PONTIFÍCIA UNIVERSIDADE CATÓLICA DO PARANÁ

CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA

CAMILA MENDES FIGUEIREDO

MODULAÇÃO DA EXPRESSÃO DE ATRIBUTOS DE VIRULÊNCIA DE *Candida albicans* POR HIDRÓXIDO DE CÁLCIO

CURITIBA

2009

CAMILA MENDES FIGUEIREDO

MODULAÇÃO DA EXPRESSÃO DE ATRIBUTOS DE VIRULÊNCIA DE *Candida albicans* POR HIDRÓXIDO DE CÁLCIO

Dissertação apresentada ao Programa de Pós-Graduação em Odontologia da Pontifícia Universidade Católica do Paraná, como requisito parcial para a obtenção do título de Mestre em Odontologia. Área de Concentração: Endodontia.

Orientador: Prof. Dr. Everdan Carneiro.

Co-orientador: Prof. Dr. Edvaldo Antônio Ribeiro Rosa.

CURITIBA

2009

Dados da Catalogação na Publicação

Pontifícia Universidade Católica do Paraná

Sistema Integrado de Bibliotecas – SIBI/PUCPR

Biblioteca Central

Figueiredo, Camila Mendes

F475m Modulação da expressão de atributos de virulência de *candida albicans* por

2009 hidróxido de cálcio = Modulating expression of virulence attributes of *candida albicans* to calcium hydroxide / Camila Mendes Figueiredo ; orientador, Everdan Carneiro ; co-orientador, Edvaldo Antônio Ribeiro Rosa. – 2009.

62 f. : il. ; 30 cm

Texto em português e inglês

Dissertação (mestrado) – Pontifícia Universidade Católica do Paraná, Curitiba, 2009

Inclui bibliografias

1. Endodontia. 2. Canal radicular. 3. Hidróxido de cálcio. 4. Candida albicans. I. Carneiro, Everdan. II. Rosa, Edvaldo Antonio Ribeiro. III. Pontifícia Universidade Católica do Paraná. Programa de Pós-Graduação em Odontologia. IV. Título. V. Título: Modulating expression of virulence attributes of *candida albicans* to calcium hydroxide.

CDD 20. ed. – 617.6342

Pontifícia Universidade Católica do Paraná
Centro de Ciências Biológicas e da Saúde
Programa de Pós-Graduação em Odontologia

TERMO DE APROVAÇÃO

CAMILA MENDES FIGUEIREDO

MODULAÇÃO DA EXPRESSÃO DE ATRIBUTOS DE VIRULÊNCIA DE *Candida albicans* POR HIDRÓXIDO DE CÁLCIO

Dissertação apresentada ao Programa de Pós-Graduação em Odontologia da Pontifícia Universidade Católica do Paraná, como parte dos requisitos parciais para a obtenção do Título de Mestre em Odontologia, Área de Concentração em Endodontia.

Orientador(a): Prof. Dr. Everdan Carneiro
Programa de Pós-Graduação em Odontologia, PUCPR

Prof. Dr. Luiz Fernando Fariniuk
Programa de Pós-Graduação em Odontologia, PUCPR

Profª Drª Luciana Mouro Sassone
Programa de Pós-Graduação em Odontologia, UERJ

Curitiba, 30 de março de 2009.

Rua Imaculada Conceição, 1155 Prado Velho CEP 80215 901 Curitiba Paraná Brasil
Tel.: (41) 3271 1637 Fax: (41) 3271 1405 www.pucpr.br ppgo@pucpr.br

DEDICO ESTE TRABALHO

Aos meus pais, Roberto e Miriam, vocês, muitas

vezes, abriram mão dos seus sonhos

para que eu pudesse realizar os meus.

À minha pequena Maya, *sometimes the smallest thing takes up*

the biggest place inside our hearts…

Ao meu marido, Abude, por ter confiado completamente

em mim, mesmo sem nunca ter olhado em meus olhos antes.

Ao meu irmão, Pedro Henrique, por ser o meu Mani,

o melhor do mundo!

AGRADECIMENTOS

Ao meu orientador Prof. Dr. Everdan Carneiro, Mestre não apenas por titulação, mas pela sua maneira calma e simples de ensinar, agradeço pelo seu apoio durante todo o curso de mestrado, não apenas profissionalmente, mas também pessoalmente.

Ao meu orientador Prof. Dr. Edvaldo Antônio Ribeiro Rosa agradeço por estar sempre presente, antes mesmo do curso de mestrado, e por seu jeito simples e direto de lidar com as situações.

À Rosimeire Takaki Rosa, pela sua infinita paciência, pela sua maneira simples e gentil de transmitir seus conhecimentos e ajudar a solucionar problemas.

AGRADECIMENTOS

À Prof. Drª. Vânia Portela Ditzel Westphalen, pela sua confiança e incentivo, pela sua dedicação e exemplo.

Ao Prof. Dr. Luiz Fernando Fariniuk, pelo seu incentivo desde os tempos do aperfeiçoamento, durante a especialização e agora, no mestrado.

Ao Prof. Dr. Ulisses Xavier da Silva Neto, por dividir seus conhecimentos, pela sua colaboração e incentivo.

Ao Prof. Dr. Sérgio Aparecido Ignácio, por tornar a estatística algo não tão obscuro.

À colega e professora Ana Cavali, pela sua competência e amizade sempre demonstrada. Você já era mestre...

Aos colegas Camila, Sandra, Vinício, Juarez, Liliane e Maria Cláudia, pela amizade e companheirismo, alguns momentos serão inesquecíveis...

À doutoranda Alessandra de Paula e Carvalho, pela sua enorme ajuda e paciência.

Aos funcionários do curso de Odontologia da PUCPR, pela sua colaboração.

“Mestre não é quem sempre ensina, mas quem, de repente, aprende.”

João Guimarães Rosa

RESUMO

Em Endodontia, o uso da medicação intracanal representa uma etapa importante para reduzir o número de microrganismos no interior do sistema de canais radiculares. Pastas à base de hidróxido de cálcio são muito utilizadas devido à sua ação antimicrobiana e capacidade de dissolução tecidual. O fungo dimórfico *Candida albicans* está comumente presente na saliva de pacientes, podendo ser carreado para o interior do canal radicular em diversas situações, como cáries, traumatismos e restaurações infiltradas. Em casos de lesão endodôntica persistente, a *Candida albicans* é considerada um microrganismo prevalente. A secreção de enzimas histolíticas tem sido considerada como um importante fator na patogênese dessa espécie. O objetivo deste trabalho foi avaliar, *in vitro*, o efeito da presença do hidróxido de cálcio na secreção de fosfatases ácida e alcalina, aspartil-proteases, esterase e hemolisina pelo fungo. Para tanto, inoculou-se uma suspensão de *Candida albicans* em frascos contendo uma seqüência de diluições seriadas de hidróxido de cálcio ou EDTA em caldo Sabouraud em condições anóxicas e normóxicas. Após 48 horas de incubação, foram realizados ensaios para determinação das atividades enzimáticas, não sendo observada diminuição na virulência do microrganismo testado quando o hidróxido de cálcio estava presente no meio.

Palavras-chave: Canal radicular; hidróxido de cálcio; *Candida albicans*; enzimas histolíticas.

ABSTRACT

In Endodontics, the use of an intracanal medication is an important step to reduce the number of microorganisms inside the root canal system. Calcium hydroxide-based pastes are widely used because of its antimicrobial potential and tissue dissolution ability. The dimorphic fungus *Candida albicans* is often present in the patients' saliva and can be carried inside the root canal system in some situations such as caries, trauma and microleaked restorations. In cases of persistent endodontic lesions, *Candida albicans* is considered to be a prevalent microorganism. The secretion of histolytic enzymes has been considered an important factor in the pathogenesis of this species. The aim of this study was to evaluate, *in vitro*, the effect of calcium hydroxide in the secretion of acid and alkaline phosphatases, secreted aspartyl proteases, esterase and hemolysin by the fungus. A suspension of *Candida albicans* was inoculated in flasks containing a sequence of serial dilutions of calcium hydroxide or 1 mM EDTA in Sabouraud broth under anoxic and normoxic conditions. After 48 hours of incubation, assays were performed in order to determine the enzymatic activities. It was not observed a decrease in the microorganism virulence when the calcium hydroxide was present in the medium.

Key-words: Root canal; calcium hydroxide; *Candida albicans*; histolytic enzymes.

MODULAÇÃO DA EXPRESSÃO DE ATRIBUTOS DE VIRULÊNCIA DE *Candida albicans* POR HIDRÓXIDO DE CÁLCIO

Camila Mendes Figueiredo*

Everdan Carneiro**

Edvaldo Antônio Ribeiro Rosa***

Vânia Portela Ditzel Westphalen**

Ulisses Xavier da Silva Neto**

Luiz Fernando Fariniuk**

Rosimeire Takaki Rosa****

*Especialista em Endodontia; Especialista em Microbiologia; Mestranda em Odontologia, Área de Concentração: Endodontia PPGO PUCPR, Paraná, Brasil.

**Especialista, Mestre e Doutor em Endodontia PUCPR, Paraná, Brasil.

***Farmacêutico, Mestre e Doutor em Biologia e Patologia Buco-Dental, PUCPR, Paraná, Brasil.

****Farmacêutica, Especialista em Microbiologia, Mestre em Biologia e Patologia Buco-Dental, PUCPR, Paraná, Brasil.

Correspondência para: Prof. Dr. Edvaldo Antônio Ribeiro Rosa

R. Imaculada Conceição, 1155, Prado Velho, 80215-901, Curitiba-PR, Brasil.

Tel.: 00 55 41 3271-1497 E-mail: edvaldo.rosa@pucpr.br

SUMÁRIO

1 ARTIGO EM PORTUGUÊS

MODULAÇÃO DA EXPRESSÃO DE ATRIBUTOS DE VIRULÊNCIA DE *Candida albicans* POR HIDRÓXIDO DE CÁLCIO

RESUMO

Objetivos. Avaliar a exacerbação ou a redução do potencial de virulência de *Candida albicans* exposta a diferentes concentrações de hidróxido de cálcio com base na secreção das enzimas fosfatase ácida e alcalina, aspartil-proteases secretoras, esterase e hemolisina.

Metodologia. A cepa SC5314 foi cultivada em atmosfera normóxia e anóxia por 48 h. Após o período de incubação, foram inoculadas em caldo *Sabouraud* (SB) puro ou contendo diluições seriadas de $Ca(OH)_2$ ou EDTA 1 mM. Após 48 h de incubação tanto em atmosfera normóxica quanto anóxica, os tubos-teste foram submetidos a ensaios para a determinação das atividades enzimáticas. Os dados obtidos foram analisados estatisticamente.

Resultados. Não foi observada diminuição da virulência do microrganismo testado quando o hidróxido de cálcio estava presente no meio. Já na presença de EDTA, houve uma tendência de aumento da secreção de enzimas quando em atmosfera anóxica.

Conclusão. A presença de oxigênio não interferiu no crescimento celular de *Candida albicans*; o hidróxido de cálcio não interferiu no potencial virulento da *Candida albicans*, nem mesmo quando em alta concentração.

1.1 INTRODUÇÃO

Uma das medicações intracanal mais utilizadas em endodontia é o hidróxido de cálcio [($Ca(OH)_2$], sendo seu uso amplamente difundido (1). É uma base forte, com pH de aproximadamente 12.5, que possui baixa solubilidade em água, sendo normalmente misturado a um veículo, formando uma pasta (1, 2). O pH elevado do hidróxido de cálcio confere-lhe propriedades como: a inativação de enzimas bacterianas, ativação enzimática tecidual e efeito mineralizador (1, 3-6). Os efeitos estão relacionados à dissociação desta substância em meio aquoso, liberando íons Ca^{2+} e OH^- (7, 8).

O hidróxido de cálcio deve ser misturado a um veículo de modo a formar uma pasta, que contenha o maior número de partículas de $Ca(OH)_2$ possível, devendo estar em contato com todas as paredes do canal radicular, preenchendo totalmente o mesmo (6).

As infecções endodônticas são polimicrobianas (9). Um microrganismo comumente presente nessas infecções, principalmente onde o conduto radicular foi exposto ao meio bucal, é a *Candida albicans* (10).

Embora muitos fatores tenham sido atribuídos ao potencial de virulência da *Candida albicans*, como a capacidade de formação de hifas, moléculas de reconhecimento na superfície e variação fenotípica (11), a secreção de enzimas histolíticas tem sido apontada como um importante evento associado à patogênese provocada por essa espécie (12-18), uma vez que essas enzimas estão associadas à destruição celular e de matriz extracelular. As cepas que produzem tais enzimas apresentam uma maior capacidade de invasão quando comparadas às cepas não produtoras.

A *Candida albicans* pode ser considerada microbiota normal da cavidade bucal, passando a causar doença quando há um desequilíbrio no organismo do hospedeiro (19, 20). O fungo presente na saliva do paciente pode ser carreado para o interior do canal radicular em dentes com cárie profunda e exposição da polpa, por traumatismos ou em casos de isolamento absoluto mal realizado (1, 21, 22). Restaurações pós-tratamento endodôntico que apresentem infiltrações também podem permitir a entrada de *Candida albicans* no interior do canal, promovendo sua recontaminação (23).

A *Candida albicans* geralmente está associada a lesões endodônticas persistentes (21, 22, 24-36). Uma das alternativas químicas para eliminar esse microrganismo é a pasta de $Ca(OH)_2$. Assim, um estudo que avalie o potencial de virulência da *Candida albicans* exposta a diferentes diluições de $Ca(OH)_2$ faz-se necessário.

1.2 MATERIAL E MÉTODOS

1.2.1 Preparo do inóculo aeróbio de *C. albicans* SC5314

As células foram crescidas em caldo Sabouraud (SB) a 37°C, 120 rpm e normóxia. Após 24 h de crescimento, foram centrifugadas e o *pellet* foi lavado três vezes com água destilada estéril e suspendido até turbidez próxima a do tubo #3 da escala de MacFarland [$9x10^6$ unidades formadoras de colônia por mililitro (UFC/mL)]. Essas células foram utilizadas como inóculo aeróbio nos experimentos subsequentes em normóxia.

1.2.2 Preparo do inóculo anaeróbio de *C. albicans* SC5314

As células foram crescidas em caldo Sabouraud (SB) a 37°C, 120 rpm e anóxia (pCO_2 10 %, pN_2 90 %). Esse procedimento foi repetido ainda mais uma vez, a fim de

que as células se adaptassem à ausência de oxigênio molecular. Após 24 h de crescimento, foram centrifugadas e o *pellet* foi lavado três vezes com água destilada estéril e suspendido até turbidez próxima a do tubo #3 da escala de MacFarland [$9x10^6$ unidades formadoras de colônia por mililitro (UFC/mL)]. Essas células foram utilizadas como inóculo anaeróbio nos experimentos subsequentes em anóxia.

1.2.3 Caldos indutores/inibidores de secreção enzimática

Alíquotas de 100 μL de inóculo aeróbio ou anaeróbio foram transferidas para tubos contendo SB acrescidos de $Ca(OH)_2$ *pro-analysis* até concentrações finais de 25 mM, 2,5 mM, 250 μM, 25 μM e 2,5 μM. A fim de se garantir ausência de cátions Ca^{+2}, foram incluídos tubos contento SB acrescido de EDTA 1 mM. Como controles positivos, foram incluídos tubos contendo somente SB. Os tubos foram incubados em atmosfera normóxica ou anóxica, em 37°C e 120 rpm.

Após 48 h de incubação, os tubos foram centrifugados a 10000 × *g* e os sobrenadantes foram recolhidos em tubos estéreis para serem utilizados nos ensaios de atividade enzimática. Os *pellets* foram duas vezes lavados com água destilada estéril e submetidos ao ensaio de determinação de biomassa pelo teste de retenção de cristal violeta.

As incubações em caldos indutores/inibidores de secreção enzimática foram conduzidas em triplicata, em dois momentos distintos.

1.2.4 Quantificação das aspartil-proteases secretoras (SAP)

Azocaseína (5 mg/mL) foi dissolvida em solução de Tris-HCl 50 mM (pH 5,0), NaCl 200 mM, $CaCl_2$ 5 mM, Triton X-100 0,05% e azida sódica 0,01%. Alíquotas de 400 μL dessa solução foram misturadas com 100 μL de sobrenadantes. Após incubação

por 2 h a 37°C, alíquotas de 150 μL de ácido tricloroacético 20% foram adicionadas. Após 30 min em temperatura ambiente, os tubos foram centrifugados a 16000 × *g* (3 min), e os *pellets* foram descartados. Os sobrenadantes foram misturados com iguais volumes de NaOH 1M e as OD_{440nm} foram medidas (27) em leitora de placas TP-reader® (ThermoPlate, Co.). Uma unidade de atividade enzimática foi definida como a quantidade de enzima necessária para incrementar a OD_{440nm} em 0,001 unidades de absorbância por minuto de digestão (28). A atividade enzimática específica foi calculada de forma a fornecer a quantidade de unidades enzimáticas por densidade óptica de cristal violeta retido pela biomassa.

1.2.5 Quantificação das esterases

Inicialmente uma curva padrão com nove amostras de oleato de sódio foi construída pela adição de volumes crescentes de oleato de sódio 90 mM [em Tris-HCl 20 mM (pH 7.0)] em solução de Tween® 80 1% e $CaCl_2$ 80 mM em Tris-HCl 20 mM (pH 7.0), com determinação das DO_{450nm}.

A atividade esterásica foi determinada empregando Tween® 80 como substrato e determinação das OD_{450nm} (29). Alíquotas de 100 μL dos sobrenadantes foram misturadas a 900 μL de solução de Tween® 80 1% e $CaCl_2$ 80 mM em Tris-HCl 20 mM (pH 7.0). Após a adição dos sobrenadantes, os aumentos das OD_{450nm} foram monitorados em função do tempo. Uma unidade enzimática foi definida como a quantidade de enzima que libera 1 μmol de ácido graxo por minuto. A atividade enzimática específica foi calculada de forma a fornecer a quantidade de unidades enzimáticas por densidade óptica de cristal violeta retido pela biomassa.

1.2.6 Quantificação da hemolisina

Para a quantificação da atividade hemolítica (30) os sobrenadantes foram diluídos seriadamente em solução de Tris-HCl 10 mM contendo NaCl 145 mM (pH 7.5). Alíquotas de 100 μL das diluições foram misturadas com alíquotas de 100 μL de eritrócitos de carneiro 1% [em Tris-HCl 10 mM contendo NaCl 145 mM (pH 7.5)] e incubadas a 37°C. Após 2 horas de incubação, as suspensões foram sedimentadas a 4°C por no mínimo 15 h. As OD_{540nm} dos sobrenadantes foram determinadas. Os controles negativos contiveram volumes iguais de tampão e suspensão de eritrócitos. Uma unidade hemolítica foi definida como a quantidade de material que lisa 50% dos eritrócitos de carneiro. A atividade hemolítica específica foi calculada de forma a fornecer a quantidade de unidades hemolíticas por densidade óptica de cristal violeta retido pela biomassa.

1.2.7 Quantificação de fosfatase alcalina

Para a quantificação da fosfatase alcalina, foi utilizado o kit Fosfatase Alcalina K019® (Quibasa Ltda, Belo Horizonte, Brasil). Alíquotas de 10 μL dos sobrenadantes foram dispensadas para tubos estéreis e a quantificação da atividade enzimática foi feita seguindo as recomendações do fabricante. Após 10 min de incubação a 37°C, os tubos foram homogeneizados e foram determinadas as OD_{590nm}. Após correções pelos fatores de calibração com solução-padrão de concentração conhecida (40 U/L), os resultados foram expressos em unidades por litro. A atividade enzimática específica foi calculada de forma a fornecer a quantidade de unidades enzimáticas por densidade óptica de cristal violeta retido pela biomassa.

1.2.8 Quantificação de fosfatase ácida

Para a quantificação da fosfatase ácida, foi utilizado o kit Fosfatase Ácida Prostática K018® (Quibasa Ltda, Belo Horizonte, Brasil). Alíquotas de 20 µL dos sobrenadantes foram dispensadas para tubos estéreis e a quantificação da atividade enzimática foi feita seguindo as recomendações do fabricante. Após 30 min de incubação a 37°C, os tubos foram homogeneizados e foram determinadas as OD_{590nm}. Após correções pelos fatores de calibração com solução-padrão de concentração conhecida (3 U/L), os resultados foram expressos em unidades por litro. A atividade enzimática específica foi calculada de forma a fornecer a quantidade de unidades enzimáticas por densidade óptica de cristal violeta retido pela biomassa.

1.2.9 Quantificação de biomassa pelo teste de cristal violeta

As massas celulares foram lavadas cinco vezes com 200 µL de NaCl 145 mM estéril. Alíquotas de 200µL de cristal violeta (CV) 0,5% foram adicionadas aos tubos (31). Após 20 min, os excessos de CV foram removidos por lavagens repetidas com água estéril. Finalmente, o CV impregnado foi liberado pela adição de 250 µL de ácido acético 33%. As OD_{590nm} foram determinadas. Os valores de absorbância dos *blanks* foram subtraídos dos valores obtidos nos tratamentos para eliminar resultados espúrios decorrentes de interferência de fundo.

1.2.10 Análise estatística

Todos os testes acima descritos foram conduzidos em triplicata, em ao menos duas diferentes situações. Os dados numéricos obtidos foram tabulados em planilhas eletrônicas Microsoft Excel® (Microsoft Co.). Os ensaios estatísticos foram conduzidos no pacote estatístico SPSS® 15.0 *for Windows* (SPSS Inc., Chicago). Os

dados foram testados quanto à sua normalidade de distribuição pelo teste de Kolmogorov-Smirnov e as diferenças foram acessadas por comparações múltiplas de Tukey HSD com um *threshold* de $p \leq 0,05$. Correlações de amostras pareadas foram obtidas pelo confrontamento dos dados de atividade específica para cada enzima e biomassa, tanto em ambiente normóxico quanto anóxico.

1.3 RESULTADOS

O crescimento celular de *C. albicans* SC 5314 em SB e em presença de oxigênio molecular (normóxia) por 48 h não apresentou divergência em relação àquele obtido em anóxia (p=0,12951) (Figura 1). Por outro lado, quando o SB recebeu a adição de EDTA, o crescimento em normóxia foi 9,37 vezes maior que em anóxia (p<0,0001). A exposição continuada às diferentes concentrações de $Ca(OH)_2$ pareceu não exercer profundas alterações na biomassa (p>0,05), embora seja perceptível uma tendência de se elevarem as biomassas, sobretudo em normóxia, quando em presença da base.

A atividade específica das aspartil-proteases (Sap) se manteve constante nos diversos tratamentos (p > 0,05), com média de atividade de 0,652 ± 0,105 $U.biomassa^{-1}$.

A atividade hemolítica específica foi mais pronunciada em anóxia para células crescidas em SB (p < 0.0001) e para células em presença de EDTA 1 mM (10,95 vezes maior; p < 0.0001) (Figura 3).

Dentre as enzimas analisadas, a esterase foi aquela que mostrou maior variabilidade nas amplitudes da atividade específica (Figura 4). Contudo, a única ocorrência de disparidade entre cultivos normóxicos e anóxicos ocorreu em SB (p =

0,0180). O $Ca(OH)_2$ não induziu alterações significativas na atividade específica da esterase. O EDTA não causou disparidades em função da presença ou não de oxigênio.

As condições atmosféricas de cultivo e a presença de $Ca(OH)_2$ não promoveram oscilações significativas na atividade específica de fosfatases ácida e alcalina (Figuras 5 e 6). A exceção foi a expressão das enzimas por células crescidas em presença de EDTA 1 mM em atmosfera anóxica, que foi superior a média

Com o intuito de se verificar a influência que as diferentes concentrações de $Ca(OH)_2$ exerceram sobre a atividade enzimática específica em função das biomassas, foram realizadas análises de correlação para amostras pareadas (Tabela 1). A falta de significância nas correlações e a baixa amplitude "*surrounding*" o zero (de -0.271 a 0.188, na tabela 1) indicam a não existência de qualquer relação direta ou indiretamente proporcional entre a quantidade de células e a atividade enzimática em função da presença de diferentes concentrações de hidróxido de cálcio. Quando os resultados dos diferentes tratamentos são compilados não se levando em consideração a presença ou ausência de $Ca(OH)_2$ ou EDTA, é possível se observar que as atividades específicas das enzimas tendem a se pronunciar de forma inversa a quantidade de biomassa quando em atmosfera anóxica (de -0.783 a -0.362, na tabela 2).

1.4 DISCUSSÃO

A *Candida albicans* não é um mero participante passivo no processo infeccioso. Ela secreta enzimas hidrolíticas (32) e tem desenvolvido uma bateria efetiva de fatores de virulência para colonizar os tecidos do hospedeiro, ultrapassar suas defesas e causar doença (16).

Este estudo demonstrou a virulência da *Candida albicans* frente a diferentes concentrações de hidróxido de cálcio, pela análise do comportamento de cinco enzimas, tanto na presença quanto na ausência de oxigênio. O EDTA (ácido etilenodiaminotetracético) foi incluído no estudo para se observar a virulência da *C. albicans* quando o íon cálcio estivesse ausente ou reduzido.

O crescimento celular de *C. albicans* SC5314 em SB e em presença de oxigênio molecular por 48 horas não apresentou divergência em relação àquele obtido em anóxia. Tal resultado é surpreendente, visto que, sendo considerada uma espécie facultativa (33), era de se esperar que a *C. albicans* tendesse a crescer de forma mais pronunciada em normóxia. É possível que após a fase de adaptação às condições normóxicas, a cepa tenha dirigido seu crescimento exclusivamente para uma forma miceliana (34), o que elevou a área de retenção do cristal violeta. Por outro lado, quando o SB recebeu a adição de EDTA, o crescimento em normóxia foi 9,37 vezes maior que em anóxia. A exposição continuada às diferentes concentrações de $Ca(OH)_2$ pareceu não exercer profundas alterações na biomassa, embora seja perceptível uma tendência de se elevarem as biomassas, sobretudo em normóxia, quando em presença do hidróxido de cálcio.

As aspartil-proteases são um tipo de enzima histolítica produzida por diversos tipos de microrganismos. Dentre os vários mecanismos de virulência presentes em *Candida albicans*, sua secreção é determinante no grau de patogenicidade do fungo (16, 35). A virulência da *C. albicans* parece estar relacionada ao nível de atividade de Sap *in vitro*. As aspartil-proteases facilitam a aderência da *C. albicans* a vários tipos de células e tecidos do hospedeiro. *C. albicans* utiliza as aspartil-proteases como enzimas ativas para modificar proteínas-alvo ou ligantes na superfície fúngica ou nas células do hospedeiro, o que pode alterar a hidrofobicidade de superfície ou levar a mudanças

conformacionais, melhorando a adesão do fungo (32). Neste estudo, a atividade de Sap se manteve constante nos diversos tratamentos. A exceção foi a expressão das enzimas por células crescidas em presença de EDTA.

A indução de hemólise é um importante fator de virulência de patógenos e que favorece o crescimento no hospedeiro em função da elevação na disponibilidade de ferro. A atividade hemolítica específica foi mais pronunciada em anóxia para células crescidas em SB e para células em presença de EDTA. Esta condição em anóxia pode se dever a *C. albicans* passar a produzir etanol e subsequentemente acetaldeído levando à sua fermentação. Os produtos dessa reação possuem atividade hemolítica (36). Ainda que sem significância estatística ($p > 0,05$), é perceptível uma tendência à manutenção desse comportamento nos tratamentos onde o $Ca(OH)_2$ foi adicionado. Tais resultados corroboram, ainda que não completamente, com os de Linares *et al*. (37) que reportaram que a atividade hemolítica de *C. albicans* se torna mais pronunciada quando é adicionada uma fonte de cálcio ao meio, embora, relatem que esse resultado não pode ser considerado absoluto, uma vez que algumas cepas mostraram inibição da atividade.

Dentre as enzimas analisadas, a esterase foi aquela que mostrou maior variabilidade nas amplitudes da atividade específica, provavelmente pela falta de especificidade pelo substrato. A despeito dessa ampla variabilidade, assim como para outros sistemas enzimáticos, o $Ca(OH)_2$ não induziu alterações significativas na atividade específica da enzima. A única ocorrência de disparidade entre cultivos normóxicos e anóxicos ocorreu em SB que não recebeu qualquer incorporação de cálcio. A princípio, poderia ser hipotetizado que uma disponibilidade muito baixa de Ca^{+2} no caldo-controle poderia ser responsável por tal diferença; contudo, a atividade específica das esterases na presença EDTA não apresentou diferenças dependentes de atmosfera de incubação. É possível que a remoção dos íons Ca^{+2} livres ou a presença do

quelante tenha normalizado a taxa de secreção e, mesmo com um número de células reduzido, a secreção se mantenha proporcional à biomassa. Tal achado é o único dentre as enzimas ensaiadas, o que parece ter valor prognóstico favorável, pois, para os demais sistemas enzimáticos, a anóxia levou a uma redução da biomassa que esteve inversamente associada a uma elevação na atividade enzimática.

Com exceção dos tratamentos onde o EDTA esteve presente, as condições atmosféricas de cultivo e diferentes concentrações de $Ca(OH)_2$ não promoveram oscilações significativas na atividade específica de fosfatases ácida e alcalina. Tal achado nos pareceu um tanto quanto inesperado, pois, assim como cepas selvagens de *C. albicans* tendem a produzir maiores quantidades de calcineurina, uma proteino-fosfatase dependente de cálcio, em condições de estresse por cálcio (38), era de se esperar que outras fosfatases também fossem produzidas em maior quantidade.

Com o intuito de se verificar a influência que as diferentes concentrações de $Ca(OH)_2$ exercem sobre a atividade enzimática específica em função das biomassas dos biofilmes, os resultados indicam a não existência de qualquer relação direta ou indiretamente proporcional entre a quantidade de células e a atividade enzimática em função da presença de diferentes concentrações de hidróxido de cálcio.

O EDTA pode ser considerado como tendo atividade antifúngica (39), uma vez que os íons cálcio apresentam um papel crítico na morfogênese, aderência e crescimento da *C. albicans*, (40). Tem sido proposto que o EDTA exerce sua elevada propriedade antifúngica quelando o cálcio presente tanto no meio de cultura quanto na parede celular (39). Entretanto, nossos resultados mostram que isso tende a ocorrer com maior intensidade em anóxia. Mesmo com redução da biomassa e consequente redução no número de células, uma elevada atividade específica para quase todas as enzimas foi

detectada. Com base nesses achados, é aconselhável uma maior ponderação na proposição do emprego de EDTA como um agente antifúngico na terapia endodôntica.

A ausência de oxigênio molecular é fator desencadeante para a maior secreção de algumas enzimas. Isso vem a corroborar com resultados publicados recentemente por parte deste grupo (33). A princípio poderia se supor que os resultados parciais envolvendo EDTA 1 mM e que foram compilados com os demais tratamentos fossem responsáveis por tais resultados; contudo, mesmo para esterase, que não apresentou discrepâncias entre cultivos normóxico e anóxico (Figura 4), os resultados indicaram uma considerável correlação positiva com significância estatística, o que nos leva a inferir que a condição atmosférica de incubação foi determinante para esse comportamento.

Neste estudo *in vitro* as diferentes concentrações de hidróxido de cálcio não reduziram a atividade enzimática da *Candida albicans*. Outros estudos baseados em fatores de virulência de *Candida albicans* se fazem necessários.

AGRADECIMENTOS

À Alessandra de Paula e Carvalho, agradecemos pelo suporte técnico.

APÊNDICE A – FIGURAS

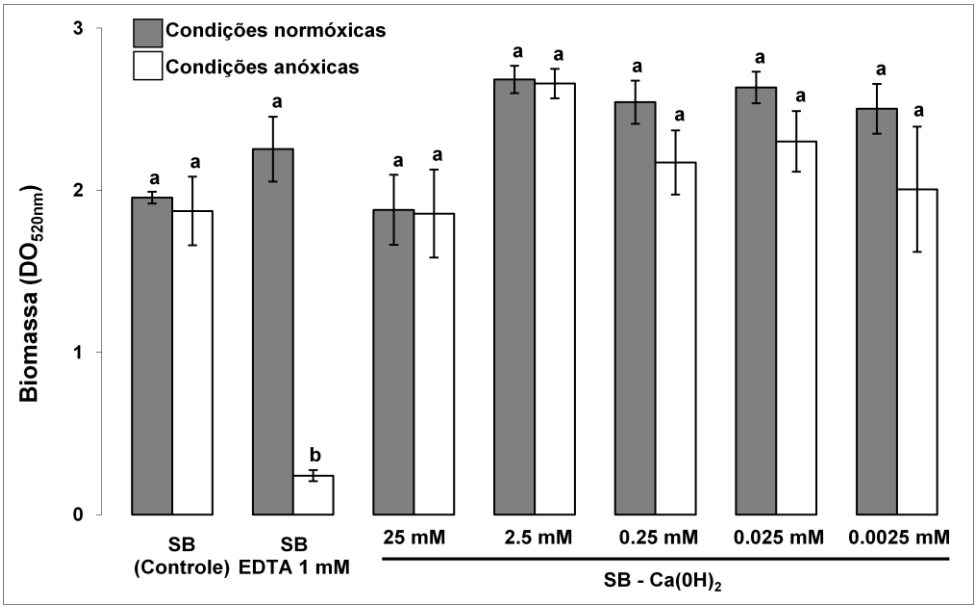

Figura 1- Biomassas em função do tipo de atmosfera: normóxica/anóxica

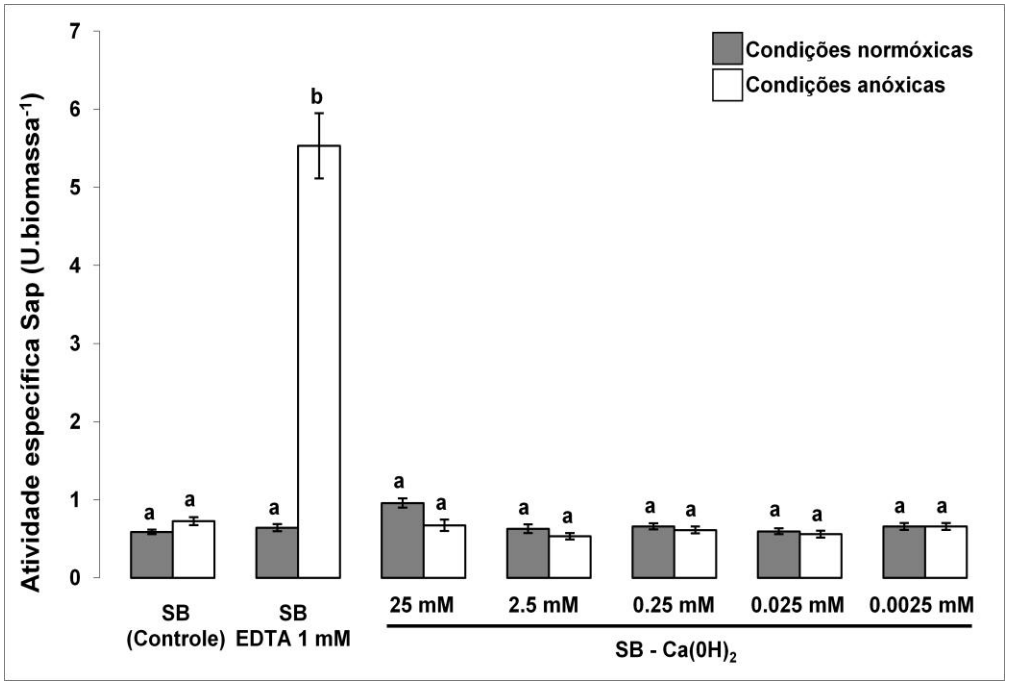

Figura 2 – Atividade específica de Sap

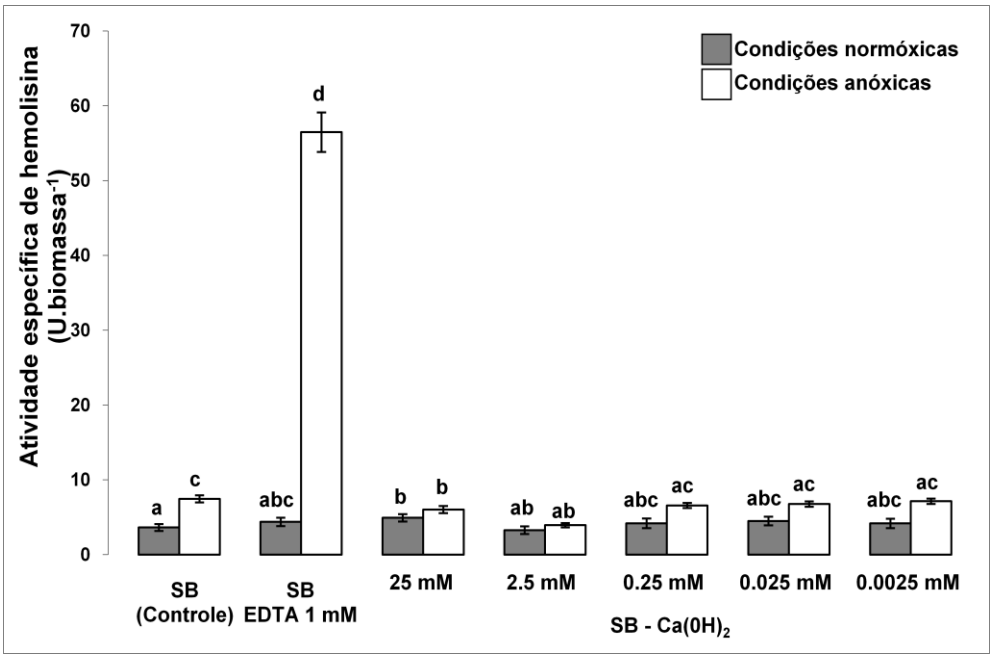

Figura 3 – Atividade específica de hemolisina

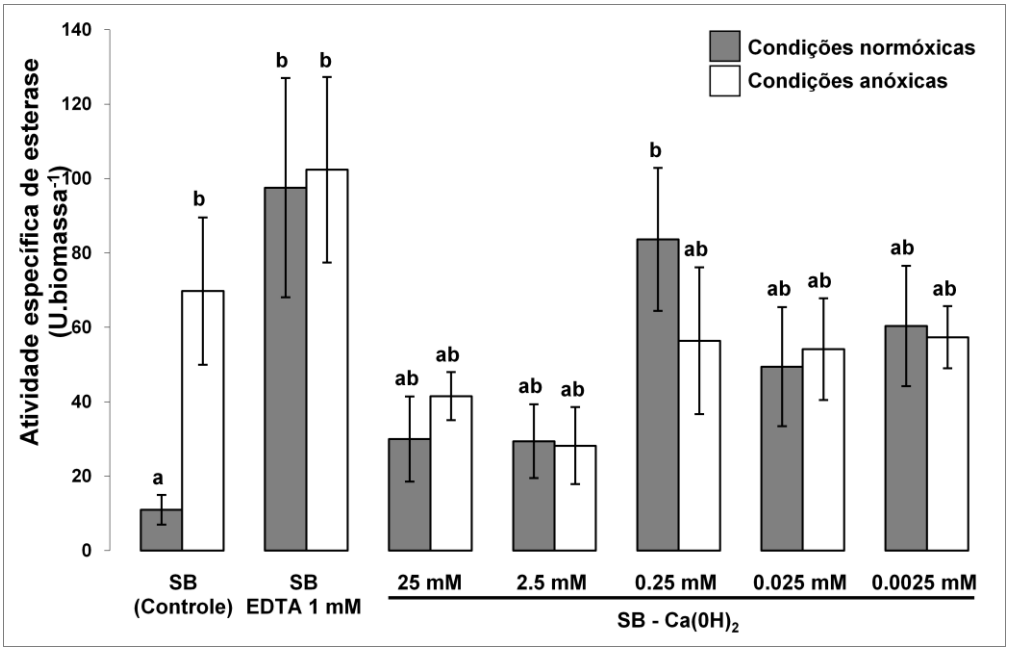

Figura 4 – Atividade específica de esterase

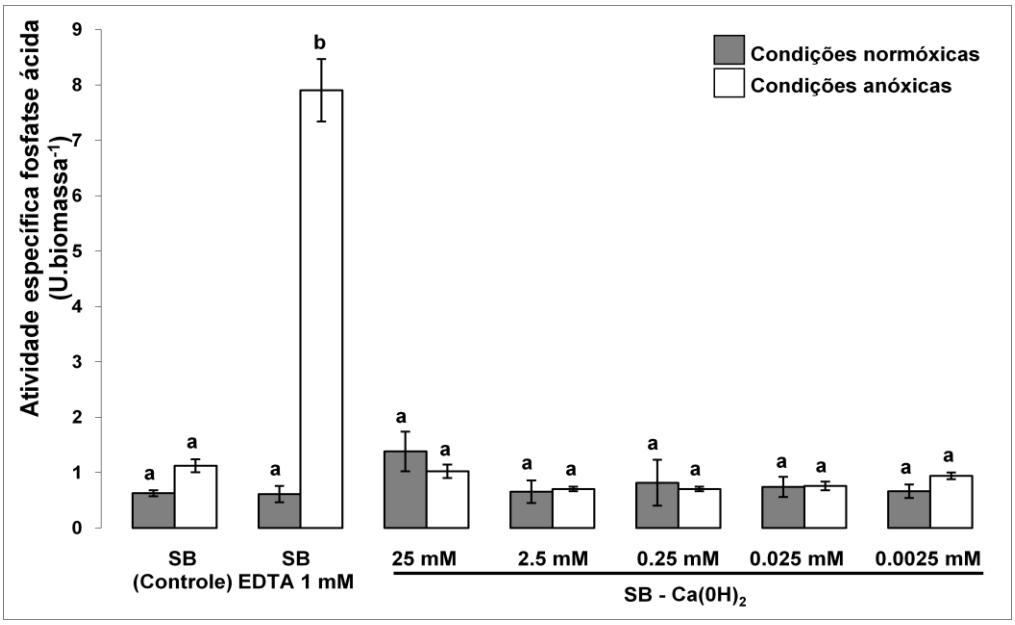

Figura 5 – Atividade específica de fosfatase ácida

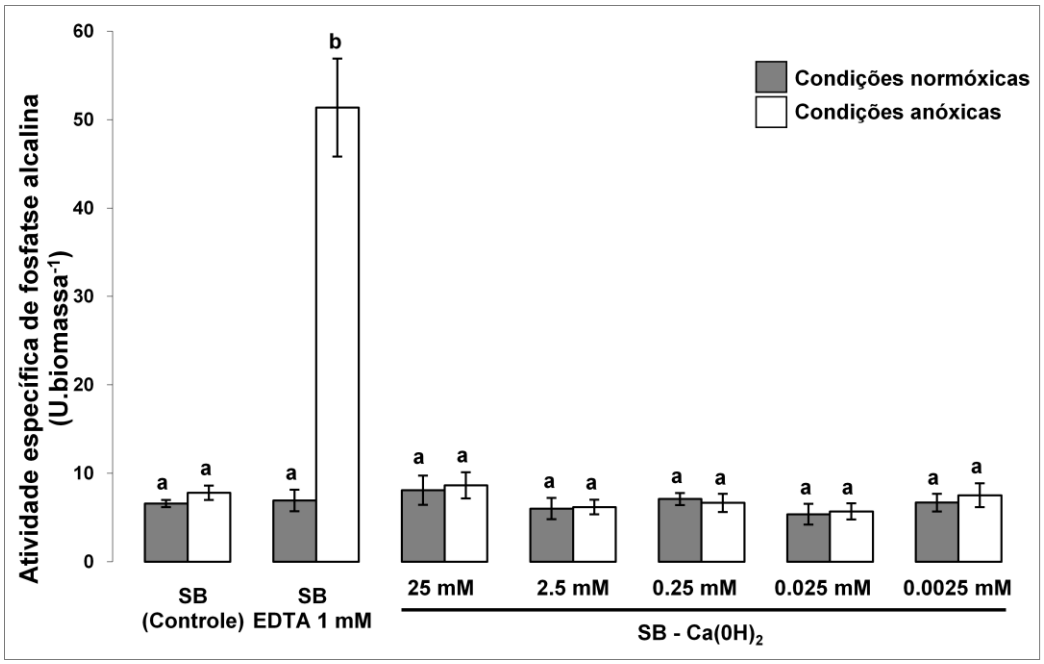

Figura 6 – Atividade específica de fosfatase alcalina

APÊNDICE B – TABELAS

Tabela 1. Correlação de amostras pareadas para atividades enzimáticas *vs* biomassa em diferentes condições atmosféricas e presença de $Ca(OH)_2$

Condições normóxicas	Correlação	Significância
Atividade enzimática em SB *vs* biomassa	0,097	0,611
Atividade enzimática em SB - 25 mM $Ca(OH)_2$ *vs* biomassa	0,162	0,393
Atividade enzimática em SB - 2.5 mM $Ca(OH)_2$ *vs* biomassa	-0,029	0,878
Atividade enzimática em SB - 0.25 mM $Ca(OH)_2$ *vs* biomassa	-0,050	0,795
Atividade enzimática em SB - 0.025 mM $Ca(OH)_2$ *vs* biomassa	-0,027	0,795
Atividade enzimática em SB - 0.0025 mM $Ca(OH)_2$ *vs* biomassa	0,113	0,888
Atividade enzimática em SB - EDTA 1 mM *vs* biomassa	0,188	0,552
Condições anóxicas	**Correlação**	**Significância**
Atividade enzimática em SB *vs* biomassa	-0,087	0,646
Atividade enzimática em SB - 25 mM $Ca(OH)_2$ *vs* biomassa	-0,189	0,318
Atividade enzimática em SB - 2.5 mM $Ca(OH)_2$ *vs* biomassa	-0,271	0,147
Atividade enzimática em SB - 0.25 mM $Ca(OH)_2$ *vs* biomassa	0,126	0,507
Atividade enzimática em SB - 0.025 mM $Ca(OH)_2$ *vs* biomassa	0,149	0,431
Atividade enzimática em SB - 0.0025 mM $Ca(OH)_2$ *vs* biomassa	0,038	0,843
Atividade enzimática em SB - EDTA 1mM *vs* biomassa	0,026	0,891

Tabela 2. Correlação de amostras pareadas para tratamentos *vs* biomassa em diferentes condições atmosféricas

Condições normóxicas	Correlação	Significância
Atividade específica de Sap *vs* biomassa	0,046	0,773
Atividade específica de hemolisina *vs* biomassa	0,762	0,000
Atividade específica de esterase *vs* biomassa	0,436	0,004
Atividade específica de fosfatase ácida *vs* biomassa	0,196	0,214
Atividade específica de fosfatase alcalina *vs* biomassa	0,702	0,000
Condições anóxicas	**Correlação**	**Significância**
Atividade específica de Sap *vs* biomassa	-0,739	0,000
Atividade específica de hemolisina *vs* biomassa	-0,783	0,000
Atividade específica de esterase *vs* biomassa	-0,362	0,018
Atividade específica de fosfatase ácida *vs* biomass	-0,743	0,000
Atividade específica de fosfatase alcalina *vs* biomassa	-0,780	0,000

MODULATING EXPRESSION OF VIRULENCE ATTRIBUTES OF *Candida albicans* TO CALCIUM HYDROXIDE

Camila Mendes Figueiredo*

Everdan Carneiro**

Edvaldo Antônio Ribeiro Rosa***

Vânia Portela Ditzel Westphalen**

Ulisses Xavier da Silva Neto**

Luiz Fernando Fariniuk**

Rosimeire Takaki Rosa****

*Endodontics Specialist; Microbiology Specialist;

**Specialist, Master and Doctor in Endodontics PUCPR, Paraná, Brasil;

***Pharmacist, Master and Doctor in OralBiolPathol, PUCPR, Paraná, Brasil.

****Pharmacist, Microbiology Specialist, Master in Oral Biol Pathol, PUCPR, Paraná, Brasil.

Correspondence to: Prof. Dr. Edvaldo Antônio Ribeiro Rosa

R. Imaculada Conceição, 1155, Prado Velho, 80215-901, Curitiba-PR, Brasil.

Tel.: 00 55 41 3271-2525 E-mail: edvaldo.rosa@pucpr.br

2 ARTIGO EM INGLÊS

MODULATING EXPRESSION OF VIRULENCE ATTRIBUTES OF *Candida albicans* TO CALCIUM HYDROXIDE

ABSTRACT

Objetives. The aim of this study was to evaluate the exacerbation or reduction of the virulence potential of *Candida albicans* exposed to different concentrations of calcium hydroxide based on the secretion of the enzymes acid and alkaline phosphatases, secreted aspartyl proteases, esterase and hemolysin.

Methods. The strain SC5314 was grown in anoxic and normoxic conditions 48 h. After the incubation period, they were inoculated in pure Sabouraud broth (SB) or containing serial dilutions of $Ca(OH)_2$ or 1 mM EDTA. After 48 h of incubation in both anoxic and normoxic conditions, the test-tubes were submitted to assays in order to determine the enzymatic activities. The data were analyzed statistically.

Results. It was not observed a decrease in the microorganism virulence when calcium hydroxide was present in the medium. In the presence of EDTA, there was a tendency to increase the enzymes secretion under anoxic conditions.

Conclusion. The presence of oxygen did not interfere in the cell growth of *Candida albicans*; the presence of calcium hydroxide did not interfere in the virulence of *Candida albicans*, even in the highest concentration.

2.1 INTRODUCTION

One of the most common intra-canal medications is a calcium hydroxide [$Ca(OH)_2$] paste, and it is widespread used (1). It is an alkali, pH around 12.5, and low soluble in water, and it is usually mixed to some liquid in order to form a paste (2). The high pH of calcium hydroxide gives it some properties such as: inactivation of bacterial enzymes, tissues enzymatic activation and mineralization induction (3-6). These effects are related with the dissolution of calcium hydroxide inside the water, releasing ions Ca^{2+} and OH^- (7, 8).

The calcium hydroxide powder must be mixed to some liquid in order to become a paste which must have as much particles of calcium hydroxide powder as possible. Also this paste must contact all the walls inside the root canal system, filling it completely (6).

Endodontic infections are polimicrobial (9). One of the microorganisms usually taking part in these infections, mainly when the root canal was exposed to the oral cavity, is the fungus *Candida albicans* (10).

Although many factors have been attributed to the virulence of *Candida albicans*, such as hyphal formation, recognizing molecules on its surface and phetotypic switching (11), the hydrolytic enzymes secretion has been pointed like an important factor associated to the disease caused by this species (12-18), once these enzymes are associated with cell destruction and extracellular matrix degradation. The strains which produce such enzymes are able to invade the host cells easier than those which do not produce.

Candida albicans can be considered normal microbiota in the oral cavity causing disease when the host has a problem (19, 20). The fungus present in the host saliva can

be carried into the root canal in the teeth with deep cavities and pulp exposition, in cases of trauma or when the absolute isolation is badly done (21, 22). Microleaked restorations after root canal treatment may also allow the penetration of *Candida albicans*, promoting the root canal recontamination (23).

Candida albicans is generally associated to persistent periapical lesions (22, 24-26). One of the alternative chemicals to eliminate this microorganism is the $Ca(OH)_2$ paste. Thus, a study to evaluate the potential virulence of *Candida albicans* exposed to several dilutions of $Ca(OH)_2$ is necessary.

2.2 MATERIAL AND METHODS

2.2.1 Preparation of aerobic inoculum of *C. albicans* SC5314

The cells were grown in Sabouraud broth (SB) at 37°C, 120 rpm and normoxia. After 24 h of growth they were centrifuged and the pellet was washed three times with sterile distilled water and suspended to a turbidity close to the tube # 3 on the MacFarland scale [$9x10^6$ colony-forming units per milliliter (CFU/mL)]. These cells were used as aerobic inoculum in subsequent experiments in normoxia.

2.2.2 Preparation of anaerobic inoculum of *C. albicans* SC5314

The cells were grown in Sabouraud broth (SB) at 37°C, 120 rpm and anoxia (pCO_2 10%, pN_2 90%). This procedure was repeated again so that the cells would adapt to the absence of molecular oxygen. After 24 h of growth they were centrifuged and the pellet was washed three times with sterile distilled water and suspended to a turbidity close to the tube # 3 on the MacFarland scale [$9x10^6$ colony-forming units per milliliter

(CFU/mL)]. These cells were used as anaerobic inoculum in subsequent experiments in anoxia.

2.2.3 Enzyme secretion inducer/inhibitor broth

Aliquots of 100 μL of aerobic or anaerobic inoculum were transferred to tubes containing SB plus $Ca(OH)_2$ pro-analysis to final concentrations of 25 mM, 2.5 mM, 250 mM, 25 mM and 2.5 mM. In order to ensure absence of Ca^{2+} ions it was included tubes containing SB plus 1 mM EDTA. As positive controls there it was included tubes containing only SB. The tubes were incubated in normoxic or anoxic atmosphere, at 37°C and 120rpm. After 48 h of incubation, the tubes were centrifuged at 10,000 X *g* and the supernatants were collected in sterile tubes to be used for testing the enzyme activity. The pellets were washed twice with sterile distilled water and subjected to the test for determination of biomass through the test retention of crystal violet.

The incubations in enzyme secretion broth inducer/inhibitor were conducted in triplicate, at two different times.

2.2.4 Quantification of secreted aspartyl proteases

Azocasein (5 mg/mL) was dissolved in 50 mM Tris-HCl (pH 5.0), 200 mM NaCl, 5 mM $CaCl_2$, 0.05% Triton X-100 and 0.01% sodium azide. Aliquots of 400 μL from this solution were mixed with 100 μL of supernatants. After incubation at 37°C for 2 hours, aliquots of 150 μL of 20% trichloroacetic acid were added. After 30 min at room temperature, the tubes were centrifuged at 16,000 X *g* (3 min) and the pellets were discarded. The supernatants were mixed with equal volumes of 1M NaOH and the OD_{440nm} were measured (27) in TP-plate reader reader® (ThermoPlate, Co.). One unit of enzyme activity was defined as the amount of enzyme required to increase OD_{440nm}

in 0.001 units of absorbance per minute of digestion (28). The specific enzyme activity was calculated to provide the amount of enzyme units per optical density of crystal violet retained by the biomass.

2.2.5 Quantification of esterases

Initially a standard curve of nine samples of sodium oleate was constructed by adding increasing volumes of 90 mM sodium oleate [in 20 mM Tris-HCl (pH 7.0)] in solution of 1% Tween® 80 and 80 mM $CaCl_2$ in 20 mM Tris-HCl (pH 7.0), with determination of DO_{450nm}.

The esterase activity was determined using Tween®80 as substrate and determining the OD_{450nm} (29). Aliquots of 100 μL from the supernatants were mixed to a solution of 900 μL of 1% Tween®80 and 80 mM $CaCl_2$ in 20 mM Tris-HCl (pH 7.0). After the addition of supernatants, the increase in OD_{450nm} was monitored within the time. An enzyme unit was defined as the amount of enzyme that releases 1 μmol of fatty acid per minute. The specific enzyme activity was calculated to provide the amount of enzyme units per optical density of crystal violet retained by the biomass.

2.2.6 Quantification of hemolysin

For quantification of hemolytic activity (30) the supernatants were serially diluted in a solution of 10 mM Tris-HCl containing 145 mM NaCl (pH 7.5). Aliquots of 100 μL of the dilutions were mixed with aliquots of 100 μL of 1% sheep erythrocytes [in 10 mM Tris-HCl containing 145 mM NaCl (pH 7.5)] and incubated at 37°C. After 2 hours of incubation, the suspensions were sedimented at 4°C for at least 15 h. The OD_{540nm} of the supernatants were determined. The negative controls contained equal volumes of buffer and suspension of erythrocytes. A hemolytic unit was defined as the

amount of material that smooths 50% of sheep erythrocytes. The specific hemolytic activity was calculated to provide the amount of hemolytic units per optical density of crystal violet retained by the biomass.

2.2.7 Quantification of alkaline phosphatase

For the quantification of alkaline phosphatase it was used the Alkaline Phosphatase Kit K019® (Quibasa LTDA, Belo Horizonte, Brazil).

Aliquots of 10 μL of the supernatants were dispensed in sterile tubes and the quantification of enzyme activity was performed following the manufacturer's recommendations. After 10 min of incubation at 37°C, the tubes were homogenized and it was determined the OD_{590nm}. After corrections by calibration factors with standard solution of a known concentration (40 U/L), the results were expressed in units per liter. The specific enzyme activity was calculated to provide the amount of enzyme units per optical density of crystal violet retained by the biomass.

2.2.8 Quantification of acid phosphatase

For the quantification of acid phosphatase it was used the kit Prostatic Acid Phosphatase K018® (Quibasa LTDA, Belo Horizonte, Brazil).

Aliquots of 20 μL of the supernatants were dispensed in sterile tubes and the quantification of enzyme activity was performed following the manufacturer's recommendations. After 30 min of incubation at 37°C, the tubes were homogenized and it was determined the OD_{590nm}. After corrections by calibration factors with standard solution of a known concentration (3 U/L), the results were expressed in units per liter. The specific enzyme activity was calculated to provide the amount of enzyme units per optical density of crystal violet retained by the biomass.

2.2.9 Quantification of biomass by the crystal violet test

The cells were washed five times with 200 μL of 145 mM NaCl sterile. Aliquots of 200 μL of 0.5% crystal violet (CV) were added to tubes (31). After 20 min, the excess of CV was removed by repeated washings with sterile water. Finally, the impregnated CV was released by the addition of 250 μL of 33% acetic acid. The OD_{590nm} were determined. The values of absorbance of the blanks were subtracted from the values obtained in the treatments to eliminate spurious results due to interference from background.

2.2.10 Statistical analysis

All the tests above were conducted in triplicate, in two different situations. The data were tabulated in Microsoft Excel® spreadsheets (Microsoft Co.). The statistical tests were conducted in the statistical package SPSS® 15.0 for Windows (SPSS Inc., Chicago). Data were tested for their normal distribution by Kolmogorov-Smirnov test and differences were accessed by multiple comparisons of Tukey HSD with a threshold of $p \leq 0.05$. Correlations of paired samples were obtained by comparing the data for each individual enzyme activity and biomass, both in normoxic and anoxic environment.

2.3 RESULTS

Cell growth of *C. albicans* SC5314 in SB and in the presence of molecular oxygen (normoxia) for 48 h showed no difference from that obtained in anoxia ($p = 0.12951$) (Figure 1). On the other hand, when SB received the addition of EDTA, the growth in normoxia was 9.37 times higher than obtained in anoxia ($p < 0.0001$).

Continuous exposure to different concentrations of $Ca(OH)_2$ seemed to not to provide profound changes in biomass ($p > 0.05$), although there is a tendency in raising the biomass, especially in normoxia, in the presence of $Ca(OH)_2$.

The specific activity of aspartyl proteases (Sap) remained constant in the different treatments ($p > 0.05$), with a mean activity of 0.652 ± 0.105 U.biomassa^{-1}. The exception was the expression of the enzymes from the cells grown in the presence of 1 mM EDTA in anoxic atmosphere, which was 8.478 times the average ($p < 0.0001$) (Figure 2).

The specific hemolytic activity was more pronounced in anoxia to cells grown in SB ($p < 0.0001$) and cells in the presence of 1 mM EDTA (10.95 times higer; $p < 0.0001$) (Figure 3).

Among all the enzymes analyzed, the esterase was the one that showed greater variability in the amplitude of specific activity (Figure 4). However, the only occurrence of difference between normoxic and anoxic cultivation occurred in SB ($p = 0.0180$). The $Ca(OH)_2$ did not induce significant changes in the specific activity of esterase. EDTA did not cause differences in terms of presence or absence of oxygen.

The atmospheric conditions of cultivation and the presence of $Ca(OH)_2$ did not lead to significant variations in the specific activity of acid and alkaline phosphatase (Figures 5 and 6). The exception was the expression of enzymes from the cells grown in the presence of 1 mM EDTA in anoxic conditions, which was higher than the average.

The atmospheric condition and the increasing concentrations of $Ca(OH)_2$ did not promote significant changes in specific activity of acid and alkaline phosphatase (Figures 5 and 6).

In order to verify the effect that different concentrations of $Ca(OH)_2$ have on the specific enzyme activity in terms of biomass, a correlation analysis was performed for paired samples (Table 1). The lack of significance in the correlation and the low extent surrounding zero (from -0.271 to 0.188, in Table 1) indicate the absence of any direct or indirect proportion between the amount of cells and enzyme activity in the presence of different concentrations of calcium hydroxide. When the results of different treatments are compiled not taking into account the presence or absence of $Ca(OH)_2$ or EDTA, it is possible to note that the specific activities of enzymes tend to be inverse to the amount of biomass under anoxic conditions (from -0.783 to -0.362, in Table 2).

2.4 DISCUSSION

Candida albicans is not a mere passive participant in the infectious process. It secretes hydrolytic enzymes (32) and has developed an effective battery of putative virulence factors to colonize the host tissue, cause disease and overcome the host defenses (16).

This study demonstrated the virulence of *Candida albicans* against the different concentrations of calcium hydroxide, analysing of the behavior of five enzymes in both presence and absence of oxygen. The EDTA (ethylenediamine-tetraacetic acid) was included in the study to observe the virulence of *C. albicans* when the calcium ion was absent or reduced.

The cell growth of *C. albicans* SC5314 in SB and in the presence of molecular oxygen for 48 h showed no difference on that obtained in anoxia. This result is surprising, since the species is considered facultative (33), it was expected that *C. albicans* tended to grow more in normoxia. It is possible that after the adaptation to the

normoxic conditions, the strain has led its growth to a mycelium form (34), what may have increased the retention area of the crystal violet. Moreover, when the SB received the addition of EDTA, the growth in normoxia was 9.37 times higher than in anoxia. Continuous exposure to different concentrations of $Ca(OH)_2$ seemed not to engage in profound changes in biomass, although there is a tendency to increase the biomass, especially in normoxia in the presence of calcium hydroxide.

The aspartil-protease enzyme is a kind of histolytic enzyme produced by various types of microorganisms. Among the various mechanisms of virulence in *Candida albicans*, its secretion is determining for the pathogenicity of the fungus (16, 35). The virulence of *C. albicans* appears to correlate with the level of Sap activity *in vitro*. Sap proteins facilitate *C. albicans* adherence to many host tissue and cells types. *C. albicans* utilizes Sap proteins as active enzymes to modify target proteins or ligands on the fungal surface or on host cells, which may alter surface hidrophobicity or lead to conformational changes, thus allowing better adhesion of the fungus (32).

In this study, Sap activity remained constant across treatments. The exception was the expression of enzymes in the cells grown in the presence of EDTA.

The induction of hemolysis is an important virulence factor of pathogens and it favors the growth inside the host due to the increase in availability of iron. The specific hemolytic activity was more pronounced in anoxia to cells grown in SB and in the presence of EDTA. This condition in anoxia may be due to the fact that *C. albicans* produce ethanol and acetaldehyde leading to its fermentation. The products of this reaction have hemolytic activity (36). Although not statistically significant ($p > 0.05$), there is a noticeable tendency to maintain this behavior in the treatments where the $Ca(OH)_2$ was added. These results support, but not completely, those from Linares *et al*.

(37) that reported that the hemolytic activity of *C. albicans* becomes more pronounced when a source of calcium is added to the medium, although, they reported that this result cannot be considered absolute, since some strains showed inhibition of activity.

Among the enzymes tested, esterase was the only one that showed greater variability in the amplitudes of the specific activity, probably by the lack of specific substrate. Despite this wide variability, as well as for other enzyme systems, $Ca(OH)_2$ did not induce significant changes in the specific activity of the enzyme. The only event of disparity between normoxic and anoxic cultivation occurred in SB that received no addition of calcium. In principle, it could be hypothesized that a low availability of Ca^{2+} in the broth-control could be responsible for this difference, however, the specific activity of esterases from EDTA did not differ depending on the incubation atmosphere. It is possible that the removal of free Ca^{2+} ions or the presence of the chelating agent has normalized the secretion rate and, even with a reduced number of cells, the secretion remains proportional to biomass. This finding is unique among the enzymes tested, which seems to have favorable prognostic value, because, for the other enzyme systems, the anoxia led to a reduction of biomass that was inversely associated with an increase in enzyme activity.

Except for treatments where the EDTA was present, the atmosphere conditions of cultivation and different concentrations of $Ca(OH)_2$ did not promote significant changes in specific activity of acid and alkaline phosphatases. This finding seems somehow unexpected, because as well as wild strains of *C. albicans* tend to produce larger quantities of calcineurin, a phosphatase-protein calcium-dependent, under conditions of stress caused by calcium (38), it should be expected that other phosphatases would be produced in a greater amount.

In order to verify the effect that different concentrations of $Ca(OH)_2$ have on the specific enzyme activity according to the biomass, the results indicate the absence of any direct or indirect proportion between the amount of cells and enzyme activity in the presence of different concentrations of calcium hydroxide.

EDTA can be considered with antifungal activity (39), because calcium ions have a critical role in morphogenesis, adherence and growth of *C. albicans*, (40) it has been proposed that EDTA exerts its high antifungal property by chelating calcium both in the culture medium and in the cell wall (39). However, these results show that this tends to occur with greater intensity in anoxia. Even with reduction of biomass and consequent reduction in the number of cells, a higher specific activity for almost all enzymes was detected. Based on these findings, it is recommended caution in the proposed use of EDTA as an antifungal agent in endodontic therapy.

The absence of molecular oxygen is the major triggering factor for secretion of some enzymes. This is corroborated with the results published recently by part of the group (33). At first glance it could be assumed that the partial results involving 1mM EDTA which were compiled with the other treatments were responsible for these results, however, even for esterase, which showed no differences between normoxic and anoxic cultures (Figure 4), the results indicate a significant positive correlation with statistical significance, which leads us to infer that the atmospheric condition of incubation was critical for this behavior.

In this *in vitro* study the different concentrations of calcium hydroxide did not reduce the enzyme activity of *Candida albicans*. Further studies based in virulence factors of *Candida albicans* are necessary.

ACKNOWLEDGMENTS

We thank Alessandra de Paula e Carvalho for technical support.

APENDIX A – FIGURES

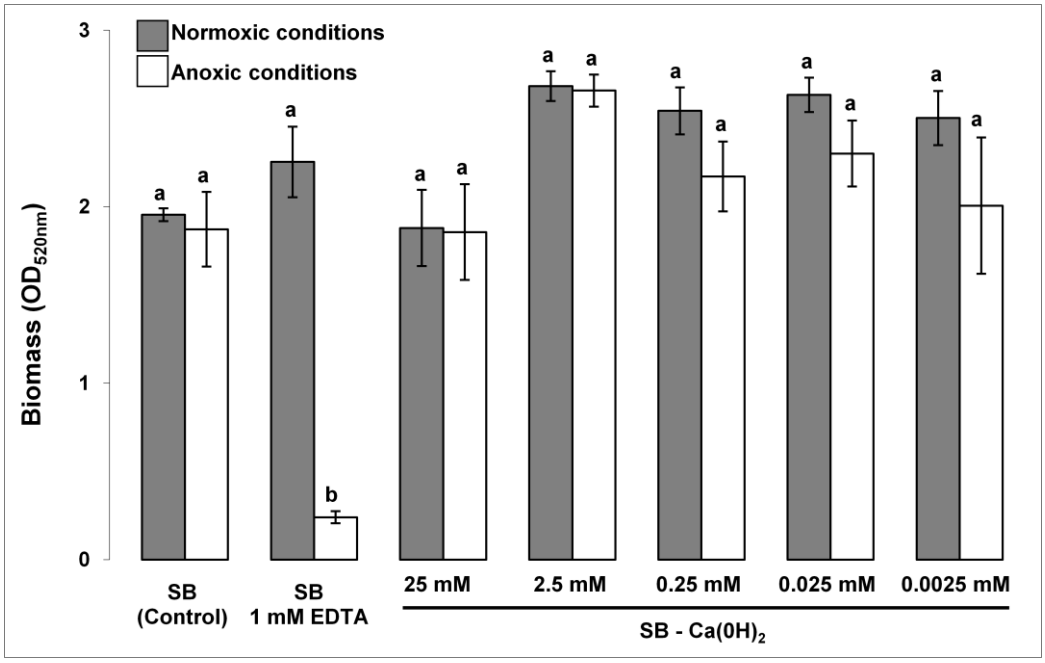

Figure 1 – Biomass in function of condition: anoxic/normoxic

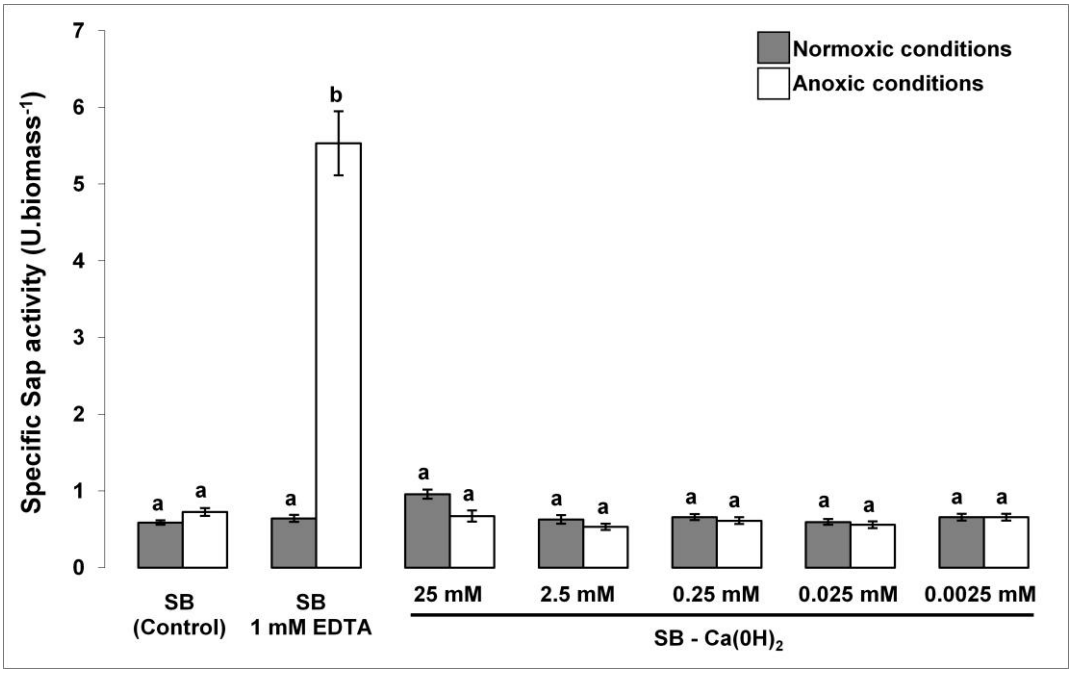

Figure 2 – Specific Sap activity

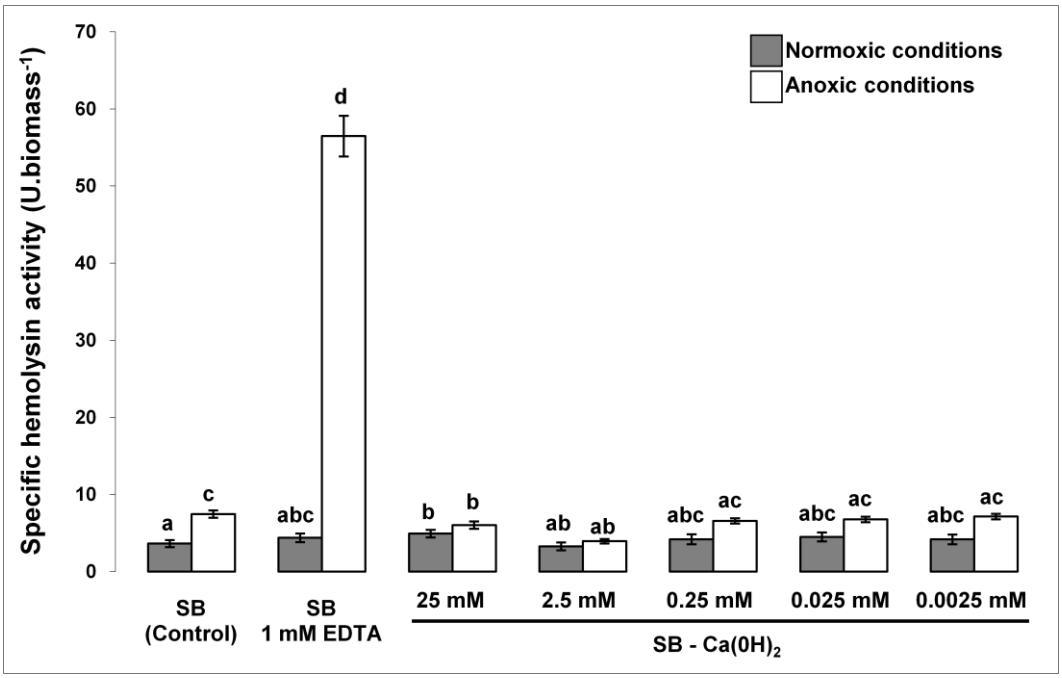

Figure 3 – Specific hemolysin activity

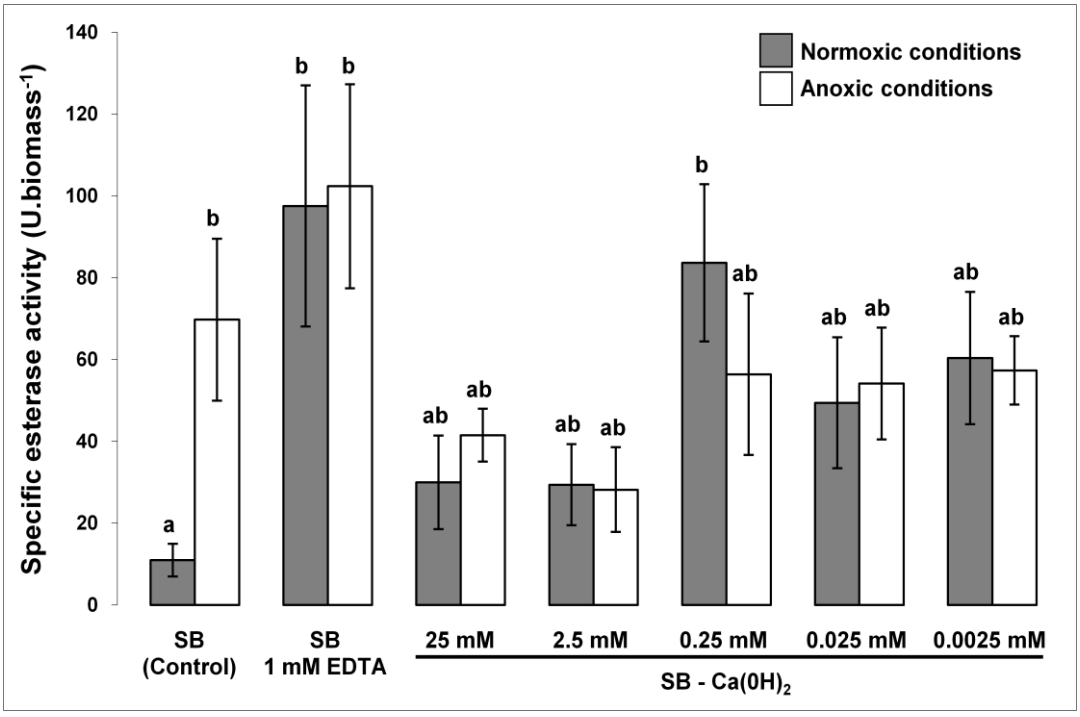

Figure 4 – Specific esterase activity

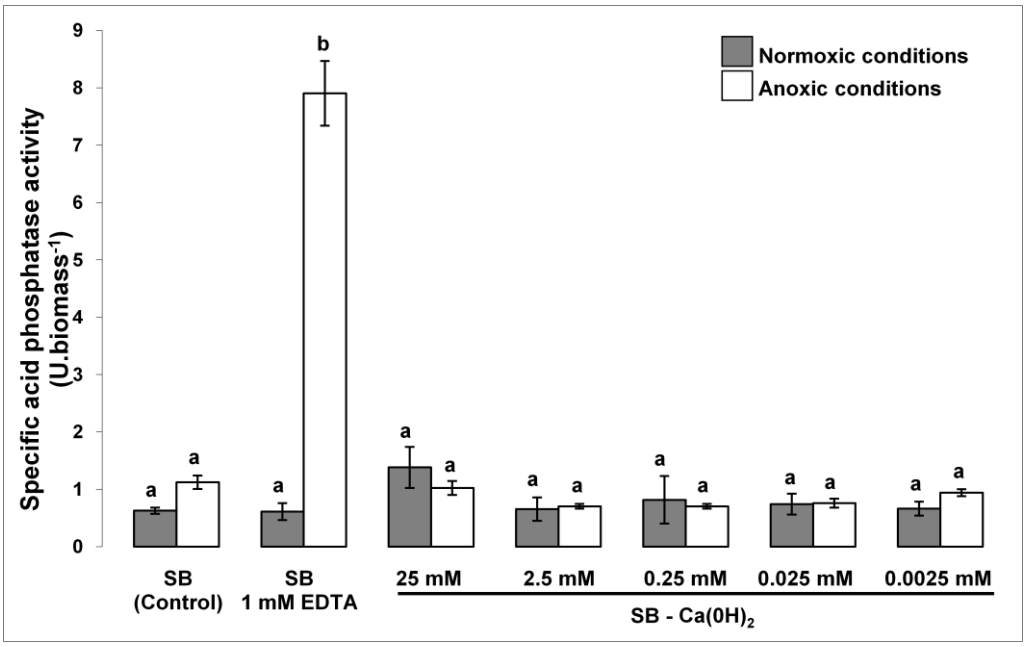

Figure 5 – Specific acid phosphatase activity

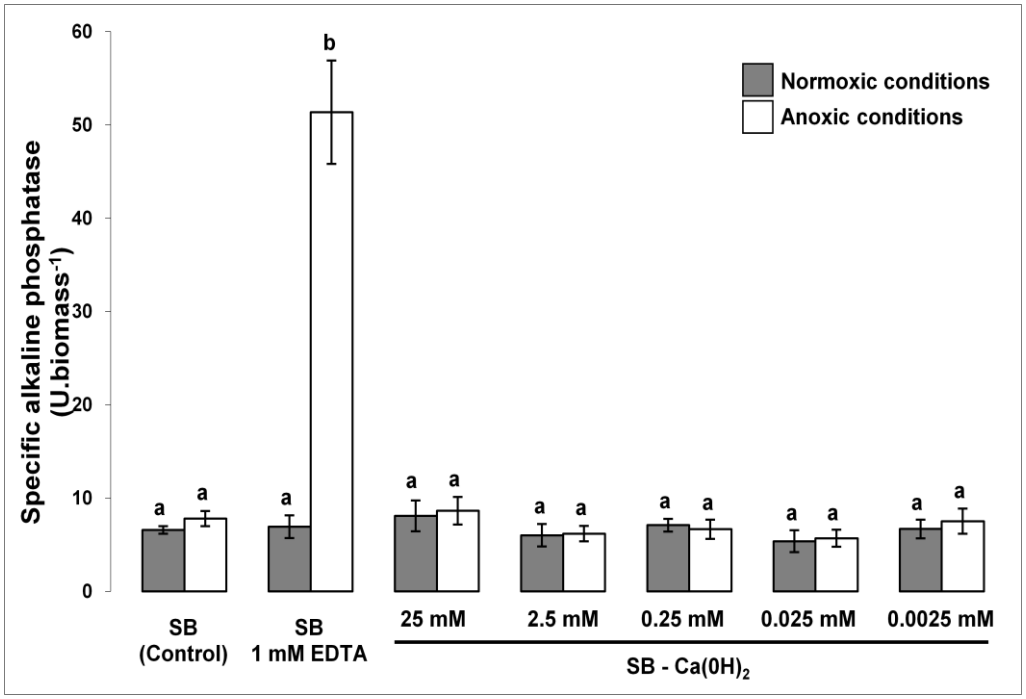

Figure 6 – Specific alkaline phosphatase activity

APENDIX B – TABLES

Table 1. Paired samples correlations for overall enzymatic activities *vs* biomass under different atmospheric conditions and presence of $Ca(OH)_2$

Normoxic conditions	Correlation	Significance
Enzymatic activity in SB *vs* biomass	0,097	0,611
Enzymatic activity in SB - 25 mM $Ca(OH)_2$ *vs* biomass	0,162	0,393
Enzymatic activity in SB - 2.5 mM $Ca(OH)_2$ *vs* biomass	-0,029	0,878
Enzymatic activity in SB - 0.25 mM $Ca(OH)_2$ *vs* biomass	-0,050	0,795
Enzymatic activity in SB - 0.025 mM $Ca(OH)_2$ *vs* biomass	-0,027	0,795
Enzymatic activity in SB - 0.0025 mM $Ca(OH)_2$ *vs* biomass	0,113	0,888
Enzymatic activity in SB - 1 mM EDTA *vs* biomass	0,188	0,552
Anoxic conditions	**Correlation**	**Significance**
Enzymatic activity in SB *vs* biomass	-0,087	0,646
Enzymatic activity in SB - 25 mM $Ca(OH)_2$ *vs* biomass	-0,189	0,318
Enzymatic activity in SB - 2.5 mM $Ca(OH)_2$ *vs* biomass	-0,271	0,147
Enzymatic activity in SB - 0.25 mM $Ca(OH)_2$ *vs* biomass	0,126	0,507
Enzymatic activity in SB - 0.025 mM $Ca(OH)_2$ *vs* biomass	0,149	0,431
Enzymatic activity in SB - 0.0025 mM $Ca(OH)_2$ *vs* biomass	0,038	0,843
Enzymatic activity in SB - 1 mM EDTA *vs* biomass	0,026	0,891

Table 2. Paired samples correlations for overall treatments *vs* biomass under different atmospheric conditions

Normoxic conditions	Correlation	Significance
Specific Sap activity *vs* biomass	0,046	0,773
Specific hemolysin activity *vs* biomass	0,762	0,000
Specific esterase activity *vs* biomass	0,436	0,004
Specific acid phosphatase activity *vs* biomass	0,196	0,214
Specific alkaline phosphatase activity *vs* biomass	0,702	0,000
Anoxic conditions	**Correlation**	**Significance**
Specific Sap activity *vs* biomass	-0,739	0,000
Specific hemolysin activity *vs* biomass	-0,783	0,000
Specific esterase activity *vs* biomass	-0,362	0,018
Specific acid phosphatase activity *vs* biomass	-0,743	0,000
Specific alkaline phosphatase activity *vs* biomass	-0,780	0,000

REFERÊNCIAS

1 – Baumgartner JC, Watts CM, Xia T. Occurrence of *Candida albicans* infections of endodontic origin. J Endod 2000;28(12):695-8.

2 – Cruz EV, Kota K, Huque J, Iwaku M, Hoshino E. Penetration of propylene glycol into dentine. Int Endod J 2001;35:330-6.

3 – Pashley DH, Kalathoor S, Burnham D. The effects of calcium hydroxide on dentin permeability. J Dent Res 1986 ;65(3):417-20.

4 – Sjögren U, Figdor D, Spangberg L, Sundqvist G. The antimicrobial effect of calcium hydroxide as a short-term intracanal dressing. Int Endod J 1991;24:119-125.

5 – Wakabayashi H, Horikawa M, Funato A, Onodera A. Bio-microscopical observation of dytropic calcification induced by calcium hydroxide. Endod Dent Traumatol 1993;4(9):165-170.

6 – Estrela C, Sydney GB, Bammann LL, Felipe Jr O. Mechanism of action of calcium and hydroxyl ions of calcium hydroxide on tissue and bacteria. Braz Dent J 1995;6:85-90.

7 – Safavi K, Nakayama TA. Influence of mixing vehicle on dissociation of calcium hydroxide in solution. J Endod 2000;16(11):649-51.

8– Yoldas O, Dogan C, Seydaoglu G. The effect of two different calcium hydroxide combinations on root dentine microhardness. Int Endod J 2004;37:828-31.

9 – Sundqvist G, Nair PN, Sjögren U, Krey G, Kahnberg KE. Intraradicular bacteria and fungi in root-filled, asymptomatic human teeth with therapy resistant periapical lesions:

a long-term light and electron microscopic follow-up study. J Endod 1990;16(12):580-8.

10 – McCullough MJ, Ross BC, Reade PC. *Candida albicans*: a review of its history; taxonomy, virulence attributes and methods of strain differentiation. Int J Oral Maxillofac Surg 1996;25:136-144.

11 – Calderone RA, Fonzi WA. Virulence factors of *Candida albicans*. Trends Microbiol 2001;7(9):327-35.

12 - Kothavade R J, Pathanki M H. Evaluation of phospholipase activity of *Candida albicans* and its correlation with pathogenicity in mice. J Med Microbiol 1998;47:99-102.

13 – Oliveira EE, Silva SC, Soares AJ, Attux C, Cruvinel B, Silva R. Killer toxin and enzyme production by *Candida albicans* isolated from buccal mucosa in patients with cancer. Rev Soc Bras Med Trop 1998;31:523-27.

14 – Ghannoum MA. Extracellular phospholipases as universal virulence factor in pathogenic fungi. Nippon Ishinkin Gakkai Zasshi 1998;39:55-9.

15 – Ghannoum MA. Potential role of phospholipases in virulence and fungal pathogenesis. Clin Microbiol Rev 2000;13:122-143.

16 – Naglik JR, Challacombe SJ, Hube B. *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. Microbiol Mol Biol Rev 2003;67(3):400-28.

17 – Shimizu MT, Almeida NO, Fantinato V, Unterkirsher CS. Studies on hyaluronidase; chondroitin sulphatase; proteinase and phospholipase secreted by *Candida* species. Mycoses 1996;39:161-7.

18 – Abu-Elteen KH, Elkarmi AZ, Hamad M. Characterization of phenotype-based pathogenic determinants of various *Candida albicans* strains in Jordan. Jpn J Infect Dis 2001;54:229-36.

19 – Wu T, Wright K, Hurst SF, Morrison CJ. Enhanced extracellular production of aspartyl proteinase, a virulence factor, by *Candida albicans* isolates following growth in subinhibitory concentrations of fluconazole. Antimicrob Agents Chemother 2000;44(5):1200-8.

20 – Siqueira Jr JF, Rocas IN, Lopes HP, Elias CN, Uzeda M. Fungal infection of the radicular dentin. J Endod 2001;28(11):770-3.

21 – Waltimo TM, Siren EK, Orstavik D, Haapasalo MP. Susceptibility of oral *Candida* species to calcium hydroxide *in vitro*. Int Endod J 1999;32(2):94-8.

22 – Egan MW, Spratt DA, Ng YL, Lam JM, Moles DR, Gulabilava K. Prevalence of yeasts in saliva and root canals of teeth associated with apical periodontitis. Int Endod J 2002;35(4):321-9.

23 – Hommez GM, Verhelst R, Clayes G, Vaneechouette M, De Moor RJ. Investigation of the effect of the coronal restoration quality on the composition of the root canal microflora in teeth with apical periodontitis by means of T-RFLP analysis. Int Endod J 2004;37(12):819-27.

24 – Waltimo TM, Siren EK, Torkko HLK, Olsen I, Haapasalo, MPP. Fungi in therapy-resistant apical periodontitis. Int Endod J 1997;30:96-101.

25 – Waltimo TM, Kuusinen M, Jäversivu A, Nyberg P, Väänänen A, Richardson M.; Salo T, Tjäderhane L. Examination on *Candida* spp. in refractory periapical granulomas. Int Endod J 2003;36(9):643-7.

26 – Adib V, Spratt D, Ng YL, Gulabilava K. Cultivable microbial flora associated with persistent periapical disease and coronal leakage after root canal treatment: a preliminary study. Int Endod J 2004;37(8):542-51.

27 – Vasquez PD, Cantera AM. A simple and rapid technique for postelectrophoretic detection of proteases using azocasein**. Eletrophoresis** 1995;16(10):1894-7.

28 – Pande M, Dubey VK, Yadau SC, Jagannadhan MV. A novel serine protease cryptolepain from *Cryptolepis buchanani*: purification and biochemical characterization. J Agric Food Chem 2006;54(26):10141-50.

29 – Plou FJ, Ferrer M, Nuero OM. Analysis of Tween 80 as an esterase/lipase substrate for lipolytic activity assay. Biotechnol Tech 1998;12:183-6.

30 – Frey J, Nicolet J. Regulation of hemolysin expression in *Actinobacillus pleuropneumoniae* serotype 1 by Ca^{2+}. Infec Immun 1998;56(10):2570-5.

31 – Peeters E, Nelis HJ, Coenye T. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. J Microbiol Meth 2008;72:157-65.

32 – Chaffin WL, López-Ribot JL, Casanova M, Gozalbo D, Martinez JP. Cell wall and secreted proteins of *Candida albicans*: identification, function, and expression. Microbiol Mol Biol Rev 1998;62(1):130-80.

33 – Rosa EA, Rached RN, Ignácio SA, Rosa RT, José da Silva W, Yau JY, Samaranayake LP. Phenotipic evaluation of the effect of anaerobiosis on some attributes of *Candida albicans*. J Med Microbiol 2008;57:1277-81.

34 – Dumitru R, Hornby JM, Nickerson KW. Defined anaerobic growth medium for studying *Candida albicans* basic biology and resistance to eight antifungal drugs. Antimicrob Agents Chemother 2004;48(7):2350-4.

35 – De Bernardis F, Chiani P, Ciccozi M, Pellegrini G, Ceddia T, D'Offizzi G, Quinti I, Sullivan P, Cassone A. Elevated aspartic proteinase and experimental pathogenicity of *Candida albicans* isolates from oral cavities of subjects infected with human immunodeficiency virus. Infec & Immun 1996;2:466-71.

36 – Ogasawara A, Odahara K, Toume M, Watanabe T, Mikami T, MatsumotoT. Change in the respiration system of *Candida albicans* in the lag and log growth phase. Biol Pharm Bull 2006;29(3).

37 – Linares CEB, Loreto ES, Silveira CP, Pozzatti P, Scheid LA, Santurio JM, Alves SH. Enzymatic and hemolytic activities of *Candida dubliniensis* strains. Rev Inst Med Trop S Paulo 2007;49(4):203-6.

38 – Blankenship JR, Heitman J. Calcineurin is required for *Candida albicans* to survive calcium stress in serum. Infect Immunol 2005;73(9):5767-74.

39 – Sen BH, Akdeniz BG, Denizei AA. The effect of ethylenediamine-tetracetic acid on *Candida albicans*. Oral Surg Oral Med Oral Pathol 2000;90(5):651-5.

40 – Klotz SA, Rutten MJ, Smith RL, Babcock SR, Cunningham MD. Adherence of *Candida albicans* to immobilize extracellular matrix proteins is mediated by calcium-dependent surface glycoproteins. Microb Pathol 1993;14:133-47.

ANEXO A – NORMAS DA REVISTA ORAL SURGERY, ORAL MEDICINE, ORAL PATHOLOGY, ORAL RADIOLOGY AND ENDODONTOLOGY

GUIDE FOR AUTHORS

Submission of Manuscripts. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology* uses an online, electronic submission system. By accessing the website http://ees.elsevier.com/tripleo authors are stepwise through the creation and uploading of the various files. When submitting a manuscript to the Elsevier Editorial System (EES), authors must provide an electronic version of their manuscript. For this purpose original source files, not PDF files, are required. The author should specify an article type for the manuscript (full length article, review article, case report, etc.), choose a set of classifications from the prescribed list provided online, and suggest the appropriate Journal section. Authors may send queries concerning the submission process, manuscript status, or Journal procedures to the Editorial Office. Once the submission files are uploaded, the system automatically generates an electronic (PDF) proof, which is then used for reviewing. All correspondence, including the Editor's decision and request for revisions will be communicated by e-mail.

International authors who are not completely fluent in the English language should seek help in the preparation of their manuscripts. Such assistance will enhance the review, improve the chance of acceptance, and greatly reduce the time until publication if the article is accepted.

If your manuscript is accepted, the Editors reserve the right to determine whether it will be published in the print edition or solely in the Internet edition of the Journal. Articles accepted for publication are subject for editorial revision.

Statements and opinions expressed in the articles and communications herein are those of the author(s) and not necessarily those of the Editor(s) or publisher, and the Editor(s) and publisher disclaim any responsibility or liability for such material. Neither the Editor(s) nor the publisher guarantees, warrants, or endorses any product or service advertised in this publication. Neither do they guarantee any claim made by the manufacturer of such product or service.

Duality of Interests. Any commercial or other associations that might create a duality of interests in connection with a submitted manuscript must be disclosed. All sources of external funds supporting the work must be indicated in a footnote, as should all corporate affiliations of the authors including author(s) relationship with a corporate entity involved with the subject of the research or product being espoused in the submission. A cover letter at the time of submission should inform the Editor of pertinent consultancies, stock ownership or other equity interests, or patent licensing arrangements. All information will remain confidential while the paper is being reviewed and will not influence the editorial decision. If the manuscript is accepted, the Editor will communicate with the authors how best to disclose the relevant information.

Publication Standards of Ethical Conduct. Submitting manuscripts for publication that contain elements of fabrication, falsification, or plagiarism constitutes a major violation of the universally accepted standards of ethical and scientific conduct.

Articles falling into the following categories are invited for submission:

Review manuscripts. Manuscripts that review the current status of a given topic, diagnosis, or treatment are encouraged. These manuscripts should not be an exhaustive review of the literature, but rather should be a review of contemporary thought with respect to the topic. Likewise, the bibliography should not necessarily be all-inclusive but rather include only seminal, pertinent, and contemporary references deemed to be most important by the author.

Clinicopathologic Conference. Papers submitted for the Clinicopathologic Conference (CPC) should present interesting, challenging, or unusual cases. The presentation should simulate clinical work-up, including a differential diagnosis. The complete diagnostic evaluation, management, and follow-up must be included. CPC articles will be organized into five parts: *Clinical presentation*-describe the clinical and imagining characteristics of the lesion. Use clinical photographs and radiographs as appropriate. *Differential diagnosis*-list and discuss lesions to be considered as reasonable diagnostic possibilities. *Diagnosis*-histopathologic findings illustrated with photomicrographs.*Management*-describe the treatment of the patient and response to treatment. *Discussion*-concentrate on the most interesting aspect(s) of the case.

Medical Management and Pharmacology Update. The Medical Management and Pharmacology Update (MMPU) is intended to provide concise, current reviews of medical problems and how they relate to dentistry. Manuscripts should include a good review of the clinical aspects of the disease, stressing the impact of the disease on the dental management and dental treatment of the patient. Emphasis should be placed on new developments, new research, or new approaches to therapy or management. Manuscripts should not be an exhaustive review of the literature, but rather a review of contemporary thought with respect to the topic. Likewise, the bibliography need not be all inclusive but rather should include only seminal, contemporary references deemed by the author to be most pertinent. The desired format for manuscripts submitted for the MMPU would include: an abstract; topic introduction/overview; epidemiology/demographics; etiology and pathogenesis; clinical presentation/physical findings; diagnosis (laboratory tests, diagnostic imaging, etc.); medical management and treatment; complications; prognosis; oral manifestations/dental implications and significance; and dental management (of patients with the disease). Manuscripts should not exceed 12 pages in 12 point, double-spaced Times New Roman (Tables and Figures count toward the 12-page limit).

Pharmacology Update is a component section of MMPU that offers the reader the opportunity to obtain concise information regarding drugs used in the practice of medicine, clinical dentistry and dental specialties. Papers submitted should present clearly and concisely background information regarding the disease or condition that is managed, the indications, rational and approved uses of the specific drugs or class of drugs, the advantages and benefits of the drug or drug class over previous drugs, mechanism of action, criteria for selection, usual dosage, pharmacokinetics, adverse effects, drug interactions, and oral health and dental management considerations.

Emphasis should be placed on new developments, effectiveness in clinical trials, therapeutic outcomes and safety. Manuscripts should reflect the contemporary thought with respect to the topic. Use of figures to illustrate the mechanism of action, and tables to presents therapeutic outcomes, drug interactions, and adverse effects are encouraged. Manuscripts should utilize the above mentioned categories for formatting the paper. Papers should not exceed 3000 words. The recommended font is 12 point, double spaced Times New Roman. A maximum of 50 references is recommended.

Clinical Notes. The Clinical Notes feature is intended to provide a forum for brief communications of a technical nature. They are not scientific papers; they may report a new instrument, technique, procedure, or, in rare situations, an interesting case report.

Copyright statement. The specified copyright statement that follows the Information for Authors in each issue of the Journal must be completed, **signed by all authors**, and faxed to the Editorial Office at (601)984-4949. If not completed in full, it will be returned to the author for completion. The copyright statement may be photocopied for submission or scanned and e-mailed.

Preparation of manuscripts. Only original manuscripts that have not been published in other forms will be considered for publication. Correct preparation of the manuscript by the author will expedite the reviewing and publication procedures. Manuscripts should be word processed double-spaced. Please note the following requirements and the instructions for online submission at:

http://ees.elsevier.com/tripleo

The article, including all tables, should be formatted in the latest version of Microsoft Word. The use of appropriate subheadings throughout the body of the text (Methods, Results, and Discussion sections) is required. Legends for figures and tables should appear after the reference list. If an illustration has been taken from published material, the legend must give full credit to the original source. Illustrations must also be submitted electronically as separate files (not embedded). File specifications are listed below in "Illustrations." Tables should be submitted as separate files (in Microsoft Word (*.doc) format.)

Routine case reports add little to our knowledge, but good case reports may occasionally be published if they meet certain criteria: (1) are of rare or unusual lesions that need documentation, (2) are well documented cases showing unusual or "atypical" clinical or microscopic features or behavior, or (3) are cases showing good long-term follow-up information, particularly in areas in which good statistics on results of treatment are needed.

Title Page. The title page of the manuscript should include the title of the article, the full name of the author(s), academic degrees, positions, and institutional affiliations. Listed authors should include only those individuals who have made a significant creative contribution. The corresponding author's address, business and home telephone numbers, fax number and e-mail address should be given.

Authorship. All persons who are identified as authors must have made substantial contribution to the manuscript through significantly contributing to the conception, design, analysis or interpretation of data; drafting or significantly revising the manuscript; and providing final approval of the manuscript. All three of these conditions must be met by each author. Persons who contribute to the effort in supporting roles should not be included as authors; rather they should be acknowledged at the end of the paper.

Abstract. An abstract of no more than 150 words, typewritten double-spaced, should precede the introduction to the article and must accompany each manuscript.

Structured abstract. A structured abstract limited to 150 words must be used for data-based research articles. The structured abstract is to contain the following major headings:*Objective(s); Study Design; Results; and Conclusion(s).* The*Objective(s)* reflects the purpose of the study, that is, the hypothesis that is being tested. The *Study Design* should include the setting for the study, the subjects (number and type), the treatment or intervention,

and the type of statistical analysis. The*Results* include the outcome of the study and statistical significance if appropriate. The *Conclusion(s)* states the significance of the results.

Methods. The methods section should describe in adequate detail the experimental subjects, their important characteristics, and the methods, apparatus, and procedures used so that other researchers can reproduce the experiment. When the paper reports experiments on human subjects, the methods section must indicate that the protocol was reviewed by the appropriate institutional review board (IRB) and that each subject in the project signed a detailed informed consent form.

Animals. Please indicate that protocols were reviewed by the appropriate institutional committee with respect to the humane care and treatment of animals used in the study.

References. References should be cited selectively. Personal communications and unpublished data are not to be cited as references; instead, are to be cited in parentheses at the appropriate place in the text. Make sure all references have been verified and are cited consecutively in the text (not including tables) by superscript numbers. Reference list format must conform to that set forth in "Uniform Requirement for Manuscripts Submitted to Biomedical Journals" (Ann Intern Med 1997;126:36-47). A copy of these Requirements may be viewed/printed online at www.icmje.org. References to articles in press must include authors' surnames and initials, title of article, and name of journal. The reference list should be typed double-spaced on a separate page and numbered in order as the reference citations appear in the text. For journal citations, include surnames and initials of authors, complete title of article, name of journal (abbreviated according to the Cumulated Index Medicus), year of publication, volume, number, and inclusive page numbers. For book citations, surnames and initials of authors, chapter title (if applicable), editors' surnames and initials, book title, volume number (if applicable), edition number (if applicable), city and full name of publisher, year of publication, and inclusive page numbers of citation.

EXAMPLES (if six or fewer authors, list all; if seven or more list first six and add *et al*):

Format for periodical references: Pullon PA, McGivney J. Computer utilization in an oral biopsy service. Int J Oral Surg 1977;6:251-5.

Format for book references: Seakins J, Saunders R, editors. Treatment of inborn errors of metabolism. London: Churchill Livingstone: 1973; p. 51-6.

Format for chapter references: Hudson FB, Hawcroft J. Duration of treatment in phenylketonuria. In: Seakins J, Saunders R, editors. Treatment of inborn errors of metabolism. London: Churchill Livingstone; 1973. p. 51-6.

Journal article on the Internet: Abood S. Quality improvement initiative in nursing homes: the ANA acts in an advisory role. Am J Nurs [serial on the Internet]. 2002 Jun [cited 2002 Aug 12];102(6):[about 3 p.]. Available from:

http://www.nursingworld.org/AJN/2002/june/Wawatch.htm

Illustrations. Illustrations should be numbered and provided with suitable legends.

A reasonable number of halftone illustrations or line drawings will be reproduced at no cost to the author, but special arrangements must be made with the Editor-in-Chief for color plates, elaborate tables, or extra illustrations. Typewritten or freehand lettering on illustrations is not acceptable. All lettering must be done professionally, and letters should be in proportion to the drawings or photographs on which they appears.

Illustrations must be submitted in electronic format. All images should be at least 5 inches wide. Images should be provided in TIF or EPS format, per the instruction for online submission at http://ees.elsevier.com/tripleo. Macintosh or PC is acceptable. Graphics software such as Photoshop and Illustrator (not presentation software such as PowerPoint, CorelDraw, or Harvard Graphics) should be used in the creation of the art. Color images need to be CMYK, at least 300 DPI, and be accompanied by a digital color proof, not a color laser print or color photocopy. Note: This proof will be used at press for color reproduction. Gray scale images should be at least 300 DPI accompanied by a proof. Combinations of gray scale and line art should be at least 1200 DPI accompanied by a proof. Line art (black and white or color) should be at least 1200 DPI with a proof.

For best possible reproduction, avoid using shading or dotted patterns; if unavoidable, submit this type of illustration in the form of a glossy photograph for best results. Use thick, solid lines and bold, solid type. Place lettering on a white background; avoid reverse type (white lettering on a dark background). Typewritten or freehand lettering is unacceptable. All lettering must be done professionally and should be in proportion to the drawing graph, or photograph. Do not send original art work, radiograph films, or electrocardiographic strips. Any special instructions regarding sizing should be clearly noted.

Legends to illustrations. Each illustration must be accompanied by a legend. These should be typed double-spaced on a separate page. If an illustration has been taken from published material, the legend must give full credit to the original source.

Tables. The tables should be typewritten double-spaced, including column heads, data and footnotes, and submitted on separate pages. Tables should be self-explanatory and should supplement, not duplicate, the text. All table reference citations should be repeats of numbers assigned within the text, not initial citations. A concise title should be supplied for each table. All columns should carry concise headings describing the data therein. Type all footnotes immediately below the table and define abbreviations. If a table or any data therein have been previously published, a footnote to the table must give full credit to the original source.

Video and Computer Graphics. Authors are encouraged to submit videos and computer-generated graphics; eg, a slide presentation with or without animation and sound. An author who wishes to supply such material should notify the editors in the cover letter and note this intention in the Author Comments area of the online submission. Although the publisher will not edit any video or computer graphic, editors and reviewers may suggest changes. All patient-identifying information must be removed or masked.

The maximum length of a video or computer graphic is 8 minutes. Longer submissions may be divided into smaller clips, each of which should be identified at the beginning of the section (eg, Video Clip 1, Graphic 1). A concise legend for each video clip or computer graphic presentation must be included with the manuscript. Videos are to be submitted in MGEG-1 or MPEG-2 (*.mpg) or QuickTime (*.mov) format. More detailed instructions can be found at http://www.elsevier.com/artwork. Videos and computer graphics accompanying a manuscript declined for publication will not be accepted separately. If the manuscript is accepted for publication, the presentation will be archived at www.mosby.com/tripleo.

Permissions. Direct quotations, tables, or illustrations that have appeared in copyrighted material must be accompanied by**written permission** for their use from the copyright owner and original author along with complete information with respect to source. Photographs of identifiable persons must be accompanied by signed releases showing informed consent. Articles appear in both the print and online versions of the journal, and wording should specify

permission in all forms and media. Failure to obtain electronic permission rights may result in the images not appearing in the the print or online versions.

NOTE: FOLLOW INSTRUCTIONS FOR ONLINE SUBMISSION AT: http://ees.elsevier.com/tripleo

Announcements. Announcements must be received by the Editorial Office at least ten weeks before the desired month of publication. Items published at no charge include those received from a sponsoring society of the Journal; courses and conferences sponsored by state, regional, or national dental organizations; and programs for the dental profession sponsored by government agencies. All other announcements selected for publication by the Editor carry a charge of $60 US, and the fee must accompany the request to publish.

Reprints. Because of the extremely high cost of preparing color articles, author reprints for articles containing color illustrations have to be prepared as overprints (overrun pages). Order forms will be sent to the **corresponding author** of articles containing color illustrations, so that overprints of those articles can be ordered the month of publication. No complimentary overprints or reprints will be provided.

Checklist for authors:

__Signed copyright transfer statement (signed by all authors) (FAXED to Editorial Office)

__Letter of submission

__Title page

__Title of article

__Full names(s), academic degree(s), affiliation(s) and titles of author(s)

__Author to whom correspondence, galleys, and reprint request are to be sent, including address and business and home telephone numbers, fax number, and e-mail address

__Structured abstract (double-spaced)

__Article proper (double-spaced)

(Figures and tables should not be part of the text of the manuscript but added as separate files)

__Statement of IRB review (stated in manuscript)

__References (double-spaced on a separate page)

__Reprint requests line (on a separate page)

__Tables (double-spaced, on separate pages)

__Legends (double-spaced, on a separate page)

__Illustrations, properly formatted (as separate files)

__Video/computer graphics, properly formatted (as separate files)

__Acknowledgments (on a separate page)

__Source of funding for research (on a separate page)

__Signed permission to reproduce previously published material, in all forms and media (scanned in as a file)

__Signed permission to publish photographs of identifiable persons from the individual specifying permission in all forms and media (scanned in as a file)

__Financial interest disclosure, if applicable (on a separate page)

__If this paper was presented at a meeting identification of organization, city, and year (on a separate page)

Updated June 2008

ANEXO B – INFORMAÇÕES SOBRE A REVISTA ORAL SURGERY, ORAL MEDICINE, ORAL PATHOLOGY, ORAL RADIOLOGY AND ENDODONTOLOGY

ISSN 1079-2104

Nível A

Fator de impacto 1.592

Organização American College of Oral and Maxillofacial Surgery, American Academy of Oral and Maxillofacial Radiology, American Academy of Oral Medicine, American Academy of Oral and Maxillofacial Pathology, and the Organization of Teachers of Oral Diagnosis

Editor James R Hupp, DMD, MD

Periodicidade mensal

Homepage http://ees.elsevier.com/tripleo