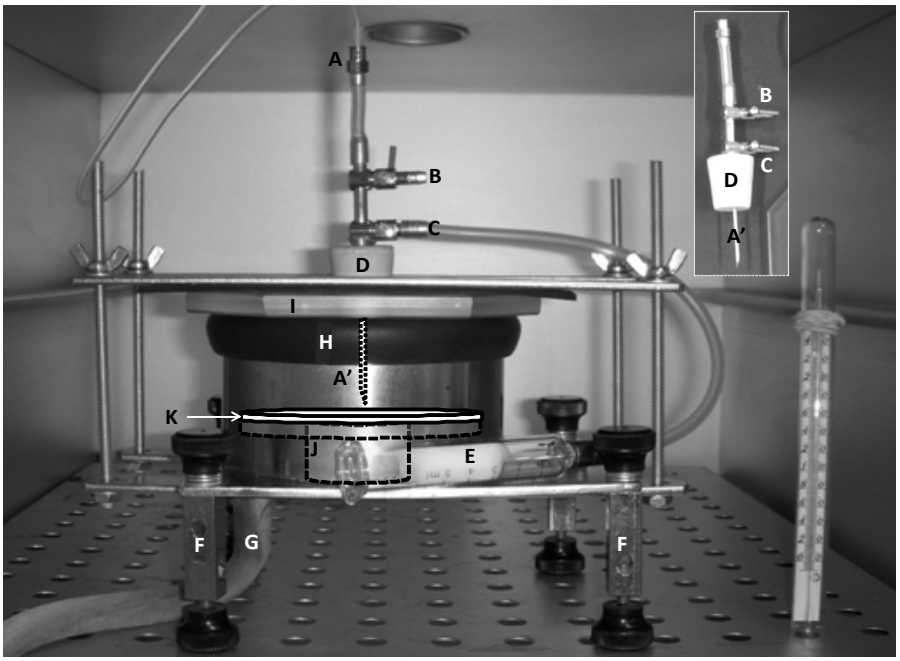

Figura 1. PEBR mounted.
A, A '= inlet needle, B, C = gas ports D = rubber stopper, E = shirt hypodermic Luer lock syringe filled with sterile cotton, F = levelers; drain G = H = rubber profile; I = glass lid with a 20 mm center hole, J = table glass of 90 mm (inside PEBR) = K sheet of filter paper used for broadcasting.

Microorganism and growth in biofilm

The strain *C. albicans* SC5314 was chosen to conduct the study due to the fact that she is a good-forming biofilms, including conditions adversas.[14] The strain was grown aerobically in YNB containing tubes at 37°C, to adapt to the environment. After 24 h, the cells were centrifuged and washed with 145 mM NaCl sterile. Cells were resuspended in NaCl 145 mM to reach a concentration of c.a. $3^{\times 107}$ blastoporos.mL^{-1} (OD600 of 0.5) to standardize the number of cells in each sample.

10 µL aliquots of this cell suspension were inoculated aseptically to sterile discs antibiogram in PEBR. The bioreactor was again mounted and connected to reservoirs Yeast Extract Peptone Glucose (YEPD) containing the metal ions at concentrations above. The broths were admitted flow 200 μL.min^{-1} and biofilms were grown aerobically at 37 $^{\circ}$ C.

Nominal proteinase activity (NPA)

After 72 h of admission continues broth, the PEBR was dismantled and antibiogram discs were carefully collected with fine-tipped tweezers metal sterile. These were transferred to polystyrene plates bottomed "U". The wells were filled with 200 μL of solution containing bovine albumin fraction V (BSA) 0.5 mg.mL^{-1}, 10 mM sodium citrate and 10 mM citric acid (pH 5.0), and incubated at 37 ° C for 4 h and 100 rpm. After the time of digestion, 100 μL aliquots of supernatants were combined with 100 μL volumes of Coomassie solution (Coomassie brilliant blue G-250 0.025%, ethanol 11.75%, 21.25% phosphoric acid) in microtitre plates flat bottom. After 5 min, the optical densities of the mixtures were determined at 600 nm. As controls were used repetitions solution Coomassie saline (blank) and repetitions of BSA solution with Coomassie solution (basal concentration). One enzyme unit was arbitrarily determined as the amount of enzyme capable of promoting digestion of a milligram of BSA per hour.

Estimation of biomass

The discs of filter paper (N = 35) containing biofilms and digested albumin were thoroughly washed by soaking in 150 mM PBS and transferred to polystyrene plates bottomed "U". The wells were filled with 200 of MTT 1 mg.mL^{-1} (150 mM in PBS). (Hawser & Douglas, 1994) after incubation for 4 h at 37 °C and

100 rpm, the MTT solution was removed by aspiration and the disks were thoroughly washed by soaking in 150 mM PBS. Absolute isopropanol (1 mL) was added to solubilize the MTT formazan produced. This formazan had its optical density measured at 540 nm.

Controls were performed with 35 antibiogram discs and moistened with uninoculated YEPD (72 h, 37 °C). The processing of MTT was conducted as above.

Specific protease activity (SPA)

The quotient of the division of biomass per NPA values normalized to 106 cells represents Specific Protease activity (SPA) for each experimental group. Thus, the SPA is the amount of enzyme capable of promoting digestion of a milligram of BSA per 106 cells per hour.

Statistics

All data were assessed for homogeneity of variance by Levene's test and analyzed by ANOVA test and a multiple comparison test of Games-Howell for heterogeneous variances, using the statistical package SPSS 18.0 (SPSS Inc., Chicago, IL). The value of $p < 0.05$ was taken as the point boundary to establish differences.

RESULTS

Estimation of biomass

On discs of filter paper (n = 35) were formed biofilms consistent. However, if their biomass showed heterogeneous regarding variances between groups. Differences between groups were determined by multiple comparisons of parametric Games-Howell.

Figure 2, lower sector shows that the biomass of biofilm "Control" was estimated to be 4.97 x 106 (± 2.67 x 105) cells. All cations significant increases in biomass, which ranged from 6.41 × 106 (± 3.53 × 105) cells (Fe^{2+}, P ≤ 0.0215) to 1.56 × 107 (± 1.34 × 106) cells (Cr^{3+} , p ≤ 1.62E-07). The mixture of cations increased the biomass estimated to 9.65 × 106 (± 5.86 × 106) cells (p ≤ 5.06E-11).

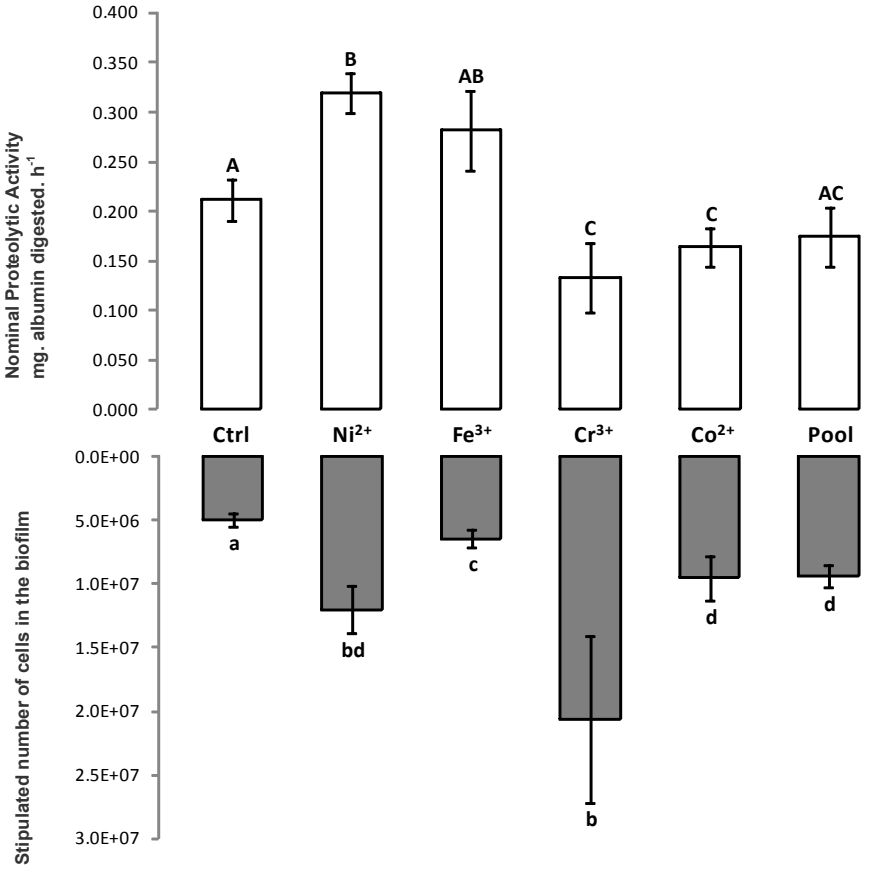

Figure 2. Nominal Proteolytic Activity and biomass of biofilms grown in the presence of metallic cations.
Different letters on the bars indicate significant differences between group means.

Proteolytic Activity Nominal (PAN)

The exposure of biofilms albumin revealed variable proteolytic activity (Figure 2, upper sector). Biofilm "Control" that secreted proteases digested the substrate speed of 0.21 (± 0.06) $mg.h^{-1}$. Only biofilms formed under the influence of Ni^{2+} showed increased proteolytic [0.31 (± 0.05) $mg.h^{-1}$, p <6.36E-09]. Moreover, Cr^{3+} [0.12 (± 0.08) $mg.h^{-1}$] and Co^{2+} [0.16 (± 0.06) $mg.h^{-1}$] proteolytic activity induced reductions (p ≤ 0.016). The Fe^{3+} [0.27 (± 0.10) $mg.h^{-1}$] and the

mixture of cations [0.17 (± 0.08) $mg.h^{-1}$] were not able to promote significant fluctuations that property ($p > 0.069$).

Specific Proteolytic Activity (SPA)

Figure 3 shows the data of nominal proteolytic activity, when normalized per 106 cells revealed that the metal ions tested tend to induce a downregulation in specific proteolytic activity ($p \leq 0.020$), except for the Fe^{3+} ($p = 0.921$).

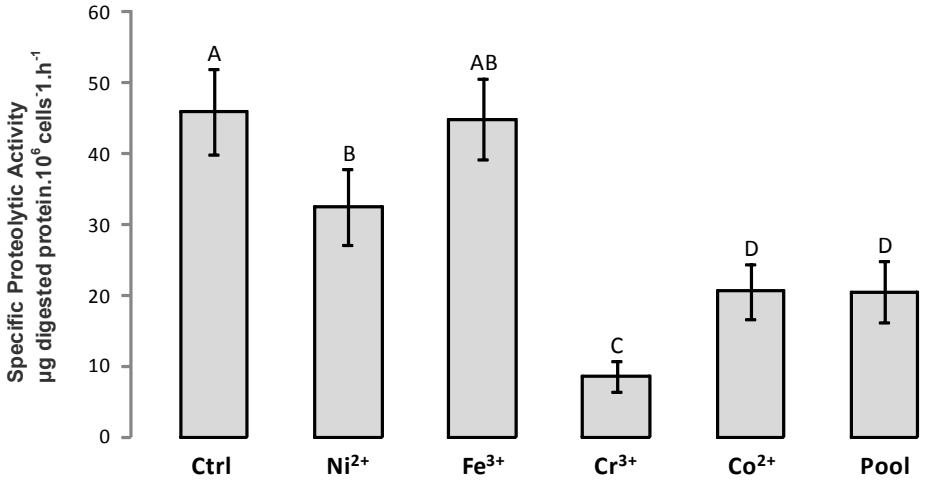

Figure 3. Specific Proteolytic activity of biofilms.
Different letters on the bars indicate significant differences between group means.

Correlations between variables

When applied the Pearson coefficient (rP) variables biomass, SPA and NPA, you can verify that the last drift with equal proportion of biomass reduction and increased NPA, regardless of the treatment to which the biofilm was being submitted (Table 1).

Table 1. Pearson coefficients for variables relationship Biomass, Nominal Proteolytic Activity (NPA) and Specific Proteolytic Activity (SPA

	Biomass	NPA	SPA
Biomass	1	negative 0.0521	negative 0,600**
NPA		1	positive 0.521**
SPA			1

** Correlações significantes com nível de 0,01 (2-*tailed*)

DISCUSSION

The installation of fixed or removable orthodontic appliances changes the oral environment with increased retentive niches, which favors, in theory, the proliferation of microorganisms. However, this proposal is insufficient to support the hypothesis that the mere presence of orthodontic appliances installed necessarily involves increases in the frequency of candidiasis in patients harboring the fungo.[15] Therefore, other variables related to insertion of orthodontic appliances must be considered.

Although the passive degradation of orthodontic appliances is a concern in matters of allergy,[16,17] the impact exerted by metal cations in biofilm formation is a topic that has only recently been considered.[10]

In this study, nutrients and metallic cations reached in the biofilm formation by diffusion capillary qualitative on filter paper with constant flow and low shear stress. Biofilms were formed on a substrate of filter paper (for antibiogram discs) in contact with a headspace atmosphere without immersion liquid-phase. In our view, this innovative approach mimics the conditions of formation of oral biofilms *bona fide* in contrast to those used by others with estatic biofilms immersed in liquid phase.[18-20] In support of this view, we obtained consistent biofilms, with coefficients variation between 0.452 and 0.925.

Not surprisingly caused us of biomass obtained prove increased when the presence of the metal ions. Our group had previously shown that the cations evaluated tend to increase biomass of biofilms of *C. albicans*.[10] However, in this study, biofilms were grown in dynamic condition in which there supply of nutrients and disposal of toxic metabolites continuously and non-intermittent. Furthermore, exposure to atmospheric molecular oxygen present in the headspace occurs in a

manner closer to that which occurs in oral mucosa. These characteristics, when considered together, reinforce the notion that the cellular content of biofilms of *C. albicans* are increased in the presence of metal ions released from orthodontic appliances, at nanomolar concentrations.

Despite the increases in biomass of biofilms exposed to cations, the SPA proved invariant (Fe^{3+}) or unexpectedly reduced (other cations and pool) compared to control. From the algebraic point of view, this is due to lack of proportionality in the NPA (Ni^{2+}) or reduction (Cr^{3+} and Co^{2+}). Under the biological perspective, these phenomena may result in inhibition of yeast-hypha morphological transition caused by various external transition metals, with consequent arrestment of the fungus as levedure.[7] Is known that yeast cells tend to secrete less proteases than micelian cells.[21.22] Based on these findings and assumptions, it is reasonable to conjecture that the metal ions at concentrations tested here, have induced a cell retention in the form of yeast leading to reduced secretion of extracellular proteases action. This phenomenon is interesting because, even with an increase in cell number induced by cations, these cells start to secrete less proteases. Proteases of extra cellular action among the major virulence factors are directly associated with fungal invasion process epitelial.[23-25] This is a sensible feature, especially when the recent installation of orthodontic appliances can damage mucous intraoral, [26,27] and predispose the patient to candidosis.

CONCLUSION

The experimental model presented here demonstrated that biofilms of *C. albicans* virulence is influenced by metal ions commonly found in the saliva of

users of orthodontic appliances. While biomass tends to be increased proteolytic activity tends to be reduced (except, Ni^{2+}). The proteolytic coefficient (SPA) showed decreased, which may contribute to reducing the predisposition to candidiasis in patients undergoing orthodontic intervention.

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